

Genome wide transcriptional profiling of *Neisseria meningitidis* in an epithelial cell line model of human nasopharyngeal colonization

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Abstract

Neisseria meningitidis (the meningococcus) is a Gram negative diplococcus which asymptotically colonizes the human nasopharynx in about 10%-30% of the population. In rare cases it can invade the bloodstream and cross the blood brain barrier to cause sepsis and meningitis. Serogroup B strains are the major cause of disease in the UK.

To gain insight into the process of colonization, I have studied the meningococcal transcriptome in a model of human nasopharyngeal colonization. As meningococcal colonization can last for weeks to months, investigating the meningococcal transcriptome over an extended period of time offers potentially important new insights into meningococcal biology unexplored in short time-course experiments as undertaken by others.

Confluent layers of a human bronchial epithelial cell line (16HBE14) were infected with a serogroup B meningococcal strain (MC58) for different time periods (4h, 24h, 96h and 21d) prior to bacterial RNA extraction. Using microarray technology, the transcriptome of host cell-associated meningococci was investigated and expression of selected genes (some previously shown to be involved in host cell interaction/colonization, and others surface expressed vaccine candidates) was validated with quantitative real time PCR.

The transcription profile at late time points (24 h, 96 h and 21 d) was compared to that at 4 h to identify genes potentially involved in prolonged bacteria host-cell association. A set of seven genes (*NMB0342-0348*) was identified which was up-regulated at later time points. By use of RT-PCR the operon structure of the locus was defined: one set of four and one pair of these genes are co-transcribed. MC58 *kanR* insertion mutants in five of these seven genes were prepared and studied in quantitative adherence and invasion assays. An impaired adherence and reduced invasion profile compared to the wild type suggests a potential role in sustained host cell association.

Declaration of Originality

I declare that the work presented in this thesis is my own and that I did not submit any of the work for the award of a degree by this or any other University. All material in this thesis, which is not my work has been clearly identified and appropriately acknowledged.

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List of abbreviations

A549	Human carcinoma alveolar epithelial cells
aa	Amino acid
ANOVA	Analysis of variance
App	Adhesion and penetration protein
ATCC	American tissue culture collection
BHI	Brain-Heart infusion medium
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary (copy) DNA
CE	Correia element
CFU	Colony forming units
Chang cells	Human conjunctival epithelial cell line
COR L23	Large cell bronchial carcinoma cell line
CSF	Cerebro spinal fluid
C ^T	Threshold cycle
Cy-3 / Cy-5	Cyanine fluorescent dyes
di H ₂ O	Deionized water
DMEM	Dulbeccos Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
DTT	dithiothreitol
ELISA	Enzyme linked immunosorbent assay
ET	Electrophoretic type
EtBr	Ethidium bromide
EtOH	Ethanol
FACS	Fluorescent activated cell sorter
FDR	False discovery rate
fH	Factor H
FHBP	Factor H binding protein
GC	Gonococcal medium
gDNA	Genomic DNA
Hib	<i>Haemophilus influenzae</i> type b
hi-fbs	Heat – inactivated fetal bovine serum
HMDS	hexamethyldisilazane
hSBA	human serum bactericidal assay
HTE E6/E7	E6/E7 transformed human tonsillar epithelial cell line
J774	Murine monocyte macrophage cell line
<i>kanR</i>	Kanamycin resistance cassette
kbp	Kilo base pairs
k Da	Kilo Dalton
K-SFM	Keratinocyte-serum free medium
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LOWESS	Locally weighted polynomial regression

MC58	<i>Neisseria meningitidis</i> serogroup B strain
MLST	Multi locus sequence type
MOI	Multiplicity of infection
MspA	Meningococcal serine protease A
MucilAir	Cultured primary human nasopharyngeal epithelial cells
MW	Molecular weight
NadA	Neisserial adhesin A
NafA	Neisserial anti-aggregation factor A
NHBP	Neisserial heparin binding protein
NhhA	Neisseria hia-homolog A
NspA	Neisserial surface protein A
nt	Nucleotide
OD ₆₀₀	Optical density at 600 nm
OM	Outer membrane
OMP	Outer membrane proteins
Opa, Opc	Opacity proteins
OMV	Outer membrane vesicles
o/n	Over night
orf	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
p.i.	Post infection
Pil	Pilin
PorA/B	Porin A/B
q-rt-PCR	Quantitative real-time reverse transcription PCR
rev / min	Revolutions per minute
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription PCR
sBHI	Isovitalex A and B supplemented Brain-Heart Infusion broth
scanning EM	Scanning electron microscope/microscopy
SD	Standard deviation
sDMEM	Supplemented DMEM with 10% hi-fbs
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sGC	Isovitalex A and B supplemented GC agar
sGC-kan50	sGC agar with 50 µg / ml kanamycin
ST	Sequence type
STM	Signature tagged mutagenesis
Taa	Trimeric auto transporter
TEER	Trans-epithelial electrical resistance
tfp	Type-IV pili
TTB	Towbins Transfer buffer
vs	Versus
v/v	Volume per volume
WT	Wild type

w/v	Weight per volume
16HBE14	SV 40 transformed human bronchial epithelial cell line
24-96 h or 24 h - 96 h	Combined transcriptional data set of 24 h and 96 h p.i.

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Chapter 1

General Introduction

1.1. History of meningococcal diseases

Illness resembling meningococcal disease described as “spotted fever” could have dated back to the 17th century. However, the first well documented meningococcal disease outbreak, which resulted in 33 deaths from a “spotted fever” in Eaux-Vives, a suburb of Geneva, Switzerland, was published in 1805 by Vieusseux (Vieusseux, 1805). With the advance of microscope and staining techniques, the Italian pathologists Marchiafava and Celli first described intracellular oval shaped micrococci in a sample of cerebrospinal fluid (CSF) from a meningococcal patient (Marchiafava and Celli, 1884). In 1887, Weichselbaum identified bacteria in the CSF of six of eight patients suffering from meningitis. He named the bacterium “diplococcus intracellularis meningitidis” (Weichselbaum, 1887) which we know today as *Neisseria meningitidis*, a member of the genus *Neisseria*, named after the German physician Albert Neisser (reviewed in Ligon, 2005).

1.2. The genus *Neisseria*

The genus *Neisseria* belongs to the family of *Neisseriaceae*, which is a member of the β -proteobacteria together with human pathogens such as *Bordetella pertussis* and *B. parapertussis*. Members of the *Neisseriaceae* include, among 13 other genera, the human (facultative) pathogens *Moraxella catarrhalis*, *Eikenella corrodens* and *Kingella kingae*. However, most organisms within the *Neisseriaceae* are mammalian commensals with a wide host range (Madigan and Martinko, 2006).

The genus *Neisseria* has the defining characteristics of being a Gram negative diplococcus and strongly oxidase positive. *Neisseria* includes the two human pathogens *N. meningitidis* and *N. gonorrhoeae* (the gonococcus). In contrast to *N. gonorrhoea* which is a mucosal pathogen (of the uro-genital tract), *N. meningitidis* asymptotically colonizes the nasopharyngeal epithelium and occasionally causes invasive disease in forms of septicaemia and meningitis. Invasive gonococcal disease is atypical. Other members of the genus *Neisseria* are mainly commensals of the human nasopharynx such as *N. lactamica*, *N. cinerea* and *N. flavescens* (Madigan and Martinko, 2006).

1.3. The ecological niche of the meningococcus

Although *N. meningitidis* can invade the human blood stream to cause infections such as septicaemia and meningitis, especially in young children (Goldschneider *et al.*, 1969a), the primary environmental niche of the meningococcus is the mucosal epithelial surface of the human upper respiratory tract. It is the relationship between bacteria and human nasopharyngeal epithelium that defines the interactive biology of meningococci with its human host. Between 10% and 30% of the healthy population are carriers (Caugant *et al.*, 1994). In nearly all cases colonization is asymptomatic, may be prolonged for weeks up to six months (Stephens *et al.*, 1999; Wilder-Smith *et al.*, 2003; Caugant *et al.*, 2006) and provides – through droplet spread – opportunities for transmission between individuals.

Invasive disease is a rare event, with an incidence in the UK and comparable developed world settings of 0.5 – 5 per 100,000. The relationship of development of invasive disease to colonization rates remains uncertain (Merz and So, 2000). Strains were isolated which i) rarely cause disease and are predominantly found as colonizer (carrier strains), ii) predominantly cause invasive disease and are rarely found as colonizer (hypervirulent strains) and iii) are associated with invasive disease but also found as colonizer (virulent strains) (Caugant *et al.*, 1994; Jolley *et al.*, 2000). Carrier strains have frequently found to be not expressing capsule and the neisserial adhesin A (NadA, described in section 1.6.7) (Claus *et al.*, 2002; Comanducci *et al.*, 2004). The well established risk of secondary cases within families in the week following a case (with a presumption that the organism has spread from the index case) supports the proposition that invasion may be an early event after bacteria arrive at the mucosal surface. However, cases have been documented in which invasive disease has followed a prolonged period of colonization with the virulent strain, a possible inference being that the balance between host and pathogen can be disastrously disturbed by e.g. viral infection or some other environmental change (Neal *et al.*, 1999).

Prolonged epithelial colonization establishes strain-specific immunity, and repeated colonization by the meningococcus and related non-pathogenic *Neisseria* (such as *N. lactamica*) throughout childhood and adolescence is considered the basis for the relative protection against invasive infection that is found in

most adults. This protection presumably derives from the sum of immune responses to products of a wide repertoire of genes encoding surface-exposed bacterial structures expressed by different strains (Neal *et al.*, 1999). However, colonization has been shown to induce strain specific immunity by production of antibodies directed against the serosubtyping antigen PorA (Jones *et al.*, 1998).

Carriage rates are lowest among toddlers and highest in adults, increasing the risk of transmission from healthy adult carriers to non-carrying young children. Carriage has been shown to be especially high among people living in close contact such as in college halls and military camps (Jones *et al.*, 1998). To fully understand the process of meningococcal colonization, invasion and host immunity, meningococcal-host cell interactions have been and are continuing to be studied in detail.

The preferred epithelial niche of the meningococcus, the human nasopharynx, is composed of columnar cells (ciliated and non-ciliated cells), mucus producing goblet cells and basal cells (Mygind and Dahl, 1998). Separated from the underlying tissue by a layer of collagen fibrils (basal membrane) the nasopharyngeal epithelium forms the outer layer of the upper respiratory tract. Functions of the epithelium include moisturizing of, and particle removal, from the inhaled air on its way into the lungs. The epithelium produces mucus and contains cells with beating cilia (Mygind and Dahl, 1998). Basal cells, in closest proximity to the basal membrane, are able to differentiate into columnar or goblet cells (Mygind and Dahl, 1998).

Columnar cells help to stabilize the epithelium and are either non-ciliated or ciliated. The latter have each ca. 100 cilia of ca. 5 μm length and 0.3 μm diameter as well as ca. 300 microvilli on each apical cell surface. Cilia beat ca. 1000 times per minute, propelling mucus up towards the throat. Proportions of ciliated cells in the epithelium can vary. Dry air, low temperatures and increasing age reduce the number of ciliated cells. In adults ciliated cells are present in the upper nasopharynx whereas in newborns the entire inner nose has ciliated cells (Mygind and Dahl, 1998).

Goblet cells produce mucus, which is released after environmental stimuli rather than by neurological signalling (Mygind and Dahl, 1998). Water is the main component of mucus. However, it contains

substantial quantities of glycoproteins and proteins such as albumin and immunoglobulins (especially IgA) as well as lysozyme and lactoferrin, both of which are involved in bacterial defence (Mygind and Dahl, 1998).

The mucociliary clearance of a particle trapped in mucus, of a healthy person, is within a range of 3 – 25 mm per minute. However, significantly lower rates have been described during cold conditions (Mygind and Dahl, 1998).

1.4. Transmission, symptoms, diagnostic and therapy of meningococcal disease

As the human nasopharynx is the primary niche of the meningococcus, transmission between individuals occurs by exchange of respiratory secretions mainly in form of droplets e.g. sneezing and coughing (DeVoe, 1982). Once transmitted to a new host, in most cases, meningococci colonize the nasopharynx asymptomatic. Rarely, invasive disease in the form of septicaemia and meningitis is developed. The early symptoms of invasive meningococcal disease are non-specific. Similar illness caused by many opportunistic pathogens, such as respiratory tract viruses, frustrates rapid and correct diagnosis. Besides symptoms such as fever and malaise, anorexia, nausea and vomiting, muscle and limb pains, headache and altered mental state (DeVoe, 1982), common to septicaemia caused by many bacterial, viral and fungal pathogens, during invasive meningococcal disease a non-blanching skin rash commonly (in 30 – 60 % of cases) occurs. The latter is the result of capillary damage allowing bleeding into the skin and is characterized by a few to many petechiae especially on the axillary folds, back and belt line (DeVoe, 1982). Tissue necrosis causing skin and muscle damage is well recognized in meningococcal sepsis, causing skin scarring and in the severest cases leading to digit or peripheral limb amputation (Van Deuren *et al.*, 2000; DeVoe, 1982).

Fulminant meningococcaemia, with a bacterial load between 2.2×10^4 – 1.6×10^8 per ml blood (Hackett *et al.*, 2002), has a high mortality rate (20 % - 80 %) due to multi organ failure, an abrupt onset and fast

and fatal progress of disease. Mortality has been reported as early as six hours after the onset of the first symptoms (Van Deuren *et al.*, 2000; DeVoe, 1982).

The lowest mortality (2 – 3 %) of meningococcal disease is observed in patients developing meningitis alone, without septicaemia. However, sequelae such as deafness, visual impairment, mental retardation and epilepsy occur in ca. 5 % of convalescent patients (DeVoe, 1982; Cartwright and Ala'Aldeen, 1997; Van Deuren *et al.*, 2000; Chin *et al.*, 2005). After crossing the blood-brain-barrier, meningococci proliferate in the subarachnoid space where humoral and cellular immune defence is absent (Simberkoff *et al.*, 1980; Zwahlen *et al.*, 1982; Brandtzaeg *et al.*, 1992). The endotoxin release from meningococci results in inflammation of the meninges by inducing production of proinflammatory cytokines such as tumor necrosis factor α (TNF α) and interleukins, which attract neutrophils (Arditi *et al.*, 1989; Brandtzaeg *et al.*, 1992; Halstensen *et al.*, 1993). Inflammation of the meninges increases the intracranial pressure, which can result in brain damage (Ashwal *et al.*, 1990).

The traditional laboratory diagnosis of meningococcal disease is by culturing the organism obtained from blood and / or CSF samples and / or nasopharyngeal swabs. Rapid diagnosis may be possible by microscopy of CSF. Processing of blood and CSF samples is dependent on local laboratory specific procedures and includes, after concentration of the sample, culture on selective media such as Thayer-Martin, a chocolate sheep blood agar which supports growth of *Neisseria* and inhibits the growth of many other bacteria by adding various antibiotics (Madigan and Martinko, 2006). Finding oxidase positive, Gram-negative diplococci confirms *Neisseria*. Serological agglutination kits are used to specify the serogroup (Madigan and Martinko, 2006).

A fast method for application on CSF samples is slide agglutination (Fox *et al.*, 2006) but the confirmation by culture of the organism is the gold standard (Madigan and Martinko, 2006). PCR targeting the IS1106 insertion sequence lying within the meningococcal genome is routinely used to confirm presence of the organism in blood and / or CSF samples. Using PCR is advantageous as it is a fast method and, in contrast

to culture, not affected by antibiotic therapy prior to sample collection (Knight *et al.*, 1992; Newcombe *et al.*, 1992).

Benzylpenicillin is bactericidal against *N. meningitidis*, and with chloramphenicol remains a first line therapy for meningococcal disease in low-income settings. In high-income settings, third generation cephalosporins with excellent tissue and CNS penetration are generally used. Resistance of meningococci to penicillin is rare but was reported in the US (Madigan and Martinko, 2006). Early antibiotic therapy is associated with a better outcome of the disease (Cartwright *et al.*, 1992). Family contacts of patients are offered prophylactic antibiotic (rifampicin or ciprofloxacin are commonly used) to prevent secondary cases (Madigan and Martinko, 2006).

1.4.1. Incidence rates and risk groups

Meningococcal disease occurs world wide at a rate of ca. 0.5 cases per 100 000 individuals. However, 15 cases per 100,000 individuals within two weeks predict major epidemics and within an epidemic, more than 100 cases per 100,000 individuals per year might be diagnosed. (WHO, 1995; Leake *et al.*, 2002). In West Africa in 1996-7 (estimated population of 270 000 000 individuals; Leake *et al.*, 2002), 213,658 cases with 21,830 deaths have been reported (Center for Disease Control and Prevention).

In Europe, incidence rates are low with an average of 0.9/100 000 (2008). Ireland, Lithuania and the United Kingdom had, in 2008, in the youngest age group (under five years of age), the highest rates in Europe with 28.7, 27.9 and 20.0 cases per 100,000 individuals, respectively (European Center for Disease Prevention and Control, 2010).

Risk groups for meningococcal disease include babies, toddlers and students. In 2008, 33 % of the reported cases were diagnosed in children under five years of age. Interestingly, more cases were reported in male than in female patients in this age group. The second largest group diagnosed with

meningococcal disease are the 15 – 24 years old. The group aged 5 – 14 years were the third largest group (European Center for Disease Prevention and Control, 2010).

In West and sub-Saharan African countries (the meningitis belt), meningococcal disease peaks during the dry season between December and June. Seasonal trends are also observed in Europe where meningococcal disease peaks in the winter months (WHO: Meningococcal meningitis, 2010; European Center for Disease Prevention and Control, 2010). Living in close proximity e.g. in military units and college halls increases transmission rates of meningococci and therefore the risk of meningococcal disease (Wilder-Smith *et al.*, 2003). For example, an outbreak occurred during a pilgrimage to Mecca (Schwartz *et al.*, 1988). In addition to the above mentioned age and life style related risk groups, genetic disorders can render individuals more susceptible to invasive meningococcal disease. Mutations in the toll-like receptor 4 and complement deficiencies such as those with a mutation in the complement component factor H contribute to a high risk of meningococcal disease (Mogensen *et al.*, 2006; Davila *et al.*, 2010; Ram *et al.*, 2010).

1.5. Classification of meningococcal strains

Strains of *N. meningitidis* are, at the highest classification level, divided into serogroups based on their polysaccharide capsule (Goldschneider *et al.*, 1969a, 1969b). There are twelve *N. meningitidis* serogroups known as A, B, C, 29E, H, I, K, L, W135, X, Y, Z of which five (A, B, C, W135, X and Y) are human pathogens (Yazdankhah and Caugant, 2004). A prevalence of serogroups in certain geographic regions has been described. Serogroups A and C are most prevalent in Africa with serogroup A causing devastating epidemics in the sub-Saharan meningitis belt. In Europe and America, serogroups B and C are most prevalent. Serogroup Y is mainly present in America and Canada whereas W135 is found worldwide (Stephens *et al.*, 2007). However, serogroup W135 caused several epidemics in the meningitis belt such as in Burkina Faso in 2002 and in Niger 2010 (World Health Organization Meningococcal Meningitis Weekly Epidemiological Record, 2003; Collard *et al.*, 2010). Serogroup X has recently caused

outbreaks in Togo and Burkina Faso between 2006 and 2010. From 2009 to 2010, cases with serogroup X increased from 4% to 35% of all confirmed meningococcal cases in this region. In Burkina Faso, the incidence rate of serogroup X meningococcal disease was 130 per 100,000 during March and April 2010 (Delrieu *et al.*, 2011).

Strains are assigned into serotypes, serosubtypes and immunotypes based on PorA, PorB and LOS variation (described in more detail in section 1.6.), respectively. Using the advance of technology, *N. meningitidis* is further classified into sequence types (ST) or electrophoretic types (ET) according to the sequence of seven house keeping genes (Table 1.1.) or to the variation in electrophoretic mobility of key metabolic enzymes, respectively (Maiden *et al.*, 1998, Caugant 1998). Jolley *et al.* (2006) recommended the nomenclature of meningococcal strains according to the serogroup: *porA* type: *fetA* type: sequence type (clonal complex). The variable regions of the *porA* and *fetA* genes are used. For example, the serogroup B strain MC58 would be named B: 15: P1.7, 16b, ST-32 (ET-5).

Table 1.1. Description of MLST genes (<http://pubmlst.org/neisseria/info/primers.shtml>)

Gene ID	Description
NMB1051	Putative ABC transporter AbcZ
NMB0823	Adenylate kinase AdK
NMB0358	Shikimate dehydrogenase AroE
NMB1458	Fumarate hydratase FumC
NMB1392	Glucose-6-phosphate dehydrogenase Gdh
NMB1341	Pyruvate dehydrogenase subunit PdhC
NMB0790	Phosphoglucomutase Pgm

1.6. Meningococcal surface structures and their genes

In the context of the present study I focus on meningococcal surface structures as what has been known for their contributions in meningococcal – host interactions and also their tested or theoretically proposed potential as vaccine candidates. Besides the capsule, *N. meningitidis* has several other surface

structures (some are shown in Figure 1.1). In the following sections meningococcal surface structures and their genes, which are known to be involved in host cell interaction, are described.

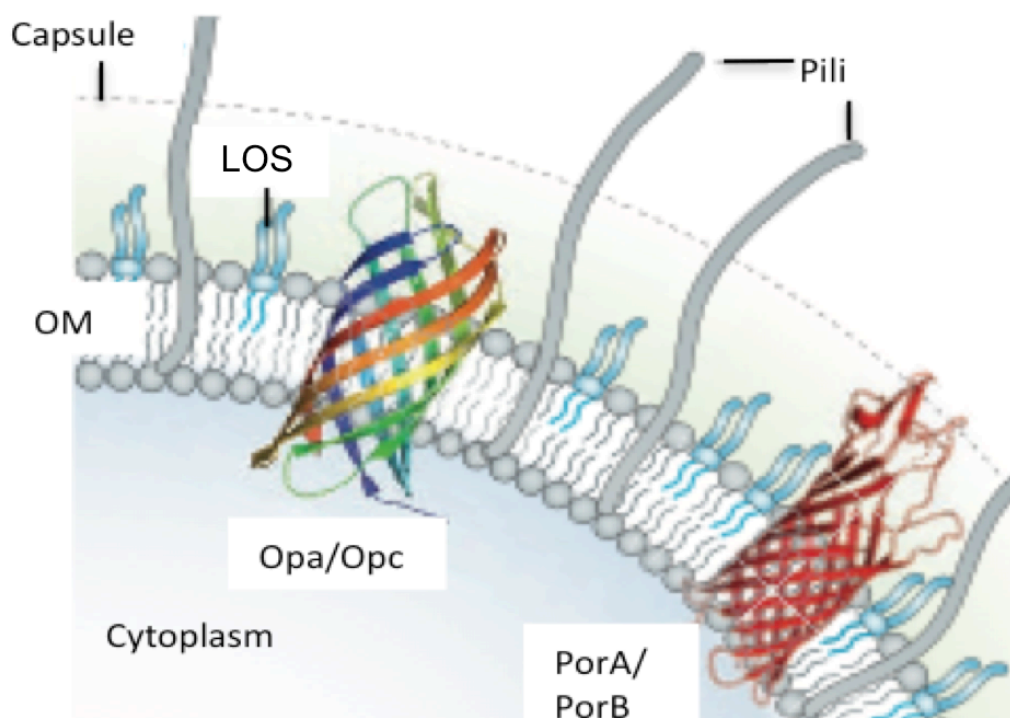


Figure 1.1. Meningococcal surface structures (adapted from Virji, 2009). Shown are the capsule, type IV pili, lipooligosaccharides (LOS), Opacity proteins (Opa and Opc) and the two major porins PorA and PorB and their position in the outer membrane (OM). A dashed line indicates that most structures are covered by the capsule.

1.6.1. The polysaccharide capsule

The meningococcus is protected from the environment by an outer shell of polysaccharide, the capsule. Based on the composition of the polysaccharide capsule, *N. meningitidis* is classified into thirteen serogroups of which five are human pathogens (serogroups A, B, C, W135, X and Y). In contrast to serogroup A, the capsules of serogroups B, C, W135 and Y contain sialic acid (Warren and Blacklow, 1962; Claus *et al.*, 1997). Sialic acid is a derivative of neuraminic acid. Sialylation of the capsule is

associated with resistance of meningococci to complement-mediated killing (Genco and Wetzler, 2010). Vaccines based on conjugated capsular polysaccharides have been successfully developed against serogroups A, C, W135 and Y (Pace *et al.*, 2009). The capsule of serogroup B contains α 2-8-linked-N-acetyl-neuraminic acid (NANA), which is identical to the neural cell-adhesion molecule (NCAM) found in the human central and peripheral nervous systems (Warren and Blacklow, 1962; Rutishauser *et al.*, 1988; Claus *et al.*, 1997). Therefore, the serogroup B capsule is poorly immunogenic but might induce autoimmunity. α 2-9 linked NANA is contained in serogroup C and α 2-6 linked NANA in serogroups W135 and Y.

There are two clusters of core genes involved in capsule expression, one set codes for capsule biosynthesis and one for translocation. In the serogroup B strain MC58, the capsule biosynthesis genes *siaB-D* and *synX* are encoded by *NMB0069*^{*}, *NMB0068*, *NMB0067* and *NMB0070*, respectively. The serogroup specific sialyltransferase *SiaD* is involved in capsule polymerization. The genes *ctrA-D*, encoded by *NMB0071-NMB0074* are involved in capsule export (Warren and Blacklow, 1962; Claus *et al.*, 1997).

Besides protecting the bacteria from adverse environmental conditions such as aridity, which potentially results in desiccation, toxins or antibacterial agents, the capsule is essential for intracellular and blood stream survival of meningococci (Gotschlich *et al.*, 1969; Kahler *et al.*, 1998; Nikulin *et al.*, 2006; Spinosa *et al.*, 2007). However, the capsule reduces meningococcal adherence to host cells and surfaces and carriage isolates are frequently capsule negative (de Vries *et al.*, 1996; Kolb-Maurer *et al.*, 2001; Claus *et al.*, 2002; Deghmane *et al.*, 2002; O'Dwyer *et al.*, 2009). A poly-cytidine tract within *siaD*, which can lead to slip-strand mispairing, is involved in the on/off expression of the meningococcal capsule (Weber *et al.*, 2006). The on/off expression of the capsule potentially contributes to the adaption to the colonization (off) and invasive disease (on) state of meningococci (Swartley *et al.*, 1997; Kahler *et al.*, 1998; Beddek *et al.*, 2009; reviewed in Van der Woude and Bäumler, 2004 and in Hill *et al.*, 2010). Increased expression of

* NMB numbers refer to strain MC58

the capsule has been shown to render meningococci resistant to otherwise bactericidal antibodies directed against PorA (Uria *et al.*, 2008).

Capsule switching, the horizontal transfer of capsule genes from strains of one serogroup to another, has been described which results in in-effectiveness of vaccine or naturally acquired antibodies against the parental strain (Swartley *et al.*, 1997; Kahler *et al.*, 1998; Vogel *et al.*, 2000; Beddek *et al.*, 2009).

Examples include the capsule transfer from serogroup C and Y to B to which vaccinated individuals are susceptible (Tsang *et al.*, 2005; Simoes *et al.*, 2009).

1.6.2. Lipooligosaccharide (LOS)

The LOS of *N. meningitidis* is composed of short phase-variable oligosaccharide chains (two to five sugars; α , β and γ) linked to lipid A - the endotoxin (Burch *et al.*, 1997; Kahler and Stephens, 1998).

Contrasting with the LOS of most other bacteria, neisserial LOS does not have attached O-antigens (Kahler and Stephens, 1998; Preston *et al.*, 1996). O-antigens are an additional set of sugars linked to the outer core of LOS, together named lipopolysaccharide (LPS). LPS is mainly found in enteric bacteria and facilitates bacterial survival in the gut (Preston *et al.*, 1996).

Twelve meningococcal LOS immunotypes have been described, each with a distinct oligosaccharide chain (Mandrell and Zollinger, 1977; Zollinger and Mandrell, 1977, 1980). LOS types L3 and L7 are most prevalent among *N. meningitidis* (Mandrell and Zollinger, 1977); L1 – L8 are associated with serogroup B and C and L9 – L12 with serogroup A (Zhu *et al.*, 2002).

Three genetic loci are involved (*lgt1-3*) in meningococcal LOS biosynthesis, composed of genes encoding for glycosyltransferases. In the sequenced serogroup B strain MC58, locus *lgt1* contains genes *lgtA* (NMB1929), *lgtB* (NMB1928) and *lgtE* (NMB1926); *lgt2* is composed of *lgtF* (NMB1704) and *rfaK* (NMB1705). *Lgt3*, comprising *lgtG*, is non-functional in strain MC58 due to a homopolymeric tract composed of 12 cytosine repeats outside of the open reading frame, which can cause a frame shift (Zhu

et al., 2002). *LgtA* and *lgtE* have been implicated to be involved in an immunotype switch from L8 to L3 (Scholten *et al.*, 1994, Berrington *et al.*, 2002). *LgtA* adds the L3 specific structure (composed of a β -N-acetylglucosamide, β -galactose and α -N-acetylneuraminic (sialic) acid) to the terminal β -galactose of L8, which is added by *lgtE* to the inner core β -glucose. *LgtB* links the L3 specific β -galactose to the β -N-acetylglucosamid (Scholten *et al.*, 1994; Berrington *et al.*, 2002). *LgtA*, *lgtB* and *lgtE* are also involved in the synthesis of immunotypes L2 and L4 (Figure 1.2).

Differences in the LOS immunotype have been described between carriage and invasive disease isolates. LOS types L3, 7 and 9 are more prevalent in invasive disease isolates, whereas LOS types L1, 8 and 10 predominate in carriage isolates, in particular when these are encapsulated (Jones *et al.*, 1992). The bias in carriage strains against L3, 7, 9 is less apparent when non-capsulate isolates are considered. A switch from one LOS type to another was reported (Jones *et al.*, 1992). A carried strain with immunotype L1, 8, 10 was found after transmission into a new host to have converted to the immunotype L3, 7, 9. This switch was associated with development of invasive disease and occurred during invasion of the nasopharyngeal epithelium (Jones *et al.*, 1992). Immunotypes L1, 8, 10 are non-sialylated, whereas L3, 7, 9 are sialylated. LOS sialylation inhibits invasion of epithelial cells and appears to be essential for survival in the bloodstream (Jones *et al.*, 1992; Estabrook *et al.*, 1997). Co-expression of different LOS types has been observed in invasive strains, expressing L8 and L1 at a low level in addition to more abundant L3 and L7 (McLeod *et al.*, 2000).

LOS can be modified by addition of phosphoethanolamine to either the lipid A or the HepII residue in the inner core (Takahashi *et al.*, 2008). Expression of *lptA*, encoding a phosphoethanolamine transferase (specific for lipid A), has been shown to modify host-cell adherence. Deletion of *lptA* decreased adherence to epithelial cell lines (Hep-2 and A549) and both human brain microvascular endothelial cells (HBMEC), and human umbilical vein endothelial cells (HUVEC) (Takahashi *et al.*, 2008). However, the role of LOS in meningococcal host cell association remains inconclusive. For example, Albiger *et al.* (2003) have shown decreased cellular adherence of a LOS-negative mutant, which was unable to invade host

cells (and take up DNA), suggesting that LOS phenotype influences functionality of pili, even when these are present in similar numbers as in wild type bacteria. Plant *et al.* (2006), in contrast, have found increased adherence in mutants with LOS truncations.

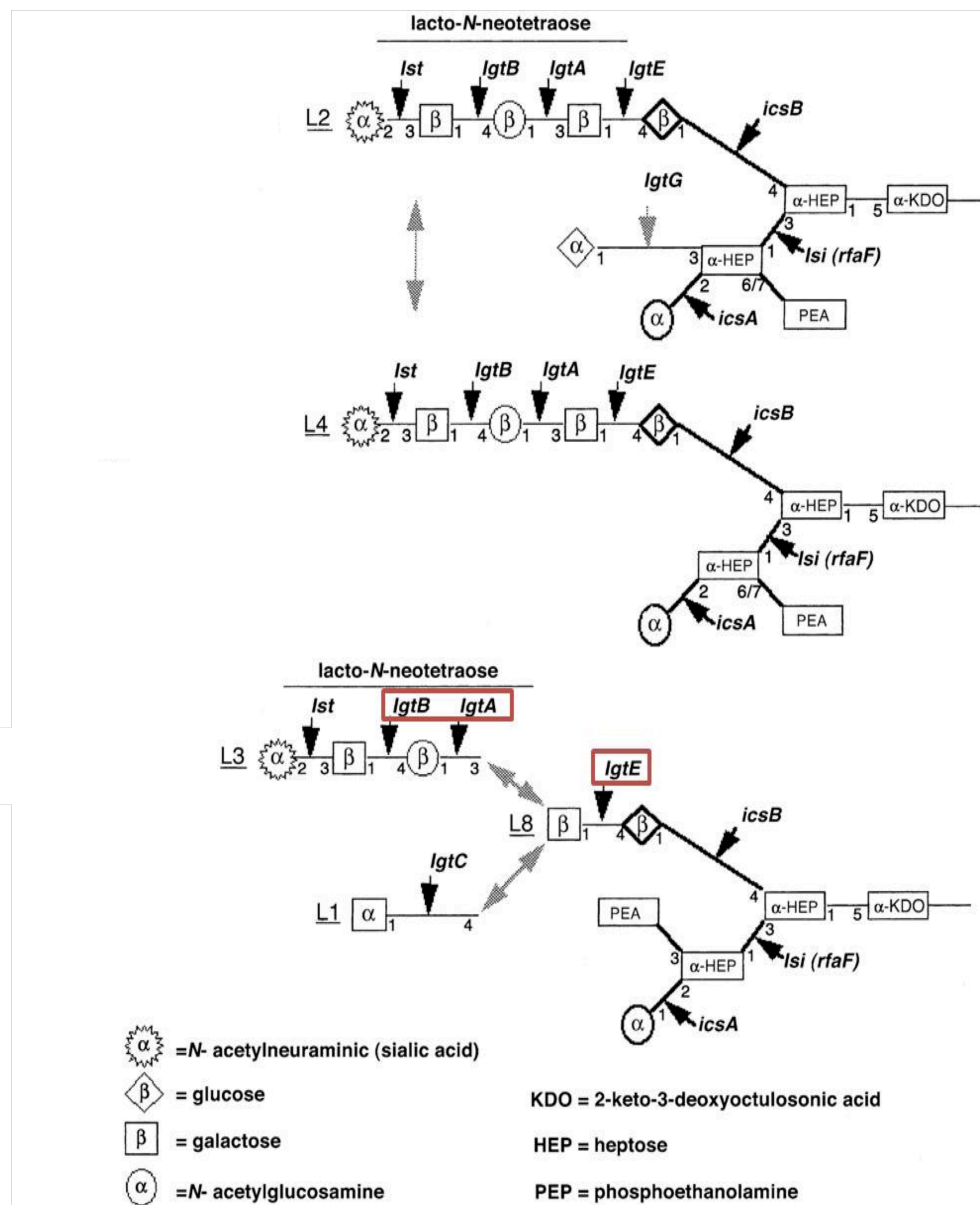


Figure 1.2. Adapted from Berrington *et al.*, (2002). Genes (indicated by black arrows) involved in different LOS immunotype synthesis. Phase variations are indicated by grey arrows. *LgtA*, *lgtB* and *lgtE*, highlighted by a red outline, are involved in switch from immunotype L3 to L8.

LOS is directly involved in virulence and the outcome of invasive meningococcal disease. Despite activating complement in the host (Brandtzaeg *et al.*, 1989b), LOS contributes to the protection of bacteria from being killed in the bloodstream (Rice *et al.*, 1987). The fatality rate and the chance of developing severe septic shock and multiple organ failure increase with increasing LOS levels (Brandtzaeg *et al.*, 1989a).

1.6.3. The type IV pilus and interaction with human epithelial cells

Bacterial pili are hair-like multimeric structures on the surface of bacteria which protrude beyond the capsule (Figure 1.1) and are involved in bacterial motility, function as adhesins, are involved in sensing the environment and in DNA uptake and are associated with virulence (Kline *et al.*, 2009). The lack of pilus expression in usually piliated bacteria significantly reduces bacterial virulence, as adherence to host cells, the initial contact of pathogen and host, is markedly decreased. In Gram-negative bacteria, type 1 pili, P-pili, type IV pili and curli have been described (Kline *et al.*, 2009). However, *N. meningitidis* only expresses type IV pili (Tfp), which will be reviewed below.

Tfp (Figure 1.1) comprise filaments of about 6 nm diameter and several μm in length (Parge *et al.*, 1995). Besides their function as adhesins, Tfp are also involved in neisserial transformation, conjugation, twitching motility and formation of bacterial aggregates on biotic or abiotic surfaces. Strong adhesion to host cells and twitching motility are commonly associated with the formation of pili bundles rather than singular pili (Nassif *et al.*, 1993; Pujol *et al.*, 1997; Merz and So, 2000).

Nassif *et al.*, (1993) have shown that expression of Tfp is necessary for meningococcal adherence to epithelial cells. In encapsulated bacteria, Tfp are the only means by which adherence to host cells is mediated (Nassif *et al.*, 1993). Pujol *et al.* (1997) were the first to report that meningococcal Tfp were down-regulated after initial contact with host cells (T84 – human colon adenocarcinoma cell line) resulting in closer contact with the host cell surface (intimate attachment). Upon intimate attachment

the host cell cytoskeleton undergoes profound phenotypic changes including polymerization of actin filaments. Meningococci apparently cross epithelial monolayers without disruption of tight junctions (Pujol *et al.*, 1997). Scanning electron microscopy of aggregates of meningococci interacting with host cells has revealed morphological changes over time (4 h and 9 h post-infection (p.i.)). At 4h p.i. bacteria appeared in small “3-dimensional” clumps on the host cell surface with Tfp clearly visible. At 9 h p.i. these pili were no longer visible, bacteria forming a diffusely adherent monolayer (“2-dimensional”) on the host cell surface. Confocal and transmission electron microscopy at 9 h p.i. showed cellular actin polymerization underneath and pedestal formation around adherent meningococci, resembling attaching and effacing (AE) lesions as described for enteropathogenic *E. coli*. With the formation of AE lesions a loss of host cell microvilli as well as absence of meningococcal pili was observed (Pujol *et al.*, 1997).

Tfp are mainly composed of the major pilin (PilE) and of several other minor subunits. Genes known to be involved in meningococcal piliation include, among others, *pilC1, C2, E, D, F, G, H, I, J, K, M, N, O, P, Q, W* (Carbonnelle *et al.*, 2006), *pilT* (Pujol *et al.*, 1999), *pilV* (Winther-Larsen *et al.*, 2001), *pilX* (Helaine *et al.*, 2005) and *comP* (Wolfgang *et al.*, 1999) with most of the genes being phase variable (Jonsson *et al.*, 1991). Of the genes involved in pilus biogenesis and function, seven (*pilT1, pilT2, PilU, PilV, pilX, pilZ* and *comP*) were identified as being non-essential for piliation but necessary for functioning of the pilus (Brown *et al.*, 2010). Some of the Tfp subunits are described in some detail below and shown in Figure 1.3.

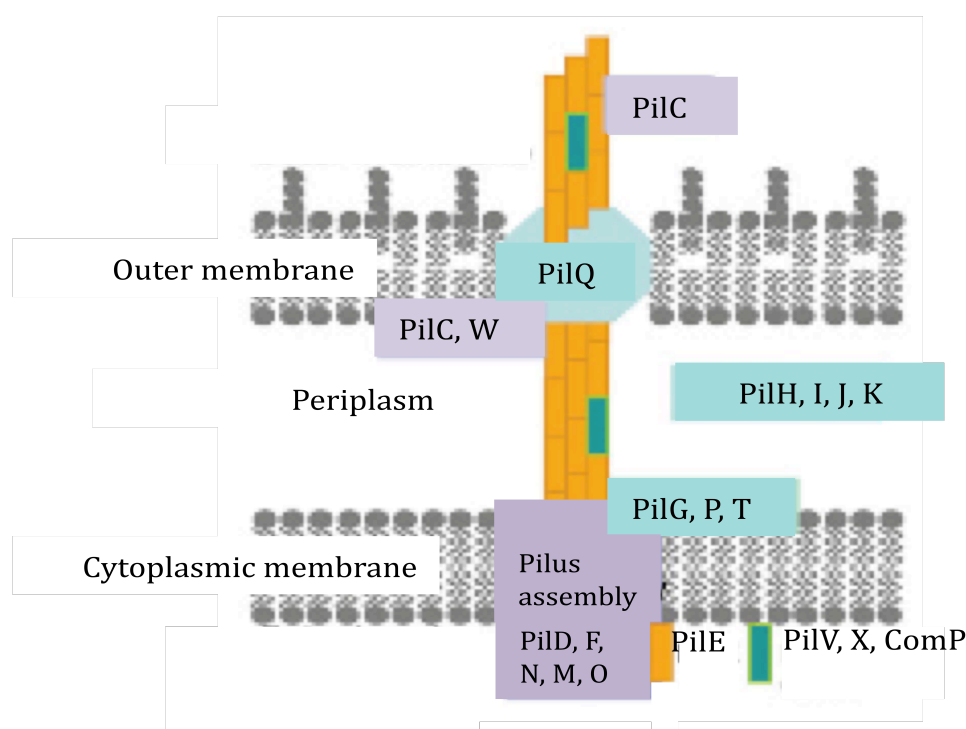


Figure 1.3. Adapted from Hill *et al.* (2010). Meningococcal type IV pilus structure and subunits and their cellular location. The major subunit PilE is shown in yellow rectangles, minor pilus subunits, chaperons and assembly proteins in green, blue and purple.

PilE (NMB0018), the pilin protein, is the highly variable, major subunit of Tfp, with the variation being in the glycosylation (Rudel *et al.*, 1995; Scheuerpflug *et al.*, 1999) and in the C-terminal globular region, where insertions, deletions and point mutations frequently occur. The C-terminal region forms a globular head (Parge *et al.*, 1995) and the N-terminal domain forms a hydrophilic α helix. On the pilus surface, pilin is further modified by O-glycosylation and phosphorylation. Pilin subunits first dimerize into a coiled-coil dimer before they polymerize into a cylinder-like structure with the C-terminal globular domain forming the pilus surface (Parge *et al.*, 1995; Merz and So, 2000). Upstream of *pilE* is the silent *pilS* cassette located, comprised of eight *pilS* genes (Tettelin *et al.*, 2000). This locus is associated with antigenic variation of *pilE* via homologous recombination of one of the eight *pilS* (silent storage) genes into the *pilE* gene (reviewed in Cahoon and Seifert, 2011).

PilD (NMB0332), the prepilin peptidase, a homologue of a gene product first described in *Pseudomonas aeruginosa*, cleaves PilE, existing intracellularly as a precursor protein and converted into its mature form in the periplasm (Lory *et al.*, 1997; Parge *et al.*, 1995; Merz and So, 2000). Translocation of PilE through the outer membrane occurs via channel formation, which involves PilD and PilQ (described below) and various other molecules such as the lipoprotein PilT (described below), PilF, PilM, PilO and secretins (Parge *et al.*, 1995; Merz and So 2000; Carbonnelle *et al.*, 2006). Tfp are assembled through the action of PilD and the minor pilus subunits PilF and PilM-O (Carbonnelle *et al.*, 2006). Deletion mutants of *pilD*, *pilF* and *pilM-O* are non-piliated but expression levels of PilE are unaffected in these mutants, indicating a defect in pilus assembly rather than in pilus subunit expression (Carbonnelle *et al.*, 2006).

PilT (NMB0052), also named PilT1, has ATPase activity and is present as hexamers (Satyshur *et al.*, 2007) in the inner membrane and the cytoplasm. Presence of ATP is essential for PilT function (Morand *et al.*, 2004). PilT has been described to be involved in twitching motility and DNA uptake (Pujol *et al.*, 1999). During twitching motility, PilT degrades the retracted pilus into its pilin subunits, which are recycled into new fibres. Maier *et al.* (2002 and 2004) demonstrated the function of PilT in twitching motility of *N. gonorrhoeae* through ATP dependent pilus retracting and disassembling. Subsequently the pilus is re-assembled by the prepilin peptidase PilD and the pilin polymerizing subunit PilF (Maier *et al.*, 2002, 2004; Carbonnelle *et al.*, 2005). The retraction force, which drives twitching motility, can reach 100 piconewtons (Merz *et al.*, 2000; Merz and Forest, 2002; Morand *et al.*, 2004).

PilT has been shown to be involved in the intimate attachment of meningococci to epithelial cells (Pujol *et al.*, 1999). Besides having lost the ability to take up DNA, a *pilT*- deletion mutant could not spread on the host cell surface and form intimate attachment. The *pilT*- deletion mutant expressed pili at all stages of infection in contrast to the wild type where pili could not be detected after the initial adherence. These results suggest that PilT is involved in intimate adherence and in the loss of pili at later stages of infection (Pujol *et al.*, 1999). In addition to the above described functions of PilT, *pilT*-deletion in several

non-piliated pilus subunit deletion mutants has been shown to restore piliation (Carbonnelle *et al.*, 2006).

PilT2 (NMB0768) was assigned to have similar functions as PilT due to 60% sequence homology to PilT. Deletion mutants of *pilT* and *pilT2* were hyperpiliated indicating an antagonistic role of PilT and PilT2 in Tfp biogenesis. In contrast to the *pilT*-deletion mutant, which lost the ability to take up DNA, *pilT2*-deletion mutants remain competent (Brown *et al.*, 2010).

PilG (NMB0333) is an integral inner membrane protein present as a tetramer (Collins *et al.*, 2007), which is another protein involved in pilus assembly (Tønnum *et al.*, 1995) and retraction (Carbonnelle *et al.*, 2006). *PilG*- deletion mutants are non-piliated, but piliation is restored in a *pilG*-/*pilT*- double deletion mutant (Carbonnelle *et al.*, 2006). Spanning the inner membrane, it was suggested that PilG links ATP hydrolysis occurring in the cytoplasm to the disassembly of the pilus fiber in the periplasm during pilus retraction. PilG is apparently functionally connected to PilT (rather than to PilD as suggested by Tønnum *et al.*, 1995), a gene product with ATP-ase activity and involved in pilus retraction mediated by PilT (Collins *et al.*, 2007).

PilC is a pilus subunit with multiple functions, mainly in adherence to host cells (Pujol *et al.*, 1999; Scheuierpflug *et al.*, 1999) and in DNA uptake (Rudel *et al.*, 1995). PilC has two homologues, PilC1 (NMB1847) and PilC2 (NMB0049) encoded by two separate genes, both undergoing phase variation (Jonsson *et al.*, 1991). In mutants expressing PilC1 but not PilC2, bacteria are non-piliated, indicating that PilC2 is essential for piliation (Jonsson *et al.*, 1991, Pujol *et al.*, 1999). PilC1, suggested to be located on the pilus tip, has been shown to be involved in adherence to host cells, even in the absence of PilC2 (Pujol *et al.*, 1999). PilC2 has been shown to be involved, alongside PilE, in DNA uptake (Rudel *et al.*, 1995).

PilQ (NMB1812) is a conserved integral transmembrane protein, which functions as a secretin. PilQ forms a gated pore in the outer membrane by aggregation of 12 subunits (homopolymer) with rotational symmetry. PilQ is essential for pilus translocation: meningococcal *pilQ*-deletion mutants do not express

pili (Collins *et al.*, 2001; Collins *et al.*, 2004) on the surface. Pilins have been found to be trapped under the outer membrane (Carbonnelle *et al.*, 2005). PilQ has also been shown to bind foreign DNA at the N-terminal region with a preference for single-stranded DNA (ssDNA) over double-strand DNA (dsDNA). The presence of the neisserial uptake sequence (Assalkhou *et al.*, 2007), a 10 bp long signal sequence (5' – GCCGTCTGAA – 3') necessary for efficient uptake of transforming DNA and initially described in *N. gonorrhoeae* (Goodman and Scocca 1988), is not required for DNA binding by PilQ (Carbonnelle *et al.*, 2005).

PilP (NMB1811) is a lipoprotein located in the inner membrane, which interacts with PilQ. PilP has a signal sequence at the conserved, folded C-terminal end, which contains a lipo-box and is thought to play a role in PilQ recognition. The N-terminus contains a lipidated cysteine residue and interacts with the central part and the N-terminus of PilQ. Localization of PilP in the inner membrane is PilQ dependent, in *pilQ*- deletion mutants PilP undergoes proteolytic cleavage (Balasingham *et al.*, 2007).

Although the exact function of PilP remains to be determined, it is thought to play a role in pilus assembly and retraction due to its interaction with PilQ. *PilP*- deletion mutants are non-piliated and non-competent. *PilP* is located upstream of, and co-transcribed with, *pilQ*. *PilP*-deletion mutants express lower levels of PilQ than wild type meningococci (Balasingham *et al.*, 2007).

PilX (NMB0890) is a minor pilus subunit present at low concentration in the bacterium. It is located on the pilus surface (along the shaft; Figure 1.3) and *pilX*- deletion mutants are unchanged in pilus morphology compared to wild-type bacteria but have impaired adherence to host cells. *PilX*- deletion mutants were not able to form bacterial aggregates (Helaine *et al.*, 2005 and 2007; Lappann *et al.*, 2006). DNA uptake and motility of meningococci were not found to be altered in *pilX*- deletion mutants compared to wild type meningococci. In *pilX-pilT*- double deletion mutants bacterial aggregation was restored (Helaine *et al.*, 2005 and 2007).

In summary, Tfp are, besides the capsule, one of the most important adhesins and virulence factors of *Neisseria* with multiple functions. Being expressed on the bacterial surface at high levels, pili or pilus

subunits might be suitable vaccine candidates. However, pilus subunits such as PilE and PilC are ruled out through their phase variability, but the minor pilus subunits ComP, PilX and PilV have been found to be highly conserved among 56 strains, suggesting that they merit further consideration (Cehovin *et al.*, 2010 and 2011).

1.6.3.1. Human CD46 – a host cell receptor for meningococcal Tfp

The human membrane cofactor protein or complement regulator protein (CD46) has been described to be a receptor for *Neisseria* Tfp (Källström *et al.*, 1997). CD46 is a 60 – 65 kDa protein which can be found on all human cells except erythrocytes (Liszewski *et al.*, 1991; Hakulinen *et al.*, 2004). Due to differential splicing, CD46 exists as two isoforms (Russel *et al.*, 1992), the membrane bound (59-68 kDa) and the soluble form (51-58 kDa) (Liszewski *et al.*, 1991; Russell *et al.*, 1992, Hakulinen *et al.*, 2004). The relative prevalence of these isoforms in an individual is genetically determined (Ballard *et al.*, 1987).

CD46 is comprised of four homologous peptide domains directly involved in complement regulation at the N-terminus, followed by a serine-threonine-proline rich region, a short region of unknown function and a transmembrane domain. The C-terminus is composed of a cytoplasmic domain (Liszewski *et al.*, 1991; Hakulinen *et al.*, 2004).

Quantitative analyses have shown that human epithelial carcinoma cell lines have higher levels of CD46 than various blood cells. HeLa and Hep-2 cells have about 100,000 molecules/cell and 250,000 molecules/cell respectively (Liszewski *et al.*, 1991). High concentrations of CD46 are also found on cells forming the blood-brain-barrier (Shusta *et al.*, 2002).

CD46 functions as cofactor of the complement inhibitor plasma serine protease factor I and binds directly to C3b and C4b of the complement system (Liszewski *et al.*, 1991; Hakulinen *et al.*, 2004). As well as *N. meningitidis* (Kallström *et al.*, 1997), other bacteria and viral human pathogens, such as

Streptococcus pyogenes (Okada *et al.*, 1995) and the Measles virus (Santoro *et al.*, 1999), use CD46 as host cell receptor.

It has been found that Tfp mediated adherence of *N. meningitidis* to human epithelial cells increases intracellular free Ca^{2+} (Källström *et al.*, 1997). In turn, the level of adherence increases with increasing intracellular free Ca^{2+} . It is thought that PilC1 triggers Ca^{2+} release as it could not be observed in *pilC1*-deletion mutants. Antibodies against CD46 were able to prevent the induction of the Ca^{2+} gradient and inhibited formation of tight adherence to host cells, suggesting that a host cell signal transduction is mediated upon pilus – CD46 interaction (Källström *et al.*, 1998).

1.6.4. Meningococcal outer membrane proteins

Meningococcal OMP were, in the pre-genomic era, assigned into class one to five, according to their migration pattern on SDS PAGE gels. Today known as major outer membrane porins PorA and PorB, RmpM and the opacity proteins Opa and Opc are the class 1, 2/3, 4 and class 5 OMP, respectively (Heckels, 1977, 1978, 1981; Klugman *et al.*, 1989; van der Ley *et al.*, 1991; Virji *et al.*, 1992b).

PorA (NMB1429) and **PorB** (NMB2039) are members of the porin family (Wright *et al.*, 2002) which are transmembrane proteins with conserved domains composed of beta sheets, forming a pore (Figure 1.1), and variable loops exposed at the bacterial surface which are immunogenic (Van der Ley *et al.*, 1991). In a three dimensional structure model of PorA (Derrick *et al.*, 1999), it was suggested that the protein forms trimeric pores in the outer membrane of meningococci. Eight loops emerge on the bacterial surface each with a different length and position. Six of the eight loops show sequence variation, while two (loop II and III) are apparently conserved among *Neisseria* species. Loop II is involved in formation and stability of PorA trimers and loop III is located in the centre of the pore, suggesting a possible role in pore function (Derrick *et al.*, 1999). Loops I and IV, also known as variable region (VR) 1 and VR2 (Derrick *et al.*, 1999), respectively, are used to classify *Neisseria* strains in to serosubtypes (Abdillahi *et al.*, 1988).

PorA and PorB have been investigated as vaccine candidates (Abdillahi *et al.*, 1988; Van der Ley *et al.*, 1991; Sacchi *et al.*, 2001; Fletcher *et al.*, 2004). PorA induces a protective immune response but due to its variability this is essentially strain-specific (Fletcher *et al.*, 2004; Sacchi *et al.*, 2001). The level of PorA expression varies in different strains and was found to be proportional to the level of the protective immune response elicited (Brodeur *et al.*, 1985; Guttormsen *et al.*, 1994). With a mutant containing class 1 outer membrane proteins of two different serosubtypes, Van der Ley *et al.*, (1992) attempted to produce a multivalent vaccine, which was shown to be protective in mice. Protective antibodies against the two serosubtypes present in the vaccine were produced (Van der Ley *et al.*, 1992). A hexavalent PorA OMV vaccine was developed which shows broader cross-protection than the monovalent vaccine (Vermont *et al.*, 2003). NonaMen, a nine-valent PorA vaccine gives protection (in mice) against nine serosubtypes (Van den Dobbelen *et al.*, 2007).

PorB (NMB2039) forms, like PorA, a trimeric β -barrel in the bacterial outer membrane and several loops on the bacterial surface (Jadhav *et al.*, 2008; Tanabe *et al.*, 2010). The β -barrel is composed of 16 β -sheets. Like PorA, loop III of PorB is located in the center of the pore (Tanabe *et al.*, 2010). PorB forms voltage dependent pores in the meningococcal outer membrane which are selective for sugars, anions and cations (Jadhav *et al.*, 2008; Tanabe *et al.*, 2010).

PorB is immunogenic and able to stimulate B cells and inhibit their apoptosis (Massari *et al.*, 2000). The latter is mediated by binding of PorB to the voltage-dependent anion channel (VDAC) of mitochondria, mimicking the binding of Bcl2 proteins to prevent cell death. Binding of the VDAC by PorB inhibits cytochrome c release, a molecule which activates the apoptotic cascade. The transport of PorB through the host cell is still unclear (Massari *et al.*, 2000).

The immunomodulating feature of PorB mediated B cell activation is thought to increase the immune response against meningococci (Massari *et al.*, 2000). It has been suggested that meningococcal PorB be used as an adjuvant for vaccines (Burke *et al.*, 2007). PorB binding to the TLR-2 ligands induces a strong immune response involving both, humoral and cellular components. High immunogenicity and the TLR-2

binding make PorB a feasible adjuvant candidate (Burke *et al.*, 2007). Wright *et al.*, (2002) discovered that recombinant (r) PorB embedded in liposomes and micelles induce a protective antibody response in mice and rPorB antisera induced complement-mediated elimination of bacteria *in vitro*.

The **class 4 OMP** of *N. meningitidis* (NMB0382) has structural homology to the gonococcal protein III and to the *E. coli* OmpA. It was termed the 'Reduction modifiable protein of the meningococcus' (RmpM) by Klugman *et al.*, (1989). OMP induces bactericidal antibodies and is present in most OMV vaccines, such as the one used in Norway (Rosenqvist *et al.*, 1999; Klugman *et al.*, 1989).

Opa (NMB0442, NMB0926, NMB1465, NMB1636) and **Opc** (NMB1053) belong to the class 5 OMP (Virji *et al.*, 1992b). Opa proteins are a family of transmembrane proteins with eight membrane spanning domains forming an intramembrane β barrel (Malorny *et al.*, 1998). The extracellular domain consists of four surface-located loops, the two central ones being hypervariable (HV-1 and HV-2). The C-terminal loop is conserved, whereas the N-terminal loop is semivariable (SV) (Malorny *et al.* 1998). Opa proteins are additional adhesins to Tfp. In encapsulated strains, the Opa proteins are masked by the capsule and it is believed that they function as adhesins and invasins only in acapsulate strains or if the capsule is downregulated (Virji *et al.*, 1993, 1996a; McNeil *et al.*, 1994; Merz and So, 1997). Opa proteins are expressed in *N. meningitidis*, *N. gonorrhoeae* and commensal strains such as *N. lactamica* and are responsible for the development of the opaque colony phenotype (Swanson 1978). Meningococcal strains have 3-4 *opa* loci, while gonococci have up to 12 (Kupsch *et al.*, 1993; Aho *et al.*, 1991; Tettelin *et al.*, 2000). *Opa* loci are subject to phase variation due to pentanucleotide repeats in the 5' domain encoding an N-terminal signal peptide (Morelli *et al.*, 1997; Hobbs *et al.*, 1998). All of the four *opa* loci in MC58 are phase variable (Tettelin *et al.*, 2000) and three (NMB0442, NMB1465 and NMB1636) have a frame shift resulting potentially in ineffective expression (Tettelin *et al.*, 2000). The expressed Opa variant in gonococci is used to trace contact in infections (O'Rourke *et al.*, 1995). However, complications due to variations in gonococcal *opa* genes were reported (Bilek *et al.*, 2009).

During *in vitro* interaction with host epithelial cells, Opa proteins bind heparin sulphate proteoglycans (HSPG) and up to four different CD66 molecules (CD66a, -c, -d and -e) located on the host cell surface (Virji *et al.*, 1996a and 1999; Bos *et al.*, 1998).

Opc is an OMP consisting of an amphipatic β -strand which spans the outer membrane 10 times and forms five surface-exposed hydrophilic and four periplasmic exposed loops (Merker *et al.*, 1997).

Monoclonal antibodies directed against the surface-exposed loops, which contain T-cell stimulating motifs, induce complement mediated killing. The Norwegian serogroup B OMV vaccine, being protective against high-level Opa-expressing serogroup A and B strains, contains Opc proteins (Rosenqvist *et al.*, 1993 and 1995). Opc is one of the major meningococcal adhesion and invasion proteins, which are not present in the closely related *N. gonorrhoeae* (Virji *et al.*, 1992b and 1993; Seiler *et al.*, 1996). The gene encoding *opc* has multiple cytidine residues between the -10 promoter sequence and the start codon. Variations in the number of these cytidine residues alters promoter binding efficiency, causing phase variation of the Opc proteins with the most effective *opc* expression when 12- 13 cytidine residues are present (Sarkari *et al.*, 1994).

1.6.5. Factor H binding protein (FHBP)

FHBP (NMB1870) is a surface expressed lipoprotein which protects meningococci from complement-mediated killing by binding to complement factor H (fH). FHBP forms two β -barrels with a connecting loop and interacts with fH domains 6 and 7 by forming hydrogen bonds (Cendron *et al.*, 2011). fH down-regulates the alternative pathway and interacts with the complement protein C3b (Weiler *et al.*, 1976).

A total of 29 gene variations (resulting in 28 amino acid variants) are known, clustered into three main sequence variants (Jacobsson *et al.*, 2006). Bambini *et al.* (2009) assigned the three variants into two groups. Consistent in both studies was that expression of *fHbp* was shown in all strains tested. However, the number of FHBP variants might be much higher. Marsh *et al.*, (2011) described 30 protein sequences

in 408 carriage isolates. By sequence typing, 423 amino acid sequences and 498 alleles of FHBP and *fHbp*, respectively, were found (Neisseria Multi Locus Sequence Typing website (<http://pubmlst.org/neisseria/fHbp>), checked on 22-6-2011). Expression levels of *fHbp* were different between strains (Fletcher *et al.*, 2004; Jacobsson *et al.*, 2006; Bambini *et al.*, 2009; Brunelli *et al.*, 2011). Strains isolated from invasive disease cases were found to have either lost the *fHbp* gene or carry a mutation resulting in failure to express FHBP. These observations have raised questions about the likely long-term efficacy of the vaccines relying on FHBP (Lucidarme *et al.*, 2010). FHBP is a component of two serogroup B vaccines currently in clinical trials (described in more detail in section 1.7).

1.6.6. The Neisseria surface protein A (NspA)

NspA (NMB0663) is structurally similar to PorA and PorB. A β -barrel composed of eight strands is embedded in the outer membrane forming four surface exposed loops. In contrast to PorA and PorB, NspA does not form a pore and the surface exposed loops are highly conserved among *Neisseria* strains (Vandeputte-Rutten *et al.*, 2003). NspA has amino acid sequence homology to neisserial Opa proteins, suggesting a possible similarity in structure of NspA and Opa proteins. Although the function of NspA is unclear, Vandeputte-Rutten *et al.* (2003) suggested a potential role of the protein in host cell invasion by binding membrane lipids at the hydrophobic residues of the surface exposed region of the protein. NspA also binds fH (Lewis *et al.*, 2010).

1.6.7. The Neisseria adhesin A (NadA)

NadA (NMB1994) is a surface expressed oligomerizing protein which was detected in a genome-wide study by Pizza *et al.* (2000) as potential vaccine antigen and has the structure of a trimeric autotransporter (Taa) (Cotter *et al.*, 2005). The C-terminal contains an outer membrane anchoring domain (Comanducci *et al.*, 2002; de Cock *et al.*, 1997). A leader peptide is present in the N-terminal

domain. The α helix in the middle-part of the protein contains coiled-coil structures with leucine zipper domains (Comanducci *et al.*, 2002) similar to UspA of *Moraxella catarrhalis* (Chen *et al.*, 1999) and YadA of *Yersinia pseudotuberculosis* (Bliska *et al.*, 1993; Eitel *et al.*, 2002). UspA is thought to induce immunity against *M. catarrhalis* (Chen *et al.*, 1999) and YadA, also known as protein 1 (Bliska *et al.*, 1993), is an outer membrane protein which mediates adhesion to host cells (Eitel *et al.*, 2002). The structure of UspA, YadA and NadA was suggested to be that of a Taa (Cotter *et al.*, 2005).

The gene *nadA* is 1086 bp (encoding 362 aa) long with a G + C content (45%) lower than average of the meningococcal genome (51.5 %), suggesting that it may have been acquired by horizontal gene transfer. Upstream of *nadA* there are nine repeats of the tetranucleotide TAAA which might contribute to phase variation of the gene (Tettelin *et al.*, 2000; Comanducci *et al.*, 2002). The *nadA* gene is present in many hypervirulent strains, rarely in carrier strains and absent in *N. cinerea*, *N. lactamica* and *N. gonorrhoeae* (Comanducci *et al.*, 2002 and 2004). Although sequence-variation in the domain encoding the coiled-coil structure has been reported, the *nadA* gene was thought to be conserved between strains suggesting it is not under selective pressure (Comanducci *et al.*, 2002). Four alleles of *nadA* are known of which *nadA1-3* are found in (hyper)virulent strains and *nadA4* solely in carrier strains. However, only 50% of the strains carry any of the *nadA* alleles (Comanducci *et al.*, 2004) and only 7% of the strains predominant in the UK express NadA (S. Kroll, pers. comm.).

NadA expression increases during the exponential growth of meningococci and is highest during stationary phase. NadA has been described as an adhesin since purified recombinant NadA bound to Chang cells *in vitro* (Comanducci *et al.*, 2002). MC58 *nadA*-deletion mutants have reduced adherence to Chang cells compared to wild-type bacteria, while expression of *nadA* in *E. coli* increased adherence and invasion to Chang cells. The host cell binding domain of NadA was shown to be in the N-terminal globular part (Capecchi *et al.*, 2005; Tavano *et al.*, 2011).

1.6.8. The Neisserial heparin binding protein (NHBP)

NHBP (NMB2132) has heparin binding activity, which has been speculated to enhance the survival of meningococci in the presence of components of human serum. Vaccination with a recombinant NHBP containing vaccine has been shown to induce bactericidal antibodies in humans. Anti-NHBP antibodies have also been found in patients recovering from invasive meningococcal disease (Serruto *et al.*, 2010). NHBP is a component of a serogroup B vaccine currently in clinical trials (described in more detail in section 1.7).

1.6.9. Macrophage infectivity potentiator-related protein

NMB0995, a macrophage infectivity potentiator-related protein has recently been named NafA for *Neisseria* anti-aggregation factor A (Kuwae *et al.*, 2011). NMB0995 is a surface expressed and sequence conserved protein of 19 kDa and has been found to induce bactericidal antibodies in a mouse model of invasive meningococcal disease (Grifantini *et al.*, 2002; Kuwae *et al.*, 2011). Using immunostaining, protein expression of NMB0995 was found to be up-regulated at 6 h post infection (p.i.) of FaDu cells (a human nasopharyngeal carcinoma cell line), but not at 2 h p.i., suggesting a role in prolonged host cell interaction (Kuwae *et al.*, 2011). A *nafA*-deletion mutant showed increased bacterial aggregation and pilus bundle formation. Expression of Pile in the mutant strain was found to be up-regulated compared to the wild type strain. In a human CD46-transformed mouse model of meningococcal disease, the *nafA*-deletion mutant showed impaired virulence compared to the wild type. These results support a role for NafA in both, host cell association and invasive disease (Kuwae *et al.*, 2011).

In meningococcal – host cell association transcriptome studies *NMB0995* has been found to be up-regulated (Grifantini *et al.*, 2002; Dietrich *et al.*, 2003) whereas the expression during a whole blood model of meningococcaemia remains unclear. Up-regulation was observed by Hedman (2009) but down-regulation by Echenique-Rivera *et al.* (2011).

1.6.10. The *Neisseria hia* homolog A (Nhha)

Nhha (NMB0992) is a conserved outer membrane protein recently characterized as a Taa (Scarselli *et al.*, 2006). As the name '*hia* homologue' indicates, it appears to be closely related to the *H. influenzae* Taa (Hia) and to the *Haemophilus* surface fibrils (Hsf), as well as to other bacterial proteins involved in host cell adhesion such as the AIDA-I of *E. coli*, UspA of *M. catarrhalis* and YadA of *Yersinia* spp. and the *Shigella flexneri* SepA invasin (St. Geme *et al.*, 1996; Peak *et al.*, 2000; Scarselli *et al.*, 2006).

Nhha is present on the bacterial surface as trimers but also dimers (Scarselli *et al.*, 2006). The protein is composed of a conserved C-terminal translocator, an adjacent passenger domain and an N-terminal leader peptide, all of which are characteristic of Taa. Similar to YadA, the neisserial translocator domain consists of a β region and the linkers, L1 and L2. The β domain, predicted to have four β strands after an α -helix, is involved in embedding the protein into the outer membrane and oligomerization on the bacterial surface. The secondary structure, four β strands and an α -helix, can form a 12- stranded beta-barrel in the outer membrane to secrete the passenger domain (Scarselli *et al.*, 2006). The linker has a transmembrane (L1) and a surface exposed region (L2) with the L1 being involved together with the β domain in the translocation process of the passenger domain (Scarselli *et al.*, 2006).

Within the passenger region of Nhha, two putative heparan-sulphate binding sites (HBS) were observed (Scarselli *et al.*, 2001, 2006) with sequence similar to the HBS of the human N-CAM, a molecule involved in cell-cell recognition via binding the extracellular matrix (ECM) components heparin and heparin-sulfate proteoglycans.

In experiments *in vitro*, Nhha promoted adherence to cultured epithelial cells and was up-regulated at 2 h p.i. (Scarselli *et al.*, 2006; Sjölander *et al.*, 2008a). In a CD46 transgenic mouse model, Nhha was shown to have a role in nasopharyngeal colonization, inhibition of bacterial phagocytosis and facilitated survival in the blood stream due to increased complement resistance (Sjölander *et al.*, 2008a).

1.6.11. Adhesion and penetration protein (App)

App (NMB1985) is a homologue of the *H. influenzae* adhesion and penetration protein (Hap), the meningococcal IgA1 protease (encoded by *iga*), and to the *E. coli* haemoglobin protease Tsh (van Ulsen *et al.*, 2001). App belongs, as NhhA, to the family of autotransporter proteins and is surface expressed. In contrast to NhhA, App has serine protease activity which is associated with the release of the protein into the culture supernatant (Hadi *et al.*, 2001; Van Ulsen *et al.*, 2001; Serruto *et al.*, 2003). The conserved serine protease motif is located at the amino terminal end and cleaves the protein at two alternative sites of the passenger domain (Serruto *et al.*, 2003). The C-terminus forms β -strands which span the outer membrane in form of a β -barrel. App mediates host cell attachment via interaction with β -integrins (Hadi *et al.*, 2001).

App has been found in all tested strains and does not undergo phase variation. App is expressed during both prolonged colonization and invasive disease (Hadi *et al.*, 2001; Serruto *et al.*, 2003). App is T-cell stimulating and induces bactericidal antibodies which make the protein a promising vaccine candidate (Hadi *et al.*, 2001; Serruto *et al.*, 2003).

1.6.12. Meningococcal serine protease A (MspA)

MspA (NMB1998), a 157 kDa protein, is a member of the meningococcal autotransporter family and is closely related to the meningococcal IgA1 protease and App. MspA was reported to be present in ST32 and ST-41/44 but not in ST-8 strains, serogroup A (Z2491), *N. gonorrhoeae* (FA1090) and in the sequenced *N. lactamica* (ST-640) genome. The serogroup C strain FAM18 contains a 180 bp insertion in the *mspA* gene and a single nucleotide mutation resulting in an early stop codon (Turner *et al.*, 2006).

In contrast to App, which is thought to be conserved and constantly expressed during colonization and during invasive disease, MspA has a poly-cytidine region which is associated with phase variation (Turner *et al.*, 2006).

MspA is, like App, cleaved at the bacterial surface (into 125 and 95 kDa fragments) and secreted into the culture medium and is involved in bacterial adherence to host cells. Anti-MspA- as well as anti-App antibodies are present in sera from patients convalescent after meningococcal sepsis. Rabbit anti-MspA serum had bactericidal activity to the majority of tested strains. However, strains Z6421 and Z4242 (ST41/44 and ST11, respectively) were not killed, although they expressed MspA (Turner *et al.*, 2006). Although inducing bactericidal antibodies against some strains, MspA is unlikely to be a vaccine candidate as it is not present and able to induce bactericidal antibodies in all tested strains (Turner *et al.*, 2006).

1.7. Meningococcal vaccines

1.7.1. Vaccines based on capsular polysaccharides for serogroups A, C, W135 and Y

Vaccines based on the meningococcal polysaccharide capsule, have been developed to prevent disease caused by serogroup A, C, W135 and Y strains. However, as purified polysaccharides of serogroup A and C did not induce a long lasting protective immune response in children and adults, and are ineffective in infants under two years of age (Gold *et al.*, 1975 and 1977; Reingold *et al.*, 1985), alternative strategies for disease prevention had to be developed. Following the success of *Haemophilus influenzae* type b (hib) protein-polysaccharide conjugate vaccines in generating robust long-standing immunity and preventing Hib disease in the youngest infants as well as inducing a herd immunity by reducing nasopharyngeal carriage (Michaels *et al.*, 1976; Granoff and Daum, 1980; Li *et al.*, 1986; Barbour *et al.*, 1995; Heath, 1998). The same approach led to introduction of serogroup C meningococcal conjugate vaccines into the UK vaccine schedule in 1999 (Fairley *et al.*, 1996; Richmond *et al.*, 1999; Borrow *et al.*, 2000; Miller *et al.*, 2001). These vaccines induce protective immune responses in infants and older individuals, and have led to a massive reduction in serogroup C meningococcal disease. Present issues are the design of an optimal schedule to maintain protection throughout life (MacLennan *et al.*, 2000; Trotter *et al.*, 2004). A quadrivalent protein-polysaccharide conjugate vaccine protective against

serogroup A, C, Y and W-135 has been licensed in the USA and Europe and is part of the US immunization schedule for adolescents (Pace *et al.*, 2009 and 2010); the vaccine developed by Baxter *et al.* (2011) will soon be available.

In 2010, a mass vaccination with a conjugated serogroup A vaccine was administered in the meningitis belt of sub-Saharan Africa. Since then, serogroup A meningococcal disease cases in this region are low and the vaccine is potentially able to prevent 10,000 deaths per year (Bishai *et al.*, 2011; LaForce and Okwo-Bele 2011).

1.7.2. Serogroup B vaccines based on OMV and OMPs

With the realization that a vaccine containing the serogroup B capsule-antigen is unlikely to protect against infection as it is composed of α -2,8-linked sialic acid which is also present in the human body, and therefore poorly immunogenic, many other potential vaccine candidates particularly surface exposed structures have been investigated. These include the serosubtyping molecule PorA, (Brodeur *et al.*, 1985; Vermont and Van den Dobbela, 2003; Van den Dobbela *et al.*, 2007), the class 2/3 outer membrane protein PorB (Burke *et al.*, 2007, Massari *et al.*, 2000), the Neisserial surface protein A (NspA NMB0663) (Martin *et al.*, 1997; Moe *et al.*, 2001), the surface exposed protein NadA (NMB1994) (Comanducci *et al.*, 2002 and 2004) and others (Fletcher *et al.*, 2004; Madico *et al.*, 2006; Pizza *et al.*, 2000).

A characteristic of the Gram-negative bacterial envelope, in particular in meningococci, is the formation of outer membrane vesicles (OMV), natural membrane blebs released by the bacteria. OMV represent an important mechanism by which bacteria interact with their surrounding environment and their production has been described as a 'Type VI' secretion system (Kuehn *et al.*, 2005). OMV contain different metabolic components such as proteins (including adhesins and invasins), enzymes and lipids (Kuehn *et al.*, 2005). Vaughan *et al.* (2006) found *N. meningitidis* OMV contained proteins from

intracellular and periplasmic space proteins. LOS, the endotoxin, able to cause a severe immune reaction due to the induction of host TNF α and interleukins, is also present in native OMV. For development of an OMV vaccine, the LOS needs to be widely removed, for example by detergent treatment or by introduction of a mutation in the gene *lpxL1*, encoding the core endotoxin lipid A (Fredriksen *et al.*, 1991).

OMV containing vaccines have been developed and licenced in Norway (Bjune *et al.*, 1992), Cuba (Rodriguez *et al.*, 1999), France (Taha *et al.*, 2007) and New Zealand (Martin *et al.*, 2006) and have been successful in the control of outbreaks by specific meningococcal clones (Granoff *et al.*, 2001). The OMV vaccines can only induce strain-specific bactericidal antibodies mainly directed against PorA (Wedegge *et al.*, 1998; Massignani *et al.*, 2003; Martin *et al.*, 2006). Such a OMV vaccine (MenZB) against the strain causing an epidemic in New Zealand has been used which protects toddlers and children but not infants (Martin *et al.*, 2006). By using meningococcal strains, genetically engineered to produce several distinct PorA proteins, hexavalent and nonavalent vaccines have been developed (Claassen *et al.*, 1996; Van den Dobbelen *et al.*, 2007).

In another attempt to overcome the problem of strain-specific immunity, a vaccine composed of 14 different recombinant Opa variants has been developed and tested in a murine model. Bactericidal antibodies were induced by all 14 Opa variants indicating a potential broad coverage of the vaccine (Callaghan *et al.*, 2011). Although being cross protective against a strain collection of different clonal complexes, it is unlikely to induce serogroup B wide protection due to the high variability of Opa proteins. Phase variation, level of expression and the lack of some / all *opa* genes in some meningococcal strains negatively affect the effectiveness of an Opa based vaccine (Callaghan *et al.*, 2011).

1.7.3. Serogroup B vaccines based on LOS

LOS has been studied as a meningococcal vaccine candidate in the past and is still under investigation (Verheul *et al.*, 1993; Quakyi *et al.*, 1999; Plested *et al.*, 2003; Mieszala *et al.*, 2003; Weynants *et al.*, 2009; Bonvehi *et al.*, 2010; Keiser *et al.*, 2011). A complication of LOS as a vaccine candidate is the content of endotoxin (lipid A), which induces severe side effects such as pain, fever and fatigue (Brandtzaeg *et al.*, 1989b and 2002; Bonvehi *et al.*, 2010). LOS detoxification by deletion of the lipid A moiety (deletion of the *lpXL1* gene) in combination with deoxycholate treatment of the OMV has been used in the vaccine developed by Glaxo Smith Kline (Weynants *et al.*, 2009). This vaccine contains modified L3,7 and L2 LOS expressed in meningococcal OMV. In preclinical studies using mice, cross-protection against immunotypes of different serogroups was shown. However, limitations of LOS as vaccine candidate are, besides the complications derived from the lipid A content, the structural similarity of the lacto-*N*-neotetraose, a tetrasaccharide of the L7 immunotype, to major human blood group antigens. This similarity might confer a risk of developing auto immunity (Mandrell *et al.*, 1988; Weynants *et al.*, 2009). In contrast to the results obtained from mouse immunizations, in a phase I clinical study with 150 healthy adult volunteers, the vaccine (composed of different L3 derived LOS expressed in OMV) did not induce a significant increase in human serum bactericidal antibodies (hSBA) titers, which abrogated further investigations (Bonvehi *et al.*, 2010).

The Walter Reed Army Institute of Research tested a native OMV vaccine (NOMV) in phase I clinical trials. NOMV is composed of native genetically modified OMV of the serogroup B strain 8570. The genes *lpXL1* and *synX*, encoding for the lipid A of LOS and the capsule biosynthesis protein respectively, were deleted. To achieve broader protection, variations of PorA factor H binding protein and opacity protein A were added to the vaccine. PorA, factor H binding protein and the opacity proteins (in detail described in sections 1.6.4. and 1.6.5.) are immunogenic but variable proteins expressed on the meningococcal surface. Immunogenicity was observed against most strains tested. The authors suggested that introducing additional PorA and factor H binding protein variants and immunotypes could further

broaden protection against additional strains of serogroup B (Keiser *et al.*, 2011). The presence of LOS was described to be crucial for induction of long lasting and cross-serogroup protective immunity (Keiser *et al.*, 2011).

1.7.4. Vaccine candidates developed from reverse vaccinology

With the completion of sequencing the *N. meningitidis* genome (Tettelin *et al.*, 2000), a new concept, termed 'reverse vaccinology' by Rappuoli (2000), was proposed to identify promising candidate vaccine antigens, i.e. surface expressed structures. Reverse vaccinology is a means of rapid DNA sequence screening *in silico* to identify potential (novel) vaccine candidates based on sequence motifs such as those, for example, predicting surface location (Rappuoli 2001). Meningococcal vaccine candidates found by reverse vaccinology include lipoproteins (Masignani *et al.*, 2003; Pizza *et al.*, 2000; Fletcher *et al.*, 2004) and some OMPs (Fletcher *et al.*, 2004; Sacchi *et al.*, 2001) such as NadA, FHBP and NHBP. Two vaccines against strains of serogroup B meningococci are currently in clinical trials (Jiang *et al.*, 2010; Toneatto *et al.*, 2011). Developed by Novartis, the four component vaccine contains NadA, FHBP, NHBP (rMenB) and the OMV used in the New Zealand vaccine (4CMenB or Bexsero). For protein stability, NHBP and FHBP were linked to two other antigens, NMB2091 and NMB1030, respectively. In phase I and II, 4CMenB showed better results than rMenB and is currently in phase III clinical trials (Toneatto *et al.*, 2011). Human serum-bactericidal antibodies (HSBA) against a collection of 15 serogroup B strains were detected post immunization with 4CMenB in healthy adults (Toneatto *et al.*, 2011). 4CMenB is currently investigated in infants. Two doses of the vaccine at 13 and 15 month of age provided protective immunity (determined by HSBA) against three reference strains, expressing each of the NadA, FHBP and NHBP variant (Prymula *et al.*, 2011).

Developed by Pfizer, the other serogroup B vaccine currently in trials contains bivalent FHBP (Jiang *et al.*, 2010). HSBA against 36 (of 45) serogroup B strains were detected post immunization. Successful immunity was connected to the level of surface expression but not to the variant of FHBP among the strains tested (Jiang *et al.*, 2010).

Both vaccines developed by Novartis and Pfizer show promising results by inducing broader protection of various serogroup B strains than the OMV vaccines licensed in Norway and New Zealand. However, complications such as variations in the vaccine antigens and level of expression contribute to the limited protection of both vaccines against a number of serogroup B strains. In contrast to vaccines based on the capsule, as developed against serogroups A, C, W135 and Y, so far no alternative surface structure has been discovered which would give universal protection against all strains of serogroup B.

1.8. Models to study meningococcal colonization

As meningococci only colonize the human nasopharynx and therefore exclusively interact with human cells, there is a limited choice of suitable models to study meningococcal colonization and disease. Human cell lines, cultured tissue explants and animals have been used as models for meningococcal - host interaction. Additionally, simple and easy to use abiotic experimental systems involving flow cells (glass surface) and Sorbarod filters (cellulose surface) have been used to study meningococcal biofilm formation.

1.8.1. Cell lines and cultured primary cells

Most cell lines are immortalised by transformation of a viral gene disrupting the signal pathways leading to the avoidance of cellular senescence. For example, the human bronchial epithelial cell line 16HBE14 was constructed by transformation of human tracheal epithelial cells with the large T antigen of simian virus 40 using a plasmid (pSVori-), resulting in inactivation of p53, a gene involved in induction of cell cycle arrest and apoptosis (Gruenert *et al.*, 1988). If not artificially immortalised, cell lines are frequently derived from cancer tissue of the target organ resulting in easy to culture and fast growing cell lines. Cultured cell lines are usually non-differentiated meaning that the *in vivo* phenotype of the desired tissue is not maintained. For example, ciliated or mucus producing cells are rarely found in cultured

human nasopharyngeal epithelial cell cultures. Cell lines have been extensively used for investigating meningococcal – host epithelial interactions, for example, A549 (adenocarcinomic human alveolar basal epithelial cells; Virji *et al.*, 1996b), COS (green monkey kidney cells COS; Virji *et al.*, 1996a), HT29 (the human colon adenocarcinoma grade II cell line; Virji *et al.*, 1996b) and HeLa (human cervical carcinoma cell line; Bos *et al.*, 1998) cells to study the interaction of meningococcal Opa proteins to human carcinoembryonic antigen cell related adhesion molecules (CAECAM), Hec-1B (human adenocarcinoma cell line; Nassif *et al.*, 1994) to study the interaction of meningococcal Tfp and host cells, and many others (reviewed by Hauck and Meyer, 2003; Carbonnelle *et al.*, 2009). HUVECs and HBMEC have been used to study meningococcal – host endothelial cell interaction and the crossing through the blood brain barrier, respectively (Nassif *et al.*, 1994; Virji *et al.*, 1994; Unkmeir *et al.*, 2002).

Primary human cells, which can maintain the phenotype of their tissue origin, have also been used in meningococcal studies. Fowler *et al.* (2006) used primary human meningeal cells to study the host immune response upon infection with *N. meningitidis* and *N. lactamica*. Human primary nasopharyngeal epithelial cells were used to study the changes in meningococcal surface components such as capsule, pili and LOS during invasion (de Vries *et al.*, 1996). Recently, MucilAir cells, an *in vitro* cultured human airway epithelium have become available commercially (www.epithelix.com), which may present new opportunities for meningococcal studies. However, their use has technical limitations, as culture is difficult for several reasons, including the need to remove the normal bacterial flora and fast growing fibroblasts from deeper tissue layers. In addition, availability of nutrients differs between *in vivo* and *in vitro* grown cells (Wiszniewski *et al.*, 2006).

1.8.2. Nasopharyngeal tissue explants

Cultured tissue explants have been used as models, which closely resemble *in vivo* conditions. In contrast to cell lines or primary cells, tissue explants are composed of several cell layers as found *in vivo* (Wiszniewski *et al.*, 2006). Their culture is complex and sources are limited and they are physically small,

limiting their use (Read *et al.*, 1995). Experiments on tissue explants may show donor (genetic and / or life style) dependent variations (Read and Goodwin, 2001). Therefore, a large number of biological replicates in studies are required. Read *et al.* (1995) and Exley *et al.* (2009) have used human nasopharyngeal tissue explants to study meningococcal colonization. Read *et al.*, (1995) described the morphological changes in meningococcal infected tissue explants over time recording mucus discharge, loss of cilia and membrane blebbing of the host epithelial layer were described. Exley *et al.* (2009) used the same model infected with a library of 576 meningococcal mutants to identify *pilE*, *NMB0103*, *NMB0313* and *NMB1829* as being essential for colonization. However, setting up explants is a complex process, sources are limited and they are physically small (<4mm²), constraining their use (Read *et al.*, 1995).

1.8.3. Animal infection models

Infant mice, rats, guinea-pigs, monkeys and chicken embryos have been used to study meningococcal colonization and disease, strain virulence and vaccine efficacy (Miller, 1933; Frasch and Robbins, 1978; DeVoe, 1982; Salit, 1984; Mackinnon *et al.*, 1992; Vogel *et al.*, 1996; Sun *et al.*, 2000). As none are the natural host of meningococci, there are limitations in their use in all cases. Infant mice and rats are the most widely used models. Lethality after intraperitoneal challenge of infant mice has been used to study meningococcal strain virulence (Miller, 1933), and supplementation with iron dextran or human transferrin has shown to increase lethality and development of fulminant meningococcaemia (Holbein 1980). After intranasal challenge, mice developed colonization in the lungs (not generally a feature of human meningococcal disease), which subsequently lead to meningococcaemia. Disease severity increased when iron dextran or human transferrin was injected intraperitoneally (Mackinnon *et al.*, 1992). Although infant mice developed meningococcaemia after intra nasal challenge, colonization of the nasopharynx (as occurs in humans), was observed throughout the experiment (three days) (Mackinnon *et al.*, 1992). To more closely model nasopharyngeal colonization, a humanised mouse model has been

developed. For example, Johansson *et al.* (2003) used a human CD46 transformed mouse to study meningococcal colonization and disease. In general, animal models are mostly useful in investigating the result (live, death, health score) of meningococcal colonization (through intranasal challenge) and are not convenient to study the process of colonization; more importantly, as the human is the only natural host of meningococci, there are limitations in the use of animal models.

1.8.4. Abiotic and biofilm models

1.8.4.1. Abiotic models

Abiotic surfaces such as glass and cellulose have been explored to study meningococcal colonization (Yi *et al.*, 2004; Lappann *et al.*, 2006; O'Dwyer *et al.*, 2009). Yi *et al.* (2004) were the first to describe biofilm formation of meningococci on a glass cover slip during static incubation in growth medium. More complex experimental models were developed by Lappann *et al.* (2006) and O'Dwyer *et al.* (2009) who used systems providing continuous flow of culture medium over a glass surface (Lappann *et al.*, 2006) and through a Sorbarod filter (O'Dwyer *et al.*, 2009).

1.8.4.2. Biofilm models

Using the definition of O'Toole *et al.* (2002), a bacterial biofilm is composed of converged microcolonies embedded in an extra cellular matrix and adhering to a surface.

N. meningitidis has been shown to form a biofilm on both abiotic (glass and cellulose), and biotic surfaces (epithelial and endothelial cell lines) (Grifantini *et al.*, 2002; Dietrich *et al.*, 2003; Yi *et al.*, 2004; Neil *et al.*, 2009; O'Dwyer *et al.*, 2009). The extracellular matrix of a meningococcal biofilm is relatively uncharacterized but has been reported to contain DNA (Lappann *et al.*, 2010), OMP and OMV (O'Dwyer *et al.*, 2009). Morphological and transcriptome analyses of meningococcal biofilms have been

undertaken. Yi *et al.* (2004) have demonstrated a difference in capacity of meningococcal carriage and invasive disease strains to form biofilms; the former is more able to do so.

Neil *et al.* (2009) investigated the mass of biofilms of strains NMB, MC58, C311 and capsule deletion mutants on monolayers of a human bronchial epithelial cell line (HBE), collagen coated and non-coated glass cover slips in a flow system. The presence of the capsule on biofilm forming wild type meningococci on HBE cells was confirmed by antibody staining (Neil *et al.*, 2009). On the other hand, Lappann *et al.* (2006) studied meningococcal biofilm formation in a flow system (glass surface) over 96 h in which it was found that only capsule negative strains were able to form a biofilm. Deletion mutants of *pilX* were described to be unable to form microcolonies but biofilm formation was not affected. In another meningococcal biofilm study, Lappann *et al.* (2010) described DNA as a component of the extracellular matrix.

1.9. The use of microarrays to study the meningococcal transcriptome during colonization

Since the complete genome sequences of whole organisms became available, methods such as microarrays have developed. Microarray analysis is a powerful tool to investigate the complete transcriptome of an organism under specific conditions in one experiment (Heller, 2002). Numerous studies have been published using microarray technology to analyse the meningococcal transcriptome (reviewed by Claus *et al.*, 2007). In the context of meningococcal – human epithelial interaction, Grifantini *et al.* (2002) studied the transcriptome of strain MC58 interacting with 16HBE14 cells over a three hour time period associating with the host cells. A profound change in the gene expression of meningococcal surface structures upon host cell contact was observed. Of the 189 up-regulated genes, five surface exposed genes (*NMB0315*, *NMB0652*, *NMB0995*, *NMB1119* and *NMB1876*), identified by FACS analysis, induced bactericidal antibodies in mice and were accordingly identified as potential vaccine candidates.

Dietrich *et al.*, (2003) compared the transcriptome of a *siaD*-deletion mutant of *N.meningitidis* strain MC58 at 6 h p.i. of human epithelial (HeLa) and endothelial (HBMEC) cell lines. Of the 72 and 48 differentially expressed genes upon infection with HeLa and HBMEC cells, 67 and 41 were found to be up-regulated, respectively. Capsule biosynthesis genes *siaB*, *siaA* and *ctrC*, *ctrD* were found to be up-regulated during epithelial infection in contrast to endothelial infection.

O'Dwyer *et al.* (2009) used a Sorbarod filter with a continuous flow of culture medium as an experimental system modelling colonization to study the transcriptome in meningococcal serogroup B strain MC58 over a 48 h biofilm-forming period. Many up-regulated genes were classified as cell wall/membrane biogenesis genes or genes encoding proteins found sequestered in OMV. The capsule and LOS genes were found to be down-regulated. Similarities were found by comparison to Grifantini *et al.* (2002) study. For example, the genes *NMB0586*, encoding for a putative adhesin, *NMB1279*, encoding for a putative membrane-bound lytic murein transglycosylase B and *NMB1533*, encoding for the OMP H.8 were found to be up-regulated in both studies.

Human nasopharyngeal colonization by meningococci can last for up to six months (Stephens *et al.*, 1999; Wilder-Smith *et al.*, 2003; Caugant *et al.*, 2006). By contrast, the longest experimental analysis of the meningococcal transcriptome in any model of colonization has been analysed at 48 h p.i. in a Sorbarod filter (O'Dwyer *et al.*, 2009) and in co-cultivation with human cells has not been reported beyond 48 h (Neil *et al.*, 2009). Previous studies have shown that the meningococcal transcriptome significantly changes upon host cell contact (Grifantini *et al.*, 2002; Dietrich *et al.*, 2003). So far, the meningococcal transcriptome has not been analysed during prolonged host cell interaction which would more closely mimic colonization than any of the other studies described above. Therefore, in the present study, the meningococcal transcriptome was investigated during long term host cell interaction.

1.10. Hypothesis and Aims

The human nasopharyngeal epithelium is the only known habitat for meningococci, from where they spread, survive and evolve as a population and occasionally invade the bloodstream to establish infection. In an individual, colonization of the nasopharynx by meningococci can be a short episode or a prolonged period, lasting for weeks to months. Understanding the interaction between host and bacteria, especially the meningococcal factors contributing to the colonization process, would yield useful knowledge for developing vaccine and therapeutics against *N. meningitidis*. In the past, most studies have been focused on a few meningococcal factors based on acquired knowledge of their cellular location and structures. With the advancing technology, such as DNA microarray it is possible to study the meningococcal genome wide transcriptional profile during interaction with cultured human epithelial cells, which has been pioneered mostly for shorter period of interactions, 4-6 hours (Grifantini *et al.*, 2003; Dietrich *et al.*, 2003). However, to fully underpin the meningococcal factors contributing to the colonization process, a much longer period of interaction may need to be investigated. My first hypothesis is that the meningococcal gene expression profile during prolonged host cell association significantly differs from that of short term and that of planktonic growing meningococci, and that genes involved in prolonged host cell association will be identified by this approach.

Meningococcal disease has been successfully prevented for serogroups A, C, W135 and Y by vaccines based on the serogroup-specific polysaccharide capsule. Besides preventing invasive disease by inducing bactericidal antibodies, nasopharyngeal carriage is also reduced in vaccinated populations, thereby building up a herd immunity. Besides these striking advantages, a vaccine preventing carriage might conceal the risk of vaccine escape mutants. Vaccine antigens expressed during colonization are likely to be under the selective pressure, which frequently leads to antigenic variation. In vaccinated individuals which have antibodies protecting from carriage and invasive disease, colonization and potentially invasive disease might still be possible by strains not expressing the vaccine antigen. A capsular switch has been associated with vaccination in *Streptococcus pneumoniae* (Temime *et al.*, 2008) and has been

observed in *N. meningitidis* from serogroup C and Y to B (Swartley *et al.*, 1997; Kahler *et al.*, 1998; Vogel *et al.*, 2000; Beddek *et al.*, 2009). My second hypothesis is that (potentially) surface expressed proteins, which are down-regulated during colonization (and therefore less likely to be under selective pressure) and up-regulated during septicaemia (essential to induce antibodies) are potential (new) vaccine candidates. By comparing the meningococcal transcriptome during prolonged host cell association to blood infection studies (Sun *et al.*, 2000; Hedman 2009), vaccine candidates which fulfil the criteria described above, should be identified.

Aims

- To identify a suitable epithelial cell line to use in an experimental system modelling meningococcal colonization for >48 h
- To determine trends over time in the genome-wide transcription pattern of the serogroup B meningococcal strain MC58 during interaction with human cells in tissue culture
- To evaluate the role of meningococcal surface structures during host cell interaction and to identify genes (up-regulated) potentially involved in prolonged host cell interaction, to inform novel candidate vaccine antigen selection (in a comparative analysis to previously published studies)
- By construction of mutants of selected genes identified in this work to investigate their role in host cell interaction

Chapter 2

Materials and Methods

2.1. Sterilization of solutions, buffers and culture medium

All solutions (including deionized (di) H₂O) and buffers which were prepared in-house and came into direct contact with cultured cells or bacteria, were sterile filtered or autoclaved. For volumes < 100 ml, this was done with a syringe (50 ml plastipak, Becton Dickinson) and a 25 mm Acrodisc syringe filter (Pall corporation, 2.2 µm pore size). For volumes > 100 ml a 500 ml filter unit (Nalgene) with a vacuum pump (ABM) was used.

Culture media for bacterial cultivation were autoclaved for 15 min at 121 °C, according to the instructions of the manufacturer (BD, Oxoid). Culture media for human cell cultures were obtained as sterile solutions.

2.2. Culture of bacteria

2.2.1. Culture of bacteria on agar plates

N. meningitidis serogroup B strain MC58, isolated in the UK from an invasive disease case (McGuinness *et al.*, 1991) and the first meningococcal strain for which the complete genome sequence was determined (Tettelin *et al.*, 2000) was used in this study, abbreviated as MC58.

Bacteria grown on agar plates were cultured statically in a dry atmosphere at 37 °C with 5% CO₂ unless otherwise stated.

Stocks of bacteria were stored in heat inactivated fetal bovine serum (hi-fbs) at -80 °C until use. Before experiments, bacteria were grown over night (o/n) on supplemented gonococcal agar (sGC) plates (BD Biosciences), which were supplemented with isovitalex A (Table 2.1) and B (Table 2.2) before pouring of plates (at 50 - 55°C) and with addition of 50 µg/ml kanamycin (Sigma) where appropriate.

Table 2.1. Components of isovitalex A

Component	Weight
Glucose (BDH)	10 g
L-glutamine (BDH)	1 g
β-NAD (DPN)	125 mg
Co-Carboxylase (Sigma)	10 mg
Thiamine – HCl (Sigma)	0.3 mg
Vitamin B12 (Sigma)	1 mg
Fe(NO ₃) ₃ (BDH)	2 mg
Para-aminobenzoic acid (PABA; Sigma)	1.3 mg

Ingredients were dissolved in 80 ml di-H₂O, sterile filtered as described before and stored at -20 °C.

Supplement A was used at a concentration of 1 ml per 100 ml culture medium.

Adenine and guanine-HCl were dissolved in 10 ml 0.1 M HCl (Sigma) placed in a boiling water bath and after cooling down to room temperature, L-cysteine and 10 ml sterile di-H₂O were added. The solution was filter-sterilised and stored at -20°C. Supplement B was used at a concentration of 1 ml in 400 ml culture medium. Supplements A and B were added to autoclaved, liquid GC agar after cooling to <60 °C.

Table 2.2. Components of isovitalex B

Component	Weight
Adenine (Sigma)	100 mg
Guanine – HCl (Sigma)	3 mg
L-cysteine – HCl (Sigma)	2.6 mg

2.3. Culture of bacteria in liquid medium

Overnight plate grown bacteria were harvested into liquid culture medium and the OD₆₀₀ adjusted to 0.2 (equivalent to 10⁸ bacteria per ml) using a spectrophotometer (S10 4D; WPA). Dependent on the specific experiment, liquid culture medium was either Brain-Heart Infusion (BHI; Oxoid) broth supplemented with isovitalex A and B, or host cell culture medium (supplemented with 10 % hi-fbs where appropriate). Bacteria in liquid culture were incubated at 37°C, on a Platform Rocker (STR6, Stuart Scientific) with shaking at 30 rev / min.

2.4. Bacterial growth curves

2.4.1. Bacterial growth curves by measuring the OD₆₀₀

Growth curves of bacteria in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% hi-fbs and in sBHI broth were performed. Bacteria were grown o/n on sGC plates and harvested into 1 ml of the corresponding liquid culture medium. The optical density was adjusted to an OD₆₀₀ = 0.02 (equivalent to 10⁷ bacteria / ml) in 5 ml of culture medium (at time T = 0). Experiments were performed in triplicates in wells of 6 well plates. Plates were incubated at 37°C in a 5% CO₂ shaking at 30 rev / min. After an initial incubation of 2 h, the OD₆₀₀ was measured every hour for 7.5 h and the average readings were plotted, showing the standard error of the mean (SEM).

2.4.2. Bacterial growth curves by CFU (colony forming units)

In anticipation of experiments using the proprietary cultured primary nasopharyngeal epithelial cells MucilAir, bacterial growth was assessed in MucilAir culture medium compared to DMEM-10%-hi-fbs. Bacteria from an o/n sGC plate were harvested into 1 ml MucilAir culture medium and the OD₆₀₀ was adjusted to 0.02. Growth estimation was followed in wells of a 24 well plate, in experiments performed in triplicate. After an initial incubation of 2 h (shaking at 30 rev / min), 10 µl samples were taken hourly and diluted to 10⁻⁴ (at 3 h – 5 h p.i.) or 10⁻⁶ (at 6 h – 7.5 h p.i.) in sterile phosphate buffered saline (PBS, Sigma). At each dilution, 10 µl were spotted in triplicate onto sGC plates and the CFU counted the next day. Average numbers of CFU / ml were plotted with the calculated SEM onto a graph. An experiment using DMEM-10% hi-fbs was performed in parallel to serve as comparison as growth of MC58 in this medium has been shown in preliminary experiments.

2.5. Culture of host cells

2.5.1. Cell line culture

The following cell lines (Table 2.3), further also referred to as host cells, were used in the present work. Cells were cultured in DMEM supplemented with 10 % (v/v) hi-fbs (Gibco), unless stated otherwise, and incubated in a dry atmosphere containing 5 % CO₂ at 37°C.

All cell lines other than MucilAir were stored in 1 ml hi-fbs supplemented with 10% DMSO (v/v, Sigma) at -80°C. For culture, vials were thawed at 37°C before transfer into a T75 tissue culture flask (Greiner Bio-one; 75 cm² culture area) containing 20 ml pre-warmed culture medium. Cells were examined every day with an inverted microscope (Olympus; 400 x magnification) until a confluent monolayer formed. The culture media was renewed every other day. All cell lines (except E6/E7 HTE and MucilAir cells) were part of the collection of this laboratory. E6/E7 HTE cells were kindly provided by M. Apicella and MucilAir cells were obtained from Epihelix-Sarl (Switzerland).

Table 2.3. Description of cell lines used in the present study

Name	Description
16HBE14	SV40 transformed human bronchial epithelial cells (Gruenert <i>et al.</i> , 1988)
HTE E6/E7	E6/E7 (human papilloma virus) transformed human tonsillar epithelial cells (which differentiate in culture to produce ciliated and mucin-producing cells; M. Apicella, pers. Comm.). Cultured in Keratinocyte-serum free medium (K-SFM; Gibco).
COR L23	Large cell bronchial carcinoma cells, a robust cell line previously used in this department for meningococcal-host cell interaction studies (Li <i>et al.</i> , 2003)
A549	Human carcinomic alveolar epithelial cells (ATCC)
Chang	Human conjunctival epithelial cell line, contaminated with a human cervical cancer epithelial cell line (Lavvapa, 1978)
MucilAir human nasopharyngeal epithelial cells	Differentiated human airway epithelial cells (nasal) isolated from a 42 year old male (non smoker); commercially available and delivered as confluent monolayers on transwells (6.5 mm diameter, 0.4 μ m pore size; Costar, Corning incorporated) cultured in serum free MucilAir culture medium without antibiotics (Epithelix-Sarl). In this study, nine wells of MucilAir monolayers were available. Experiments were mainly performed for comparison / validation of results obtained with the cell lines (www.epithelix.com)
J774	Murine monocyte macrophage cell line (ATCC)

2.5.2. Propagation of cell lines

Confluent monolayers were washed with 5 ml sterile PBS (Sigma) – EDTA (0.02 % v/v; BDH) and then 5 ml trypsin - EDTA (0.05 % v/v trypsin, 0.02 % v/v EDTA, equivalent to 0.5 mg/ml trypsin and 0.2 mg/ml EDTA; Gibco) was added to the flask, before incubation for 15 min at 37 °C to detach cells. Cells were harvested by centrifugation (1500 g, 10 min) after which the pellet was gently re-suspended in 3 ml cell culture medium. Cells were seeded into three T75 flasks (VWR) at a density of ca. 50 000 cells per flask, or, when required, into tissue culture plates (Greiner; 24-well or 6-well plates with 1.9 cm² and 9.6 cm² growth area, respectively) at a density of 5000 cells per cm² growth area. Culture medium was changed every other day (20 ml for T75 flasks, 1 ml per well of a 24-well plate and 3 ml per well of a 6-well plate) until

monolayers were confluent. Cells in 24- or 6-well plates were incubated in a moisturized box (a tissue moisturized with di-H₂O was placed at the bottom of a plastic box) to prevent evaporation of the culture medium from culture plates. The lid was kept loose to ensure that the incubator atmosphere (5 % CO₂) was maintained around the plates.

2.5.3. Culture of MucilAir cells

Confluent monolayers of differentiated human nasopharyngeal epithelial cells (MucilAir; Epithelix-Sarl) were delivered on 6.5 mm transwells (Costar 3470, Corning incorporated), containing ca. 500 000 cells per monolayer. As described by the manufacturer, each monolayer was composed of basal cells, mucus-producing goblet cells and ciliated cells, derived from primary cells obtained from the nasopharynx of a 42 year old male donor (Product analysis; Batch number MD009601, Epithelix-Sarl) and had a shelf life of a minimum of 6 months. Cultures were maintained in serum free MucilAir culture medium without antibiotics (Epithelix-Sarl). The transwells were placed in wells of a 24 well plate containing 0.7 ml MucilAir culture medium, generating a liquid-air interface on the surface of the monolayer. Monolayers were carefully washed daily with 100 µl sterile PBS to remove mucus and were allowed to adjust to the culture conditions five days prior to experiments. Plates were kept in a moisturized box as described above to prevent evaporation of culture medium. The culture medium was changed every other day.

2.6. Characterisation of host cell monolayers

2.6.1. The trans-epithelial electrical resistance

Trans-epithelial resistance (TEER) is a property which can be monitored to determine the formation of tight junctions in an epithelial cell monolayer. In this study, the TEER was measured using a Millicell-ERS device (Millipore). Values of >1000 Ωcm² indicate formation of tight junctions (Millicell-ERS manual). For measurements, cells were cultured in transwells and culture medium added to the upper and lower chambers. Measurements were taken with electrodes in either side of the monolayer.

COR L23, Chang and 16HBE14 cells were each seeded into transwells (3 μm pore size, 4.2 cm^2 effective growing area; Becton Dickinson) placed in wells of 6-well plates at a density of ca. 5000 cells per cm^2 in 3 ml culture medium. Another 3 ml of culture medium was added into the bottom of the well. The electrodes were equilibrated and stored in DMEM for 24 h prior to measurements. The first measurement was taken on the 2nd day after inoculation of the cells and subsequently on each following day until the TEER values came to a plateau. Blank values, from a transwell containing only culture medium, were subtracted from those obtained from wells containing cells to give corrected measurements, which were taken twice at each time point and the average was used for further calculations. According to the instructions of the manufacturer (Millicell-ERS manual), the electrical resistance in Ωcm^2 was calculated by multiplying the corrected measurement by the effective growth area of the transwell (4.2 cm^2).

2.6.2. Western blot analysis of host cell protein CD46

CD46 was described to be a receptor for *Neisseria* Tfp (Källström *et al.*, 1997). To confirm expression of CD46 by the host cell lines, western blots using CD46 specific antibodies were performed.

2.6.2.1. Whole-cell protein extraction

Host cells (16HBE14, COR L23, Chang, A549 and J774) were grown in triplicate to confluent monolayers wells of 6-well plates and washed three times with PBS. Whole cell protein preparations obtained after addition of 5 ml lysis buffer and shaking of the bottle until lysis of cells (Weyand *et al.*, 2006; Table 2.4). Protein concentrations were measured with the Nanodrop (ND-1000 Spectrophotometer) and samples stored on ice until further use. Where comparison was to be made with monolayers infected with MC58, monolayers were processed at 24 h p.i. Two negative controls were included: plate grown MC58 - extracted by resuspending the overnight growth of MC58 in lysis buffer and proteins extracted from the mouse cell line J774, which is known to be human-CD46 negative.

Table 2.4. Components of lysis buffer (Weyand *et al.*, 2006)

Component	Volume or weight
Tris-HCl (BDH)	50 mM
NaCl (BDH)	150 mM
Triton X-100 (BDH)	1 % (v/v)
Sodium deoxycholate (BDH)	1 % (w/v)
SDS (BDH)	0.1 % (v/v)
EDTA (BDH)	5 mM
Sodium orthovanadate (Sigma)	0.264 mM
NaF (Sigma)	50 mM
Protease inhibitor cocktail (Roche)	1 tablet

2.6.2.2. Protein electrophoresis for SDS-PAGE gels

For each sample, two reactions were set up, for Western blot analysis and conventional staining of protein bands (Instant Blue staining; Expedeon Protein Solutions), respectively. To 20 µg of each protein sample (6.5 µl), 2.5 µl SDS sample buffer (NuPAGE, Invitrogen) and 1 µl 0.5 M dithiothreitol (DTT; Sigma) were added. Samples were heat-denatured by incubation at 70°C for 10 min. Samples were electrophoresed in 10 % Bis-Tris gels (NuPAGE, Invitrogen) in an Xcell Sure Lock Electrophoresis Cell (Invitrogen) for 35 min at 200 V. A molecular mass protein ladder (Benchmark prestained protein ladder; Invitrogen) was included on each occasion. Separate, identical gels were used for SDS-PAGE and instant blue staining.

2.6.2.3. Instant Blue staining to visualize protein bands on SDS-PAGE

After electrophoresis, the apparatus was dismantled and one of the two gels was stained overnight in 20 ml Instant Blue staining solution, shaking at 100 rpm on an orbital shaker (SO1, Stuart Scientific). The next day, the gel was repeatedly washed with tap water in a shaking water bath until bands on the gel were visible. Gels were examined for the presence / absence of specific bands. Using a Gel Dryer (model 583, Bio-Rad), gels were fixed on a chromatography paper (3MM, Whatman) for permanent preservation.

2.6.2.4. Western blot

For western blot analysis, the second electrophoresed gel was used. Protein bands were blotted onto a Hybond C-extra membrane (Amersham Biosciences) as follows. The membrane was soaked in Towbins Transfer buffer (TTB, Table 2.5) and placed directly onto the gel.

Table 2.5. Components of Towbins Transfer buffer

Component	Weight or volume
Tris (BDH)	3.03 g
Glycine (BDH)	14.4 g
Methanol (BDH)	200 ml
di-H ₂ O	800 ml

TTB-soaked chromatography papers (3MM Chr, Whatman) were placed under and on top of the membrane and the Xcell Sure Lock Electrophoresis Cell (Invitrogen) chamber was assembled as described by the manufacturer and filled with TTB. Protein transfer was carried out for 1 h at 30 V before the apparatus was dismantled and the membrane washed in PBS containing 0.1% Tween 20 (v/v; BDH). Subsequently, the membrane was incubated overnight at 4°C shaking at 100 rpm on an orbital shaker (SO1, Stuart Scientific) in 20 ml blocking solution (3% BSA in PBS containing 0.1% Tween 20 (v/v)) before incubation for 1 h shaking at room temperature in 10 ml blocking solution containing 2 µl 1st antibody (monoclonal antibody 2F1 directed against the cytoplasmic tail Cyt-1 of CD46, generously provided by Dr. N. Weyand (Weyand *et al.*, 2006). The membrane was washed four times in PBS containing 0.1 % Tween 20 (v/v) for 45 min before incubation in 10 ml blocking solution containing 2 µl 2nd antibody (anti-mouse-HRP; BD) for 1 h with shaking at room temperature.

For visualization of bands, the advanced ECL chemo-luminescence kit (ECL; Amersham) was used according to the instructions of the manufacturer. Briefly, the membrane was incubated in developing solution before an ECL Hyperfilm (Amersham) was placed in the dark for 3 sec onto the membrane before image development.

2.7. Bacteria – host cell experiments

2.7.1. Infection of confluent host cell monolayers

Overnight plate-grown bacteria were harvested into liquid culture medium and the OD₆₀₀ adjusted to 0.2. Monolayers of host cells were infected at a multiplicity of infection (MOI) of ca. 100:1, i.e. 100 bacteria per cell. Table 2.6 shows the volume of OD₆₀₀ = 0.2 used to infect monolayers. From growth curves, it was estimated that 1 ml of OD₆₀₀ = 0.2 contains ca. 10⁸ bacteria (and therefore it can be calculated that there are ca. 10⁷ bacteria in 100 µl). The actual MOI was experimentally confirmed by determination of the CFU per ml. Therefore, 100 µl of the OD₆₀₀ suspension were diluted to 10⁻⁶ in cell culture medium and three times 100 µl of the dilution were plated out and cultured in triplicate on sGC plates. The CFU were counted the next day and the MOI calculated by multiplying the average number of CFU with the dilution factor and dividing the result by the number of cells per monolayer.

Table 2.6. Volume of OD₆₀₀ = 0.2 bacteria suspension used to infect monolayers of host cells at a MOI of 100:1 depends on the number of cells forming a monolayer

Cell culture device	Growth area	Number of cells in a confluent monolayer	Volume of OD ₆₀₀ = 0.2 bacteria suspension used for infection	Volume of culture medium *
24 well plate (Greiner)	ca. 2 cm ²	ca. 1-2 x 10 ⁵	100 µl	1 ml (2 ml)
6 well plate (Greiner)	ca. 10 cm ²	ca. 10 ⁶	1 ml	3 ml (5 ml)
T 25 culture flask (VWR)	25 cm ²	ca. 2.5 x 10 ⁶	2.5 ml	10 ml
T 75 culture flask (VWR)	75 cm ²	ca. 7.5 x 10 ⁶	7.5 ml	25 ml (50 ml)

*numbers in brackets show culture volume used in over night experiments >24 h

2.7.2. Visualizing adherence of bacteria to host cells by scanning electron microscopy

2.7.2.1. Sample preparation of infected and non-infected 16HBE14 cells

Cells were grown to confluent monolayers on sterile 13 mm glass cover slips (BDH) in the wells of 24-well plates and infected as described in the previous section. Slides were prepared at 4 h, 10 h, 24 h, 48 h, 72

h, 96 h and 21 days p.i. As controls, confluent, non-infected monolayers of 24 h and 21 days of age were prepared. Experiments were performed in duplicate. Monolayers were fixed overnight at 4°C in 3% glutaraldehyde (v/v; BDH). The next day after washing slides three times for 5 minutes with 0.1 M PBS, 300 µl per well secondary fixative of 1% osmium tetroxide (OsO_4 ; Sigma) solution in 0.1 M PBS was added and incubated and the slides incubated at room temperature for 30 min. After further washes (three times for 5 min with PBS and three times for 5 min with sterile di- H_2O), the fixed preparations were dehydrated gradually by incubation for 10 min each sequentially, in 50%, 75%, 85%, 95% (v/v) aqueous ethanol solutions, and lastly, twice for 10 minutes in 100% ethanol (v/v; BDH). Samples were critical point dried by incubation in liquid CO_2 three times for 15 min using a Critical Point Drying apparatus according to the instructions of the manufacturer (Emitech K850). The principle of critical point drying is, that an atmosphere is created in which no surface tension occurs during the transition of liquid to gaseous state of a medium. As for water, this atmosphere would involve high pressure (3212 psi) and temperature (374°C), which would destroy the sample. Therefore, the sample is first dehydrated in ethanol which is subsequently replaced by liquid CO_2 . Critical point drying with CO_2 is possible at 31 °C and 1072 psi. (Emitech K850 Critical Point Drier instruction manual 2007, document number A4800148). After critical point drying, slides were mounted on 15 mm diameter aluminium scanning EM stubs (Jeol) and coated for 75 sec with palladium-gold using a Mini Sputter Coater (Polaron SC7620). Coating increases the image quality as electrons of the electron beam (part of the microscope) would not interact with any of the noble metals and therefore more electrons would bounce off the sample surface (the scanning EM image is produced by detection of electrons bouncing off the sample surface). In addition, charged structures of the sample are covered, preventing electron uptake of the sample surface, thereby increasing the image quality (Sputter Coater technical brief, Quorum Technologies 2002; document number TB-Sputter). To evenly coat the samples with a thin layer of a noble metal, samples are placed in the sample chamber of a sputter coater apparatus (Mini Sputter Coater; Polaron SC7620). A vacuum is created and the sample chamber is repeatedly flushed with Argon, a noble gas. An electric field is induced between the gold-palladium cathode and the anode. Argon, having heavy atoms, serves

as ionisation gas meaning by bombarding the gold-palladium cathode, negatively charged gold-palladium ions are induced migrating to the anode and coating the sample which is placed next to the anode. (Sputter Coater technical brief, Quorum Technologies 2002; document number TB-Sputter). For the coating process a current of ca. 20 mA and a constant pressure of 4×10^{-2} mbar were used. Representative images at a magnification of 2 000 – 30 000 X were taken at a minimum of three locations using a Jeol JSM-6390 scanning electron microscope.

2.7.2.2. Scanning electron microscopy of MucilAir cells

As MucilAir cells were cultured on transwells, the sample preparation differs from that described above (Table 2.6).

Transwell grids were carefully cut out of the plastic frame in a sterile bath of PBS without letting the membrane dry. Membranes were fixed using glutaraldehyde and Osmium-tetroxide before gradual dehydration in aqueous ethanol dilutions as described above. Instead of critical point drying, samples were chemically treated with hexamethyldisilazane (HMDS) as follows. Membranes were placed onto a dust free polyphenylene (PPE) surface (the lid of a 24-well plate) and generously immersed in HMDS in a fume cabinet. Gently agitation every 15 sec until all the HMDS had evaporated prevented the membranes fixing onto the plastic surface. Samples were carefully mounted onto 15 mm diameter aluminium scanning EM stubs, coated with gold-palladium and examined with the Jeol JSM-6390 scanning EM.

2.7.3. Determination of viable epithelial cells per 16HBE14 monolayer

To determine changes in the number of viable 16HBE14 cells in infected and non-infected monolayers over time, cells were grown in three wells of a 24-well plate. Host cell monolayers were infected with MC58 as described before and maintained for 24 h, 96 h and 21 d. Non-infected monolayers served as the controls at each time point. To terminate experiments, monolayers were washed and trypsinised with 1 ml trypsin-EDTA as described above (Section 2.5.2). Ten μ l of detached cells from each well were

mixed with 10 μ l Trypanblue (Invitrogen) and 10 μ l of the cell – dye mixture loaded into an Improved Neubauer Haemocytometer (Reichert). Using a light microscope (Standard 20, Zeiss), the total number of cells, and the number of dead (stained) cells was counted. The number of cells per ml (equivalent to the number of cells per monolayer) was calculated as directed by the manufacturer. Subsequently, the relative number of viable cells was determined by subtracting the relative number of dead cells from 100 %.

2.7.4. Quantitative analysis of bacteria – host cell interactions

2.7.4.1. Host cell association assays

Cells were grown to confluent monolayers in 24-well plates and infected as described above . Bacteria – host cell co-cultivation was for 1 h, 4 h, 24 h, 48 h, 72 h, 96 h or 21 days, respectively. Infected monolayers were washed 3 times with 1 ml sterile PBS and fresh culture medium added at 4 h p.i. and subsequently twice a day where appropriate. Experiments were repeated in three independent biological replicates, and each performed in at least in triplicate (technical replicates) to allow calculation of standard deviation (SD) and standard error of the mean (SEM).

To terminate the experiments, infected monolayers were washed three times with 1 ml PBS and 1 ml 1 % saponin (Sigma) in sterile H₂O was added. After incubating the plate for 10 min at 37°C, the suspension was rigorously pipetted to disrupt bacteria – host cell contacts and to lyse host cells. From each well, 100 μ l were diluted either 10⁻³-fold (4 h experiments), 10⁻⁴-fold (24 h and 48 h experiments), 10⁻⁵-fold (72 h and 96 h experiments) or 10⁻⁶-fold (21 days experiments) in cell culture medium. For each dilution, three aliquots of 100 μ l were plated on three sGC plates and CFU counted the next day. For each time point, the average number of cell associated CFU per monolayer, SD and the standard error of the SEM were calculated. For comparison of results the Student's t-test was calculated using excel formula (paired, two-tailed).

2.7.4.2. Host cell invasion assays

Gentamicin protection assays were performed as described by Jones *et al.* (1992) with some modifications to determine the number of invasive CFU.

Host cells were grown to confluent monolayers in 24-well plates and infected as described above (Table 2.6). Infected monolayers were washed three times with 1 ml PBS and fresh culture medium added 4 h p.i. and subsequently twice a day for experiments >4 h. All experiments were performed in at least triplicates to allow calculation of SD and SEM.

To terminate experiments, infected monolayers were washed 3 times with 1 ml sterile PBS as described above, after which 1 ml culture medium containing 150 µg / ml gentamicin (Sigma) was added to each monolayer. After incubation for 1 h at 37°C in an atmosphere containing 5% CO₂, monolayers were washed three times with 1 ml PBS to remove gentamicin. Host cells were lysed with 1% saponin as described above (Section 2.7.2.1) and three aliquots of 100 µl were directly cultured on three sGC plates for 4 h and 24 h experiments. For 48 h and 72 h experiments, lysates were diluted in cell culture medium 10-fold and 10⁻³-fold for 96 h and 21 days experiments before three aliquots of 100 µl were cultured for enumeration after growth on sGC agar plates. For each time point, CFU were counted the next day and the average number of invasive CFU, SD and SEM per monolayer calculated.

2.8. Separation of cell-associated bacteria from host cells for preparation of meningococcal RNA

For microarray and quantitative real time PCR, high quality RNA of host cell adherent bacteria is required, ideally, free of contaminating human RNA which may lead to non-specific background signals. To achieve this, host cells can either be lysed, or bacteria and host cells separated before bacterial RNA extraction. Methods tested were i) the use of saponin, a detergent isolated from the soap tree (*Quillaja saponaria*), lyses eukaryotic but not prokaryotic cells (Dourmashkin *et al.*, 1962). Saponin was used in a meningococcal transcriptome study (Griffantini *et al.*, 2002); ii) the use of RLT buffer (Qiagen), a comparable method to saponin. RLT buffer is part of the RNeasy kit (QIAGEN) and contains guanidine thiocyanate, which lyses eukaryotic cells and inactivates RNases, thereby facilitating subsequently

extraction of intact RNA. RLT buffer was used to lyse host cells in an *E. coli* transcriptome study (Di Cello *et al.*, 2005); iii) the use of trypsin-EDTA to separate bacteria from host cells by digesting proteins.

Bacteria are recovered in the supernatant of a differential spin. This method was used in a meningococcal-host cell transcriptome study by Dietrich *et al.* (2003).

The three different methods were compared and the optimum method was determined by comparing quality and quantity of extracted RNA samples. For evaluating the three different methods, confluent monolayers of 16HBE14 cells in T75 flasks were infected with MC58 and incubated for 4 h.

2.8.1. Differential lysis of host cells and bacteria using saponin

After 4 h of bacteria – host cell co-cultivation, monolayers were washed three times with 5 ml PBS before 10 ml ice cold saponin (1 % in H₂O) was added. After incubation on ice for 20 min the detached cell suspension was vortexed for 1 min at 2500 rpm. To protect RNA, 20 ml RNeasy Protect (QIAGEN; Sodium-acetate ammonium-sulfate solution) was added before centrifugation at 3500 g for 10 min at 4°C. The supernatant was discarded and the pellet, containing bacteria, was resuspended in 100 µl sterile PBS and 1 ml Trizol (Gibco) was added. Samples were stored at -80°C o/n and RNA extracted the next day.

2.8.2. Differential lysis of host cells and bacteria using RLT buffer (QIAGEN)

This method was described by Di Cello *et al.* (2005) to extract *E. coli* K1 RNA after co-cultivation with human brain micro endothelial cells (HBMEC) using RLT-lysis buffer (QIAGEN). Briefly, infected monolayers were washed three times with PBS before 5 ml RLT buffer was added. Host cells immediately lysed. The host cell-bacteria suspension was vortexed for 1 min at 2500 rpm before centrifugation at 3500 g for 10 min. The pelleted bacteria were resuspended in Trizol and RNA extracted as described below.

To assess survival of meningococci after treatment with RLT, 100 µl of the host cell - bacteria suspension was diluted up to 10⁻⁶ and 10 µl of the dilutions (neat to 10⁻⁶) were plated onto sGC plates in triplicate. CFU were counted the next day.

2.8.3. Separation of bacteria and host cells using trypsin-EDTA

Trypsin-EDTA treatment and differential centrifugation were used to separate adherent MC58 from host cells as described by Dietrich *et al.* (2003) with several modifications. After washing the infected monolayer three times with 5 ml PBS, 10 ml 10 x concentrated Trypsin-EDTA (0.5% trypsin, 0.2% EDTA, equivalent to 5 mg/ml trypsin and 2 mg/ml EDTA; Sigma) was added. After 5-8 min incubation at 37°C in a 5% CO₂ atmosphere, 10 ml of ice-cold PBS was added before a differential spin (a slow centrifugation where cells are pelleted but bacteria remain in the supernatant) at 300 g for 3 min at 4°C to separate bacteria from host cells. The supernatant was recovered and 10 ml RNeasy Protect (QIAGEN) added to conserve the RNA. Samples were centrifuged at 3500 g for 10 min at 4°C and the pellet was resuspended in 100 µl sterile PBS before 1 ml Trizol (Gibco) was added. Trizol is a commercially available product which contains phenol and guanidine-isothiocyanate and is designed to protect RNA during lysis of cells (eukaryotic and prokaryotic). Samples were stored at -80°C o/n and RNA extracted the next day. To determine the number of adherent bacteria and to detect possible contamination, three aliquots of 100 µl of the trypsin-EDTA treated cell-bacteria suspensions were diluted 10⁻⁴- fold in DMEM, and 100 µl of each dilution was cultured on sGC plates in triplicate. The next day the average cell adherent CFU per monolayer was calculated as well as SD and SEM.

2.9. Purification of bacterial and cellular nucleic acids and amplification methods

2.9.1. Bacterial and cellular RNA extraction

2.9.1.1. RNA extraction of host cell-associated bacteria

After adding the RNA protection reagent Trizol to bacteria separated from host cells and overnight storage at -80°C, samples were slowly thawed on ice and vortexed at 2500 rpm for 1 min (MS1 minishaker, IKA). Using lysis matrix B tubes (MP Bio) samples were fast-prep processed (Bio 101 Thermo Electron Corporation; FP120) three times at a speed of 6.0 for 40 s with 5 min incubation on ice in between. To the supernatants 300 µl chloroform (BDH) was added. Samples were vortexed at 2500 rpm for 1 min followed by centrifugation at 12 000 g for 15 min, resulting in three distinct layers of each

sample in the tube. The aqueous layer was transferred into 2 volumes of RNase free, 100 % ethanol (BDH). RNA was purified using an RNeasy kit (Qiagen) with DNase on-column treatment as described by the manufacturer (Qiagen). RNA was eluted with 40 µl RNase-free di-H₂O. RNA concentration and quality was assessed using the Nanodrop and bioanalyzer technology as described below.

2.9.1.2. RNA extraction of plate-grown bacteria

Bacteria growth of an o/n plate was resuspended in 100 µl PBS before 1 ml Trizol was added. Using lysis matrix B tubes RNA was extracted as described in Section 2.9.1.1.

2.9.1.3. RNA extraction of host cells

Confluent monolayers of host cells, grown in 24 well plates, were incubated in 1 ml Trizol for 5 min before vortexing for 1 min at 12 000 g. To each sample, 300 µl chloroform was added and the samples further treated as described in Section 2.9.1.1.

2.9.2. Qualitative and quantitative assessment of extracted RNA

RNA concentrations were measured using the Nanodrop (ND-1000 Spectrophotometer). Besides the RNA concentration, the A260/280 and the A260/230 ratios were measured to assess RNA purity. Values < 2.0 for the A260/280 ratio indicate protein and / or phenol contamination; values < 2.0 for the A260/230 ratio indicate carbohydrate and / or phenol contamination (Nanodrop technical Support Bulletin 2007). Samples with a concentration of more than 20 ng/µl were further analysed by capillary gel electrophoresis (Bioanalyzer 2100 Agilent) using the total prokaryotic RNA 6000 Nano Assay with the RNA 6000 Nano Kit (Agilent) as described by the manufacturer. This technique allows qualitative analysis of prokaryotic rRNA. The amount of contaminating human rRNA can be determined. Samples showing clear bands of bacterial 23S and 16S rRNA, a 23S to 16S ratio of 1.5 – 2.0 and ideally, an RNA integrity number (RIN) of >8 and minimal human RNA contamination (when samples were obtained from host

cell-association experiments) were used in further experiments. The RIN indicates the level of degradation of RNA. Values between 8 and 10 are regarded as non-degraded.

2.9.3. Bacterial DNA extraction

Bacteria were heat-killed in 100 μ l PBS for 1 h at 56 °C prior to DNA extraction. DNA was extracted using the QIAamp DNA Mini kit (Qiagen) following the instructions of the manufacturer. DNA yield, A260/280 and A260/230 ratios were measured using Nanodrop.

2.9.4. Preparation of cDNA for reverse transcription PCR

For reverse transcription PCR (RT-PCR) RNA samples were DNaseI treated, reverse transcribed and then subjected to PCR. In addition to the DNase on-column, a second DNaseI treatment was performed to remove remaining DNA.

2.9.4.1. DNaseI treatment

In order to prevent false positive signals in the RT-PCR reactions due to presence of DNA, samples were DNaseI treated. To 8 μ l RNA (containing 0.5 - 1 μ g RNA), 1 μ l DNase buffer (Sigma) and 1 μ l DNaseI (Amplification Grade 1 U / μ l; Sigma) were added. Reactions were gently mixed and incubated for 20 min at room temperature before 1 μ l DNase stop solution (Sigma) was added.

Using Nanodrop, the RNA concentration was measured before measurement of the potentially remaining DNA using the Qubit dsDNA BR assay (Invitrogen) as described by the manufacturer. Samples containing > 10 ng/ μ l DNA were repeatedly DNaseI treated as described above.

2.9.4.2. Reverse transcription

To denature potential secondary structures of the RNA, reactions were incubated at 70°C for 10 min and cooled on ice for at least 1 min to prevent re-formation of secondary RNA structures. In each reverse transcription reaction, 0.5 μ g DNaseI treated RNA was used, 1 μ l gene-specific reverse primer (5

pmol/ μ l) and 1 μ l of dNTPs (10 mM/ μ l each; Qiagen) were added to the RNA sample and the volume adjusted to 14 μ l with RNase-free H₂O. Reactions were incubated at 65°C for 5 min and snap cooled on ice before collection at the bottom of the tube by brief centrifugation. Next, 1 μ l dithiothreitol (DTT, 0.1 M; Sigma), 4 μ l 5x first strand buffer (Invitrogen) and 1 μ l Superscript III reverse transcriptase (200 U/ μ l; Invitrogen) were added. Reactions were incubated at 25°C for 10 min followed by 2 h at 50°C using a Master Cycler (Eppendorf).

As a control for each RNA sample, 0.5 μ g RNA was RNase treated by adding 1 μ l RNase (100 μ g/ μ l; Qiagen) and incubating at 37° for 20 min. Control samples were reverse transcribed as described above.

2.9.5. Polymerase Chain Reaction (PCR)

PCR reactions were set up as shown in Table 2.7. Dependent on the nature of the sample, either genomic DNA, cDNA or heat-inactivated bacteria were used as templates (due to safety regulations, bacteria had to be heat-inactivated for 30 min at 56°C). Heat inactivated bacteria could be used as template in PCR reaction as the HotStar Taq-polymerase (Qiagen) was used which needs an initial activation step at 95°C which also breaks up bacterial cells and releases their DNA. Primers used in this study are shown in Table 2.8. Primer sequences were either designed using the MacVector program or primer sequences were taken from previous studies.

PCR cycles were as follows:

- 95°C for 5 min followed by 40 cycles of:
- 95°C for 30 s
- 53°C for 1 min
- 72°C for 1 min per kbp of amplified product, followed by a final elongation at 72°C for 10 min.

Next to a DNA ladder (dependent on product size 1 kb or 100 bp DNA ladder Invitrogen),

10 μ l of PCR products were subjected to electrophoresis on a 1% agarose gel (supplemented with 5 μ l ethidium bromide, (1% aqueous solution, Fluka Biochemika) for 40 min at 100 V.

Table 2.7. PCR reactions

Component	Volume
Template (DNA or cDNA or heat-inactivated bacteria)	1 µl (100 ng DNA or cDNA)
Forward and reverse primer (50 pmol/µl each)	1 µl of each
10 x PCR buffer (QIAGEN)*	5 µl
HotStar Taq Polymerase (5 U/µl; QIAGEN)*	0.75 µl
DMSO (Sigma)**	2.5 µl
Nuclease free H ₂ O	Adjust to a total volume of 50 µl

*For amplification products >2000 bp and for products sent for sequencing, 0.75 µl Polymerase Mix (Expand Long Template PCR System; Roche) was used with the corresponding 10x buffer (Roche); ** DMSO was only used for amplification of *NMB0348* products.

Table 2.8. Primers used in PCRs and RT-PCRs and the length of the amplified product..

Amplified Gene ID	Forward primer name and sequence 5'-3'	Reverse primer name and sequence 5'-3'	Product length (bp)	Primer used in PCR or RT-PCR
Human CD46	CD46-f- CCTGCAAATGGGACTTAC G*	CD46-r- AAAAACCTTATCGCATTCAAAC*	448	RT-PCR
NMB0342 / NMV_0379	NMB0342f- TAGCAGGGCTTTGGCTTCT TCG	NMB0342r- CGATCCCTATGTTTCATGCAG	1242	PCR
NMB0342	NMB0342rt-f GGATTGGTGCTGATTGT GGTATTC	NMB0342rt-r TCGCCTTCAAGCCGTTTTTAC	148	RT-PCR
NMB0343	NMB0343rt-f AACACCTCAAACGGCTG GAGAC	NMB0343rt-r TGCATGAACATAGGGATCGTCTTC	170	RT-PCR
NMB0344 / NMV_0381	NMB0344f- TCGCCACCTATACCGTTAC	NMB0344r- CTGGACTTCCTGCGTACC	1467	PCR
NMB0344	NMB0344rt-f GGACATTATGCCGTTTTGG TCG	NMB0344rt-r TGGAAATACTCGTCAGGGGTAGCC	155	RT-PCR
NMB0345 / NMV_0382	NMB0345f- ATGTTGCTGGCAACAGAC G	NMB0345r- GCGGTTTTTTCTGTTTCATGG	1652	PCR
NMB0345	NMB0345 rt-f AAGTGGTCAATACCGTGG TCGCAC	NMB0345 rt-r AGGATTTGCCCCAACTGGAC	292	RT-PCR
NMB0346	NMB0346 rt-f GGATAAGGATGTCCAAAA CCGC	NMB0346 rt-r AAACCGTCAAAAGCCTGCTCGTCG	267	RT-PCR
NMB0347 / NMV_0384	NMB0347f- GCAGATCATGCAGCAGGC	NMB0347r- TGCTCGCTTCGTTGAACA	1478	PCR
NMB0347	NMB0347 rt-f ATAGCGACACAGAAAAAG ATGC	NMB0347 rt-r AGCGTAAACTGCGACACCAG	139	RT-PCR
NMB0348 / NMV_0385	NMB0348f- CCTTTTCAGACGGCATTGC	NMB0348r- GAGTGGAAACGAGCAGGTCT	1645	PCR
NMB0348	NMB0348 rt-f GAGGCATACGGTTACAAC GAGG	NMB0348 rt-r GAAATCGGCAACGGTTTGG	210	RT-PCR
kanR	kanR-f- CGGGATCCGCCGTCTGAA CCAGCGAACCATTGAGG **	kanR-r- ACGCGTCGACGCTTTTATAGACATCT AAATCTAGG**	1500	PCR

*primer sequences were obtained from McLaughlin *et al.* (2003); **primer sequences were obtained from Pelicic *et al.* (2000)

RT-PCR primers were designed to amplify a short product (<500 bp) within the coding sequence, whereas primers used in PCRs were designed outside of the coding region of the gene of interest

2.9.5.1. Sample preparation for sequencing

PCR products were excised from the gel with a sterile scalpel and purified using a gel extraction kit (Qiagen). Purified products were diluted with EB buffer (Qiagen) to a concentration of 10 ng per 100 bp product length. The gene specific reverse primer was diluted to a concentration of 3 μ mol, and together with the purified PCR product, the sample volume was adjusted to 10 μ l and sent for sequencing to the MRC Genomics Core Laboratory, Imperial College London. Sequences were aligned with the available gene sequence of interest using BLAST 2 sequences, a software tool for sequence analysis (available under <http://blast.ncbi.nlm.nih.gov/Blast>).

2.10. Microarray experiments

2.10.1. The 'Pan - Meningitis' microarray slide used in the present study

The microarray slide used in this study was the 'Pan-Meningitis' array designed by the Bacterial Microarray Group at St. Georges Hospital (B μ G@S) and manufactured by Agilent (initially Oxford Genetic Technology). Each array is composed of ca. 8500 40 to 60-mer oligonucleotides representing the genomes of four *N. meningitidis* strains: MC58 (serogroup B), Z2491 (serogroup A), FAM 18 (serogroup C) and 053442 (serogroup C) with MC58 being the reference strain (core genome). Three different oligonucleotides have been used per gene. Each spot was composed of one kind of oligonucleotide. As the four *Neisseria* strains show a high sequence similarity, the three oligonucleotides per gene were designed in homologous regions present in all four strains (Figure 2.1).

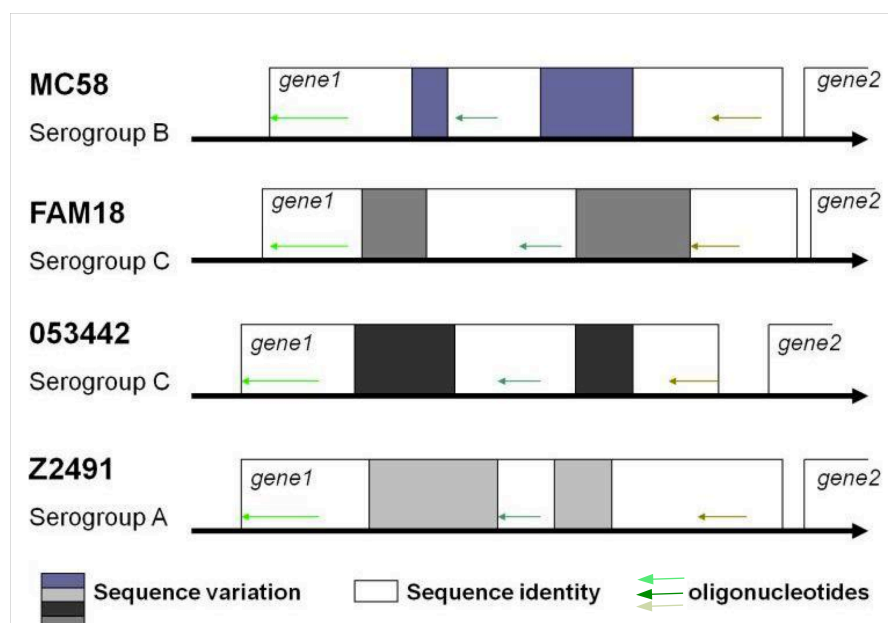


Figure 2.1. Schematic diagram of a homologous gene (termed gene 1) found in all four *Neisseria* strains, showing regions of sequence identity (white boxes) where the three oligonucleotides per gene (indicated by green arrows) were designed.

For each oligonucleotide, a maximum of three base pair mismatches between the four strains was allowed. For genes where this procedure was not possible due to sequence variations between the four strains, strain specific spots were incorporated.

Each array contained 9000 spots, including control spots representing human, mouse and *E. coli* genes. On each slide (similar to a microscopic glass slide), eight arrays were printed (Figure 2.2), allowing multiple samples to be hybridized in parallel and under the same hybridisation conditions, minimizing the technical variation between samples. Due to the design of the slides, in this study, four replicates of each time point of meningococcal host cell interaction were analyzed.

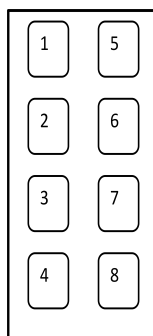


Figure 2.2. Schematic graph of a microarray glas slide with eight arrays (numbered 1 - 8).

2.10.2. Sample preparation for microarray analysis

2.10.2.1. Bacterial genomic DNA and cDNA labelling with Cy3 and Cy5

In the present study, competitive two-colour (Cy5/Cy3) hybridization of microarrays was performed. A mixture of sample cDNA (of bacterial host cell co-cultivation experiments) and genomic MC58 DNA was hybridized to arrays, with the genomic DNA serving as an internal reference. MC58 genomic DNA was Cy3 labelled and MC58 sample cDNA Cy5 labelled. For labelling of MC58 genomic DNA, 1 μ l random hexamer primers (3 μ g/ μ l; Invitrogen) were added to 50 ng MC58 DNA and the volume adjusted to 41.5 μ l with di-H₂O. The samples were incubated at 95°C for 5 min transferred to ice to cool for 1 min and then briefly centrifuged to collect the sample at the bottom of the tube. To each sample, 5 μ l 10 x REact 2 buffer (Invitrogen), 1 μ l dNTPs (containing 5 mM dA/G/TTP and 2 mM dCTP), 1.5 μ l Cy3 dCTP and 1 μ l Large Fragment DNA Polymerase I (5 U / μ l; Invitrogen) were added. Samples were incubated at 37°C for 90 min in the dark.

For each array hybridization 1 μ g RNA was reverse transcribed and labelled with Cy5 (Amersham). To each RNA sample, 1 μ l random primers (3 μ g/ μ l) was added and the volume adjusted to 11 μ l with water. Samples were incubated at 72°C for 5 min and immediately cooled on ice before a brief centrifugation. For the reverse transcription reaction, 5 μ l first strand buffer, 2.5 μ l DTT (100 mM, Invitrogen), 2.3 μ l dNTPs (containing 5 mM dA/G/T-TP and 2 mM dCTP), 1.7 μ l Cy5 labelled dCTP (Invitrogen) and 2.5 μ l SuperScript III reverse transcriptase (200 U/ μ l) were added. Samples were incubated at 25°C for 10 min followed by 42°C for 90 min in the dark.

2.10.2.2. Preparation of Cy3-Cy5 labelled DNA mixture

For array hybridisation, a DNA mixture composed of Cy3 labelled genomic DNA and Cy5 labelled cDNA was prepared. One Cy3 labelled genomic DNA sample was combined with a Cy5 labelled cDNA sample and purified (from excess random primers, dNTPs and dye) using a MinElute DNA column (Qiagen) according to the instructions of the manufacturer. DNA was eluted in 20 µl H₂O and Cy3 and Cy5 concentrations measured using the Nanodrop technology.

To 18 µl labelled DNA, 4.5 µl 10 x blocking agent (Agilent) and 22.5 µl 2 x hybridisation buffer (Agilent) were added and the mixture incubated for 3 min at 95°C. Subsequently, samples were incubated at 37°C for 30 min and samples collected at the bottom of the tube by brief centrifugation.

2.10.3. Hybridisation of microarray slides

Each array was hybridised with a previously prepared DNA-cDNA sample. Forty µl of the prepared sample was placed into the middle of one of the eight wells of a gasket slide, a glass slide containing eight wells in the same format as the array slide, each well (and the array of eight) surrounded by sealing gaskets. After loading the gasket slide, the microarray was carefully placed on top and held in place by a metal clamp. Slides were incubated o/n in a rotating (20 rpm) hybridisation oven (Agilent) at 65°C.

The next day, the slide-gasket sandwich was disassembled in a trough containing 400 ml Oligo aCGH wash buffer 1 (part of the chip-on-chip wash buffer kit, Agilent). The array was washed in 400 ml fresh Oligo aCGH wash buffer 1 for 5 min and transferred to 400 ml pre-warmed (37°C) Oligo aCGH wash buffer 2 (Agilent) for 1 min. These washing steps removed non-specific background signals and stabilized the hybridized spots. The slide was removed from the buffer and immediately scanned after air drying for 1 min.

2.10.4. Microarray slide scanning, quality control and statistical analysis

2.10.4.1. Microarray slide scanning

Slides were scanned using the gene array laser scanner (GenePix-4000B, Axon) and the raw scan data pre-analysed using the feature extract software (Agilent) customized for the BμG@S Pan-Meningitis array. This software is an automated data generating and quality control program that distinguishes hybridized spots from the background, detects spot intensities, calculates Cy5 / Cy3 ratios and flags outliers. Scanning and feature extraction was performed by a member of the BμG@S group. Files generated by the feature extraction software were imported into Genespring GX 11 (Agilent) for further analysis.

2.10.4.2. Quality control of hybridised and scanned microarrays in Genespring GX 11

Data from replicate arrays were compared to establish constituency of results. That is to say, replicate samples at time X were compared spot by spot and the results amalgamated to produce a correlation coefficient between arrays. Comparing samples analyzes at different time points allowed assessment of similarities and differences, which were visualized in correlation maps. In addition, heat maps of each array were prepared and replicates of each time point aligned to visualize potential outliers. According to the level of expression, each gene is represented as a coloured bar in heat maps. Outliers can be easily identified by aligning two arrays and by comparing the colour of the same gene in different replicates.

2.10.4.3. Statistical analysis of microarrays using Genespring GX 11

Genespring GX11 is the latest version of a software program to analyse microarrays. The term technology in Genespring GX11 is used to describe the whole statistical analysis process. The technique is developed according to the array design (for example organism, spot location, replicates of spots) and the hybridization process (for example two-colour or one colour). In this study, the technique designed by BμG@S for the Pan-Meningitis array was used. Dependent on the technique, the Genespring GX11 user has limited choices of normalization, post hoc tests and filter settings.

After importing the data obtained after scanning and feature extraction into Genespring GX11, replicates of each sample were grouped according to the experimental condition or time point and the signal intensities of the spots averaged. The measurements were flagged for detected spots (after subtraction of the background intensity) only.

Entities, the technical term for array spots in Genespring GX 11, were filtered on expression of the raw data. This process filters spots on their signal intensities by applying percentiles. A saturated spot would be in the 100th percentile, but the setting of the lower percentile is chosen to reflect the background signal obtained from areas between spots to limit misinterpretation of background signals. The software's default filter settings are 20th -100th percentile but as in this study background intensities were found to be low and only a minority of spots saturated, it was decided to lower the minimum threshold to the 5th percentile.

Subsequently a 'gene-level experiment' was automatically created, applying normalization steps, including (locally weighted least squares regression) LOWESS normalisation. The normalisation steps and their parameters are dependent on the designed technique and could not be influenced by the Genespring GX 11 user. The creation of a 'gene level experiment' is a technical term describing the combining of all spots representing the same gene (in this case each array had three spots per gene, each composed of a different oligonucleotide). All subsequent analyses were performed at the gene level rather than spot-level.

Before further statistical analyses were applied, data were filtered on MC58 expression data. Data were subjected to 1-way analysis of variance (ANOVA), to detect differentially expressed genes under any of the experimental conditions or time points. As a post-hoc test, the Student-Newman-Keuls (SNK) was performed (alternatively the more stringent Tukey-test could have been used). Based on the mean, data are ranked and pair-wise compared. The p-value was calculated asymptotically (default setting) and the Benjamini Hochberg false discovery rate was applied. Data of each time point were paired as shown in Figure 2.3.

In the resulting data set, the p-value cut-off was set to $p < 0.01$ (in all pairs of comparison). Data were filtered on the fold change of expression (fc) and the cut-off was set to ≥ 2.0 in at least one pair of comparison. This resulted in a list of < 500 statistically robust differentially expressed genes for analysis.

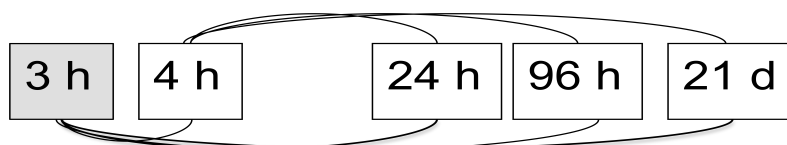


Figure 2.3. Data pairs of microarray experiments. To estimate the effect of the presence of host cells on the meningococcal transcriptome and for comparison with other studies, 4 h, 24 h, 96 h and 21 days data were compared to 3 h DMEM data. To draw inferences about sequential changes in the meningococcal transcriptome on host cell association, 24h, 96 h and 21 days data were compared to 4 h data.

2.10.5. Grouping of differentially expressed genes into functional groups and pathways

To overview the data exported from GX 11, genes were assigned into groups and gene pathways using the COGs (clusters of orthologous groups of proteins, available at <http://www.ncbi.nlm.nih.gov/COG/>; Tatusov *et al.*, 1997 and 2003) and KEGG databases (Kyoto Encyclopedia of Genes and Genomes, available at <http://www.genome.jp/kegg/pathway.html>; Kanehisa, 1996).

Gene lists obtained from Genespring GX 11 analysis were inspected for known surface-expressed genes (many of them described in the introduction) and for any hypothetical or putative surface components (identified as such in the NCBI *Neisseria meningitidis* MC58 Genome database; http://www.ncbi.nlm.nih.gov/sites/genome?Db=genome&Cmd=Retrieve&dopt=Protein+Table&list_uids=155).

2.10.6. Comparison of the meningococcal gene expression profile during host cell interaction to previous meningococcal transcriptome studies

Gene expression profiles were compared to data generated in other meningococcal transcription studies (Grifantini *et al.*, 2002, Dietrich *et al.*, 2003, Hedman *et al.*, 2009, O'Dwyer *et al.*, 2009). To make comparisons, data from each study had to be normalized to a similar sample: to 3 h DMEM for the Grifantini *et al.* (2002) and Dietrich *et al.* (2003) studies and to the median signal intensity of each gene for Hedman *et al.* (2009) and O'Dwyer *et al.* (2009) studies. For comparisons, members of all differentially-regulated gene lists were classified as up- or down-regulated genes. Correlation coefficients were calculated for all differentially expressed genes and separately for each up-and down-regulated list. Using Venn diagrams, three gene lists at a time were compared.

2.10.7. Quantitative real-time reverse transcription PCR (q-RT-PCR)

To gain supportive evidence of the expression profile of single genes obtained by microarray experiments, q-RT-PCR was performed on RNA of three independent biological replicates (not previously used for microarray analysis) at each time point. Of each time point, meningococcal RNA was extracted as described in Section 2.9.1.1. In addition, RNA of bacteria cultured for 3 h in DMEM-10% hi-fbs without host cells was extracted in triplicates as described in Section 2.9.1.1.

The concentration and quality of extracted RNA was measured using the Nanodrop and Bioanalyzer, as described in Section 2.9.2. Contaminating DNA concentrations were measured using Qubit dsDNA BR assay (Invitrogen) kit as described by the manufacturer. Samples with a DNA concentration of < 10 ng/μl were used in further experiments (preliminary data have shown no inference when samples had < 10 ng DNA). Of each RNA extraction, 1 μg was reverse transcribed using random hexamer primers (Invitrogen; 50 ng/μl) as described before (Section 2.9.4). For the q-RT-PCR, 2.5 ng cDNA, 1 μl gene specific primer-probe solution (Applied Biosystems, 20 X concentrated; containing gene-specific forward and reverse primers and a gene-specific dye-labeled probe) and 10 μl TaqMan Universal PCR Master Mix (Applied Biosystems) were pipetted into a MicroAmp Optical 96-well reaction plate (Applied Biosystems). The

volume was adjusted to 20 μ l with sterile nuclease free H₂O and the plate covered with a MicroAmp 96-well Optical Adhesive Film (Applied Biosystems). Reactions were collected at the bottom of the wells by centrifugation for 30 sec at 500 g. q-RT-PCR reactions were performed in a Step1Plus Real Time PCR System (Applied Biosystems) with the cycles set as follows: 95 °C for 10 min (initial enzyme activation and cDNA denaturing) followed by 40 cycles of 95 °C for 15 sec (denaturing) and 60 °C for 1 min (annealing and elongation).

Q- RT-PCR was performed in quadruplicate on each of the three biological replicates of each time point, which resulted in a total of 12 q-RT-PCR replicates per time point and gene. A representative plate set-up is shown in Table 2.9. For each gene, a forward and a reverse primer, as well as a reporter probe (sequences shown in Table 2.10) were obtained (Applied Biosystems).

Gene expression levels were calculated using the $\Delta\Delta C_T$ method (Schmittgen TD and Livak KJ 2001 and 2008; Custom TaqMan Gene Expression Assays Protocol, Applied Biosystems) which requires an endogenous control (housekeeping gene, expected to be expressed at a constant level) and a calibrator (a sample serving as comparison point). In this study, expression levels of 16S rRNA served as the endogenous control and were measured on each plate (and of each sample). The 3 h-DMEM samples were used as calibrator.

Table 2.9. Representative plate set-up for q-RT-PCR.

Gene ID	3hDMEM-I	3hDMEM-II	3hDMEM-III	3hDMEM-IV	24h adh.-I	24h adh.-II	24h adh.-III	24h adh.-IV	96h adh.-I	96h adh.-II	96h adh.-III	96h adh.-IV
Gene 1												
Gene 2												
Gene 3												
Gene 4												
16S												
Gene 5												
Gene 6												
Gene 7												

Each of the three biological replicates (e.g. 3h DMEM-I, II and III) of each time point was analysed in quadruplicate (column 1-4) per gene. In addition to the genes of interest shown in Table 2.10, expression of 16S rRNA was analysed on each plate (calibrator sample).

Table 2.10. Primer and probe sequences used in q-RT-PCR experiments

Gene ID	Forward primer 5'-3'	Reverse primer 5'-3'	Reporter probe 5'-3'
<i>NMB0067-siaD</i>	CCTAGAAGCCCTAATAATATAACT CCTAAAAAATTACTTT	AGGAGTAGTGGCCTGTAAA AGACA	CAGCCTGCTCATCTCT
<i>NMB0342</i>	ACAGCCGCTTCATTATGTGGAA	GCTGCCCCACAGGAACA	CCAGCCAAAACAAAAC
<i>NMB0344</i>	GCCGTTTTGAAGGCGTAAGC	GCCGCCTGAAAACAAATCT TTGAG	AACCGCCAGAAAAC
<i>NMB0345</i>	GACAAGAAACCGTCCTTCAAAAC C	GGCGATATGCAATGCGTAT GC	CCGTTCAAGCCATATTT
<i>NMB0995</i>	GCAGCAGGCAGTCGAAGT	CGAGGTTGTTGACGTAGTT GCA	AAGGCTACGCCATCA CG
<i>NMB1040</i>	GCAAGCTACCTCACGCTCAAT	CGGATTGTCGGGATGGTGT T	CCGTGTCCGTCTTCG
<i>NMB1429-porA</i>	GGATGTGTATTATGCCGGTCTGA AT	GGCGTGTCTCGCATATTTAA AGG	TTTTGCCGGGAACTAT
<i>NMB1822-pglD</i>	GCCCTGTACGCCATCGA	TCGGTGTGAGGCGTTTT	TTCGCGCAATTCCTTT
<i>NMB1926-lgtE</i>	TGCCTTTATCGTTCGTTTGAAAC	AGCAAAGGAAATGACCGAT TCTCA	CCGGTCTGACAATAAC
<i>NMB2003</i>	ACCGACGCGCTGATGTC	GTCGGCAATCAAATCCAAA TAGCT	CCTGACGCTGTTCCCTC
<i>16S</i>	GCTAATACCGCATACGTCTTGAGA	TCGGCCGCTCGAATAGC	AAGGTCCCCTGCTTTCT

2.11. Site directed mutagenesis of MC58 by insertion of a kanamycin resistance cassette (*kanR*) into the target gene

Specific MC58 gene insertion mutants were prepared by transformation using genomic DNA of serogroup C strain 8013 previously transformed by insertion of a kanamycin resistance cassette (encoding aminoglycoside phosphotransferase) into the target gene (homologous to the target gene of strain MC58), prepared and kindly provided by Dr. Vladimir Pelicic (Pelicic *et al.*, 2000).

For transformation, MC58 was harvested from an o/n sGC plate into sBHI broth (Oxoid) and the OD₆₀₀ adjusted to 0.1. Using a 24 well plate, 270 µl of bacteria suspension and 30 µl of genomic 8013 DNA (carrying a *kanR*-cassette in the target gene) at a concentration of 100 ng/µl were added. After incubation for 4 h statically at 37°C in an atmosphere containing 5% CO₂, the transformation reaction was plated onto a sGC agar containing 50 µg/ml kanamycin (sGC-kan50). Six colonies were picked after

o/n incubation and separately cultured (shaking at 30 rev per min on a Platform Rocker STR6; Stuart Scientific) in 3 ml sBHI broth for 6 h. Bacteria from cultures showing growth after 6 h ($OD_{600} > 0.2$) were harvested by centrifugation and the pellet resuspended in 100 μ l sterile PBS. Gene-specific PCR and sequencing of the PCR product was used to confirm correct insertion of the *kanR*-cassette. Stocks of the remaining resuspended bacteria were stored at -80 °C until further use.

Once correct mutations were established in each case, genomic MC58 mutant DNA was used to retransform wildtype MC58, again selecting for kanamycin resistance. This step was taken to minimise the risk that any other strain 8013 genes inadvertently carried over in the first transformation with the *kanR*-cassette remained in the strains to be used in the present study. Gene specific PCR and sequencing of the PCR product was used to confirm correct insertion of *kanR*-cassette into the target gene.

Chapter 3

Establishing and characterizing a prolonged
meningococcal colonization model using cultured
human epithelial cells

3.1. Introduction

Many different cell lines have been used to study meningococcal host epithelial cell interaction, for example Chang (Virji *et al.*, 1992, 1993, 1995; Serruto *et al.*, 2003; Tavano *et al.*, 2011), 16HBE14 (Grifantini *et al.*, 2002), COR L23 (Li *et al.*, 2003), E6/E7 HTE (Neil *et al.*, 2009) and A549 (Virji *et al.*, 1996b; Takahashi *et al.*, 2008). The cell lines available in the present study for assessing bacterial host cell association were 16HBE14, COR L23, Chang, A549, E6/E7 HTE and MucilAir cells as described in Table 2.3 (Chapter 2). In this chapter, the selection of an appropriate cell line for a genome wide meningococcal transcriptional analysis of long-term host cell interaction is described. The qualities sought for the cell line to carry forward were i) the ability of MC58 to associate to the cell line over a period up to 21 days; ii) the formation and presence of tight junctions of the host cell monolayer, a characteristic of the nasopharyngeal epithelium in-vivo (Mygind and Dahl, 1998); iii) the expression of the putative meningococcal pilus-binding receptor CD46; iv) the ability of the cell line to survive prolonged co-cultivation with MC58; v) the relation of invasive to host cell adherent bacteria; and vi) visualizing meningococcal host cell adherence of the selected cell line using scanning electron microscopy.

A minimum of 1 µg of RNA from host cell-associated bacteria RNA is required for microarray analysis if RNA amplification and its attendant problems in data interpretation is to be avoided. To achieve this, at least ca. 10^7 CFU must be recovered, equivalent 2×10^5 host cell-associated bacteria per cm^2 host cell monolayer in a T75 flask (our unpublished preliminary results).

3.2. Results

3.2.1. Quantitative host cell association assays

Cell association was assessed over 21 days by quantitative host cell association assays were used to determine the total CFU of cell-associated bacteria. Therefore, plate grown bacteria were resuspended in cell culture medium and confluent host cell monolayers were infected at a MOI of 100:1. As the host cells were lysed, using saponin, to prepare cultures for bacterial enumeration at each time point, the recovered CFU represent the total number of adherent and invasive bacteria. Accordingly, parallel host cell invasion assays were performed to allow – by subtraction, the amount of host cell adherent bacteria to be determined.

Quantitative host cell association experiments were performed on each cell line for up to 21 days (time points: 1 h, 4 h, 24 h, 48 h, 72 h, 96 h and 21 days p.i.). To obtain statistically robust results, each experiment comprised three independent biological replicates with at least three technical replicates (Figure 3.1).

Numbers of recovered CFU were compared pairwise at each time point between different cell lines and the same cell line at succeeding time points. Significant differences in host cell adherent CFU were calculated using the Student's t-test (paired, two-tailed) and are shown in Table 3.1.

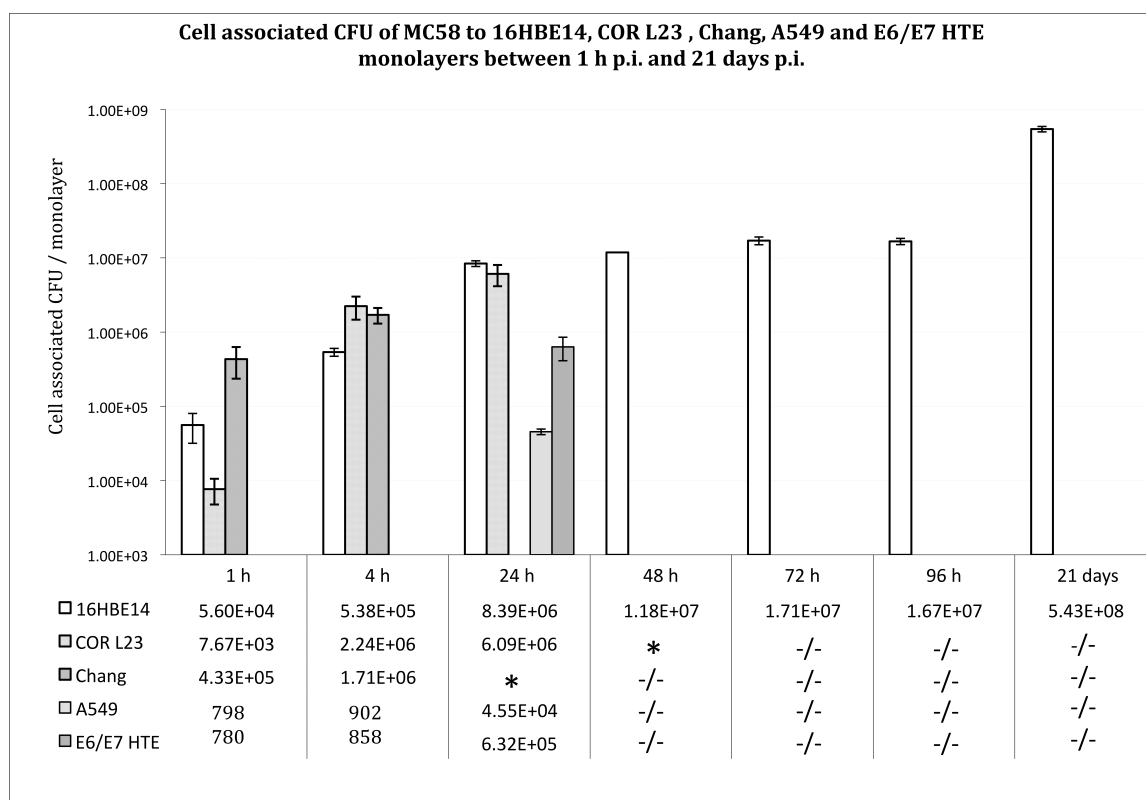


Figure 3.1. Cell associated CFU of MC58 recovered from host cell association experiments with 16HBE14, COR L23, Chang, A549 and E6/E7 HTE cells. Each experiment was performed as three biological replicates with at least three technical replicates. Error bars indicate SEM. Results with A549 and E6/E7 HTE cells at 1 h and 4 h p.i. were too low to be shown in the histogram at the chosen scale. *Disintegration and morphological deterioration of Chang monolayers occurred at 8 – 12 h p.i. and of COR L 23 monolayers at 28 – 30 h p.i., therefore CFU assays were not performed (indicated as -/-).

Table 3.1. P-value calculations for the adherent CFU to different cell lines sorted for each time point. Differences in cell associated CFU per cell line and per time point reaching significance ($p \leq 0.05$) are shown in black. P-values ≥ 0.05 are shown in grey. P-values were calculated using t-tests (paired, two-tailed).

Cell line 1	Cell line 2	P-value
1 h p.i.		
Chang	16HBE14	0.1032
Chang	COR L23	0.1148
Chang	A549	0.0950
Chang	E6/E7 HTE	0.0950
4 h p.i.		
Chang	16HBE14	0.9669
Chang	COR L23	0.3279
Chang	A549	0.0137
Chang	E6/E7 HTE	0.0136
Chang	Chang (1 h p.i.)	0.0132
COR L23	16HBE14	0.4189
COR L23	A549	0.0473
COR L23	E6/E7 HTE	0.0436
COR L23	COR L23 (1 h p.i.)	0.0402
16HBE14	A549	0.0019
16HBE14	E6/E7 HTE	5.61×10^{-4}
16HBE14	16HBE14 (1 h p.i.)	4.23×10^{-4}
A549	A549 (1 h p.i.)	0.3205
E6/E7 HTE	E6/E7 HTE (1 h p.i.)	0.6030
24 h p.i.		
COR L23	A549	0.0184
COR L23	E6/E7 HTE	0.0137
COR L23	COR L23 (4 h p.i.)	0.0092
16HBE14	COR L23	0.1039
16HBE14	A549	7.00×10^{-4}
16HBE14	E6/E7 HTE	5.63×10^{-4}
16HBE14	16HBE14 (4 h p.i.)	4.23×10^{-4}
A549	E6/E7 HTE	0.0080
A549	A549 (4 h p.i.)	0.0235
E6/E7 HTE	E6/E7 HTE (4 h p.i.)	1.52×10^{-4}
48 h p.i.		
16HBE14	16HBE14 (24 h p.i.)	0.0031
72 h p.i.		
16HBE14	16HBE14 (48 h p.i.)	0.0557
96 h p.i.		
16HBE14	16HBE14 (72 h p.i.)	0.0105
21 days p.i.		
16HBE14	16HBE14 (96 h p.i.)	0.0026

At **1 h** p.i., the host cell associated CFU recovered from Chang cells (4.33×10^5 CFU / monolayer) appeared to be higher when compared to 16HBE14 (5.60×10^4 CFU / monolayer), COR L23 (7.67×10^3 CFU / monolayer), A549 and E6/E7HTE cells (798 and 780 CFU / monolayer, respectively). However, the number of CFU recovered from Chang cell experiments at this time point was not found to be significantly higher compared to the other cell lines tested (p-values are shown in Table 3.1.).

At **4 h** p.i., the number of cell associated CFU had significantly increased in experiments performed with COR L23 (2.24×10^6 CFU / monolayer) Chang (1.71×10^6 CFU / monolayer) and 16HBE14 cells (5.38×10^5 CFU / monolayer) compared to 1 h p.i. data. Significant differences between the CFU of these three cell lines at 4 h p.i. by pair wise comparisons were not observed. However, compared to A549 (902 CFU / monolayer) and E6/E7 cells (858 CFU / monolayer) the numbers of cell associated CFU from Chang, COR L23 and 16HBE14 cells were significantly higher. A significant increase of cell associated CFU between 1 h p.i. and 4 h p.i. was not observed for A549 and E6/E7 HTE monolayers.

At time points between **8 h** and **12 h** p.i., and subsequently every 24 h, infected cell lines were monitored by light microscopy. At 8 h to 12 h p.i., Chang cell monolayers repeatedly disintegrated, as some cells were detached from the bottle surface and showing signs of cell death, including membrane blebbing and loss of circular shape (data not shown). Therefore, cell association experiments were not performed for time points >8 h with Chang cells.

At **24 h** p.i., significantly higher numbers of cell associated CFU were recovered from 16HBE14 cells (8.39×10^6 CFU / monolayer) than from E6/E7 HTE (6.32×10^5 CFU / monolayer; $p = 5.63 \times 10^{-4}$) and A549 (4.55×10^4 CFU / monolayer; $p = 7.00 \times 10^{-4}$) cells. There was no significant difference of recovered CFU from 16HBE14 and COR L23 cells (6.09×10^6 CFU / monolayer; $p = 0.1039$) observed.

By comparing numbers of CFU recovered from the same cell line at 24 h p.i. and 4 h p.i., a significant difference was observed for COR L23 ($p = 0.0092$), 16HBE14 ($p = 4.23 \times 10^{-4}$), A549 ($p = 0.0235$) and E6/E7 HTE cells ($p = 1.52 \times 10^{-6}$). Numbers of recovered CFU from A549 and E6/E7 HTE cell lines were

consistently below 10^6 CFU / monolayer at ≤ 24 h p.i. which was not sufficient to yield 1 μ g RNA.

Therefore, A549 and E6/E7 HTE cell lines were excluded from further cell association experiments.

At time points between 28 h and 32 h p.i., COR L23 cells repeatedly showed a similar pattern of cell death as that observed for Chang cells after 8 h – 12 h p.i. Therefore, COR L23 cells were excluded from further cell association experiments.

Cell association experiments were continued with 16HBE14 cells with assays at time points from 48 h to 21 days p.i. A significant increase of cell associated bacteria was observed for time points 48 h ($p = 0.0031$) and 21 days p.i. ($p = 0.0026$). P-value reflects significance of difference between consecutive assay results.

3.2.2. Trans-epithelial electrical resistance (TEER)

As the technologies have not been established in our laboratory to investigate the formation of tight junction on monolayers of epithelial cells grown on a plastic-surface, in the present study the ability to form tight junctions was tested for each cell line using a transwell system. It was also an intention to use transwells for validation of the findings from microarray analysis.

In order to test if the 16HBE14, COR L23 and Chang cell monolayers form tight junctions, one of the characteristics of human nasopharyngeal epithelium, the TEER of host cell monolayers grown in transwells was measured using Millicell-ERS (Millipore) electrodes (Figure 3.2). Only in the case of 16HBE14 monolayers was an increase of the TEER observed above the threshold of the formation of tight junctions ($>1000 \Omega\text{cm}^2$; Millicell-ERS manual) could be observed. This was observed from the time the monolayer grew confluent (48 h post seeding) until the end of the experiment (72 h). Although a slight increase in the trans-epithelial electrical resistance of COR L23 and Chang cell monolayers was observed after 48 h, values for both cell lines remained below the threshold for tight junctions throughout the experiment.

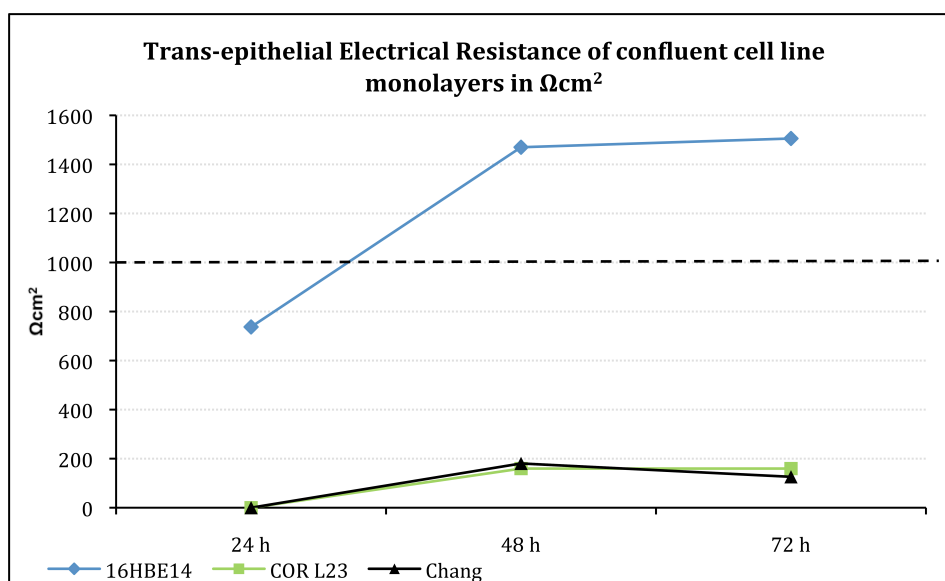


Figure 3.2. The TEER of 16HBE14, COR L23 and Chang cell monolayers over time. TEER values of a transwell without host cells were subtracted from each measurement. The presence of tight junctions is proposed at values of $>1000 \Omega\text{cm}^2$ indicated by the dashed line in the graph. The results showed that tight junctions were formed by 16HBE14 monolayers after 24 h of seeding but not by COR L23 or Chang cells.

3.2.3. CD46 western blot and rt-PCR

CD46 was suggested to be a receptor for meningococcal pili (Johansson *et al.*, 2003). Therefore, the expression of CD46 by A549, Chang, COR L23 and infected and non-infected 16HBE14 cells was assessed by immunoblotting (western blotting) and RT-PCR.

The Western blots were performed using an antibody directed against the cytoplasmic tail 1 (Cyt-1) of CD46 (Weyand *et al.*, 2006). CD46 protein could be detected in A549, Chang and 16HBE14 cells all presenting with bands in the range of 51 – 68 kDa (Figure 3.5).

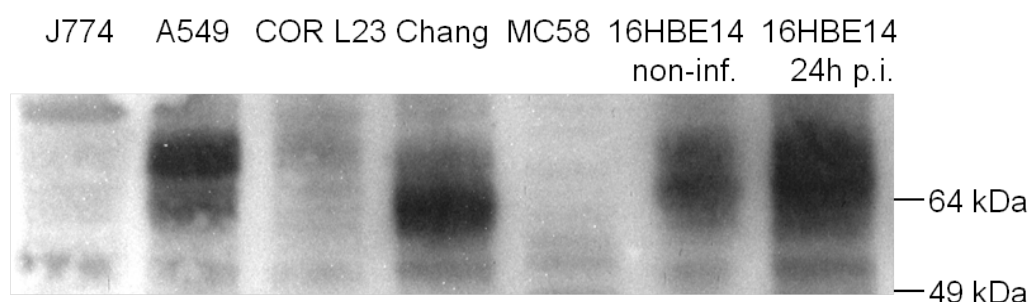


Figure 3.3. Western blot of electrophoretically-separated and membrane blotted whole cell protein extracts (20 μ g) for Cyt-1 (double band 51 – 68 kDa) detection of CD46. Western blots were performed on extracts from A549, COR L23, Chang and 16HBE14 cells (infected and non-infected). As negative controls J774, a mouse macrophage cell line, and MC58 protein extracts were used. Proteins of infected 16HBE14 cells were extracted at 24 h p.i.

Infected 16HBE14 cells gave a stronger signal than non-infected 16HBE14 cells, indicating a possible up-regulation of CD46 upon infection with MC58. The varying molecular weight (MW) between different cell lines could be explained by the prevalence of the large (59 - 68 kDa) or the small isoform (51 - 58 kDa) respectively (Liszewski *et al.*, 1991, Russell *et al.*, 1992, Hakulinen *et al.*, 2004). The high MW isoform appeared to be more abundant in protein extracts of A549 cells whereas the small isoform appeared to be more intense in protein extractions of Chang cells. In non-infected 16HBE14 cells a band of ca. 64 kDa, representing the high MW band was detected. Proteins of infected 16HBE14 cells showed a band at the range of both isoforms, which might be due to a high concentration of CD46 in infected 16HBE14 cells.

In order to detect *CD46* up-regulation upon infection with MC58, RT-PCR of non-infected and infected (24 h) 16HBE14 cells was performed using primers amplifying a 448 bp product (McLaughlin *et al.*, 2003) in the middle of the 1179 coding region of *CD46*.

Expression of *CD46* by RT-PCR could be detected in infected 16HBE14 cells but not in non-infected 16HBE14 cells indicating an up-regulation of *CD46* in 16HBE14 cells upon infection with MC58 (Figure 3.4).



Figure 3.4. RT-PCR for *CD46* expression of non-infected 16HBE14 (middle lane) and infected (24 h) 16HBE14 (right) monolayers besides a DNA marker (left). A band representing the amplified region of *CD46* (448 bp) was detected in infected 16HBE14 cells.

3.2.4. Viability of infected and non-infected 16HBE14 monolayers

MC58 infected and non-infected control 16HBE14 cell monolayers were stained with trypan-blue to determine cytotoxicity of MC58 (Figure 3.5). The monolayers were infected for 72 h, 96 h and 21 days and the viability of host cells at each time point was determined qualitatively by inspecting stained monolayers under a light microscope and quantitatively by using a counting chamber and calculating the relative number of viable cells. Infected 16HBE14 monolayers showed no visible difference to control monolayers over 21 days.

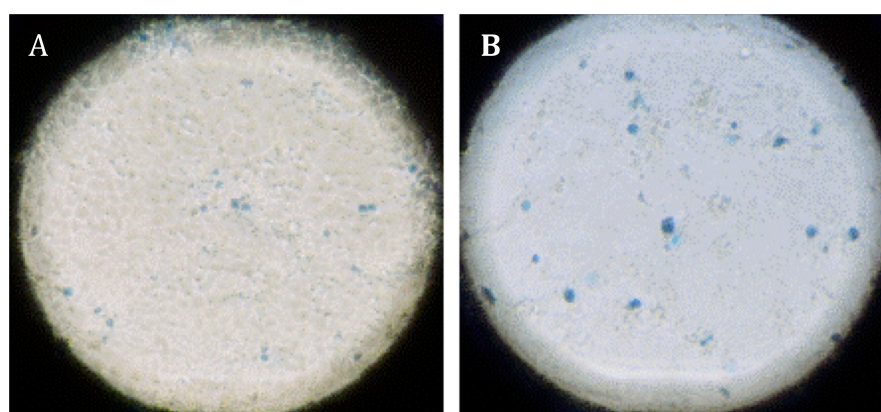


Figure 3.5. Representative images of a trypan-blue stained infected (A) and non-infected (B) 16HBE14 monolayer at 72 h p.i. at a magnification of 100.

3.2.5. Quantitative host cell invasion assays

In order to determine the extent of bacterial invasion of 16HBE14 cells over 21 days, gentamicin protection assays were performed (Figure 3.6). An increase in bacterial invasion of the monolayers over time (from 10 invasive CFU / monolayer at 4 h p.i. to 5.29×10^5 CFU / monolayer at 21 days p.i.) was observed. However, compared to the total numbers of cell associated CFU as shown in Figure 3.3., data from the invasion assays indicate that cell association of MC58 is mainly in form of adherence. Table 3.2 shows the percentage of invasion for each time point. The proportion of internalized bacteria was consistently $\leq 0.1\%$ over time.

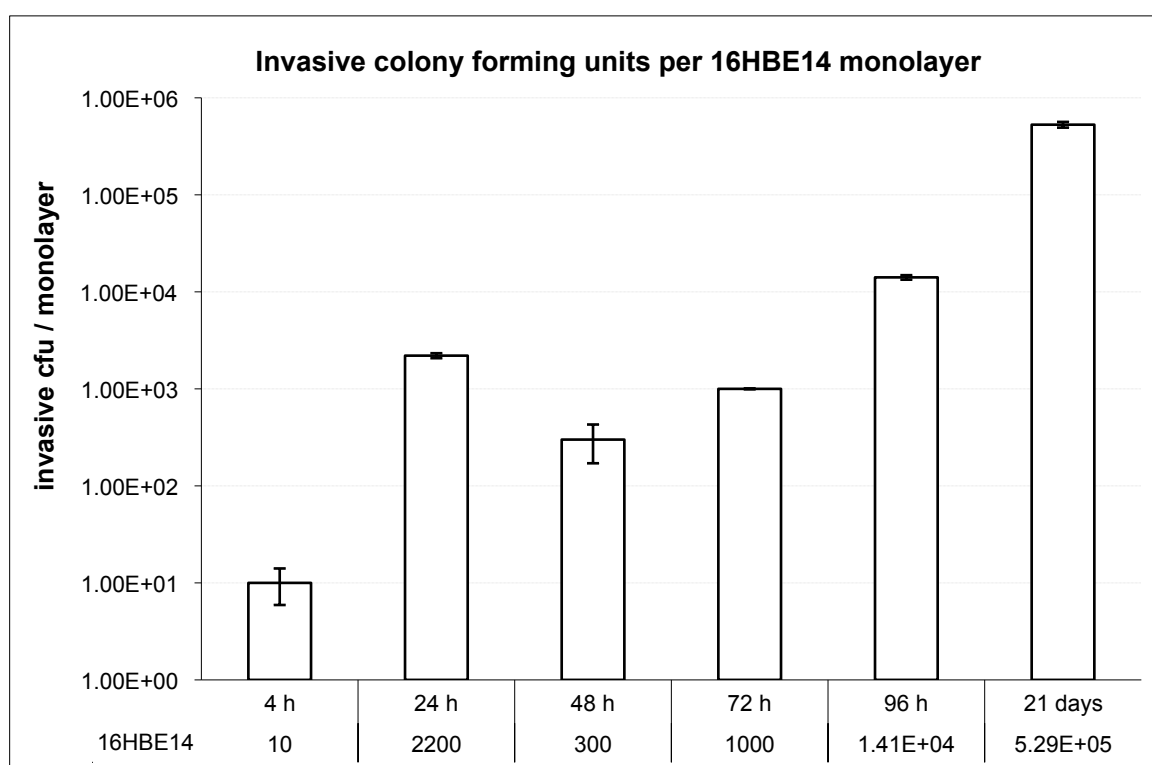


Figure 3.6. Invasive CFU per 16HBE14 monolayer (ca. $1-2 \times 10^5$ cells) over time. Experiments were performed with three biological replicates and at least three technical replicates. Data shown are representative of one experiment. Error bars indicate the SEM.

Table 3.2. Numbers of cell associated and invasive CFU / monolayer over 21 days and the relative number of invasion / monolayer.

Time point	4h	24h	48 h	72 h	96 h	21 days
Numbers of total cell associated and invasive (in brackets) CFU / monolayer	5.38x10 ⁵ (10)	8.39x10 ⁶ (2200)	1.18x10 ⁷ (300)	1.71x10 ⁷ (1000)	1.67x10 ⁷ (14100)	5.43x10 ⁸ (5.29*10 ⁵)
Percentage of invasion	0.02 %	0.03 %	0.003 %	0.006 %	0.08 %	0.1 %

3.2.6. Visualizing host cell adherent meningococci over time using scanning electron microscopy – an introductory description

Scanning electron microscopy (scanning EM) was used to visualize the bacterial - host cell and bacterial – bacterial interactions during prolonged co-cultivation. Increasing numbers of adherent bacteria were observed over time, consistent with the adherence experiments reported in the previous section (3.2.1).

In this section, morphological changes of host cells and adherent bacteria over time are presented.

Experiments were performed on 16HBE14 cells (4 h, 10 h, 24 h, 48 h, 72 h, 96 h and 21 days p.i.) and on the cultured primary human nasopharyngeal MucilAir cells (24 h and 96 h p.i.; obtained from Epithelix, Switzerland; described in Chapter 2).

Morphological changes of host cell associated meningococci encompassed the transition from single bacteria to bacterial aggregates or microcolonies (Mikaty *et al.*, 2009). A biofilm, aggregated bacteria embedded in an extracellular matrix, (Costerton *et al.*, 1999; Neil *et al.*, 2009; Lappann *et al.*, 2010), was visible at later stages of co-cultivation. As negative controls, non-infected 16HBE14 monolayers (24 h and at 21 days post seeding) and MucilAir monolayers were used.

On the host cell surface, structures evolving upon infection with meningococci were observed which were described to be potentially involved in maintaining meningococcal colonisation (Mikaty *et al.*, 2009). These host cell structures include elongated filaments in close proximity to adhering bacteria as well as larger membrane ruffles (Edwards *et al.*, 2000; Higashi *et al.*, 2009). Similar filamentous

structures have been reported to appear on infection of human primary cervical epithelial cells with gonococci (Edwards *et al.*, 2000).

Representative scanning EM photographs were taken after scanning the samples several times.

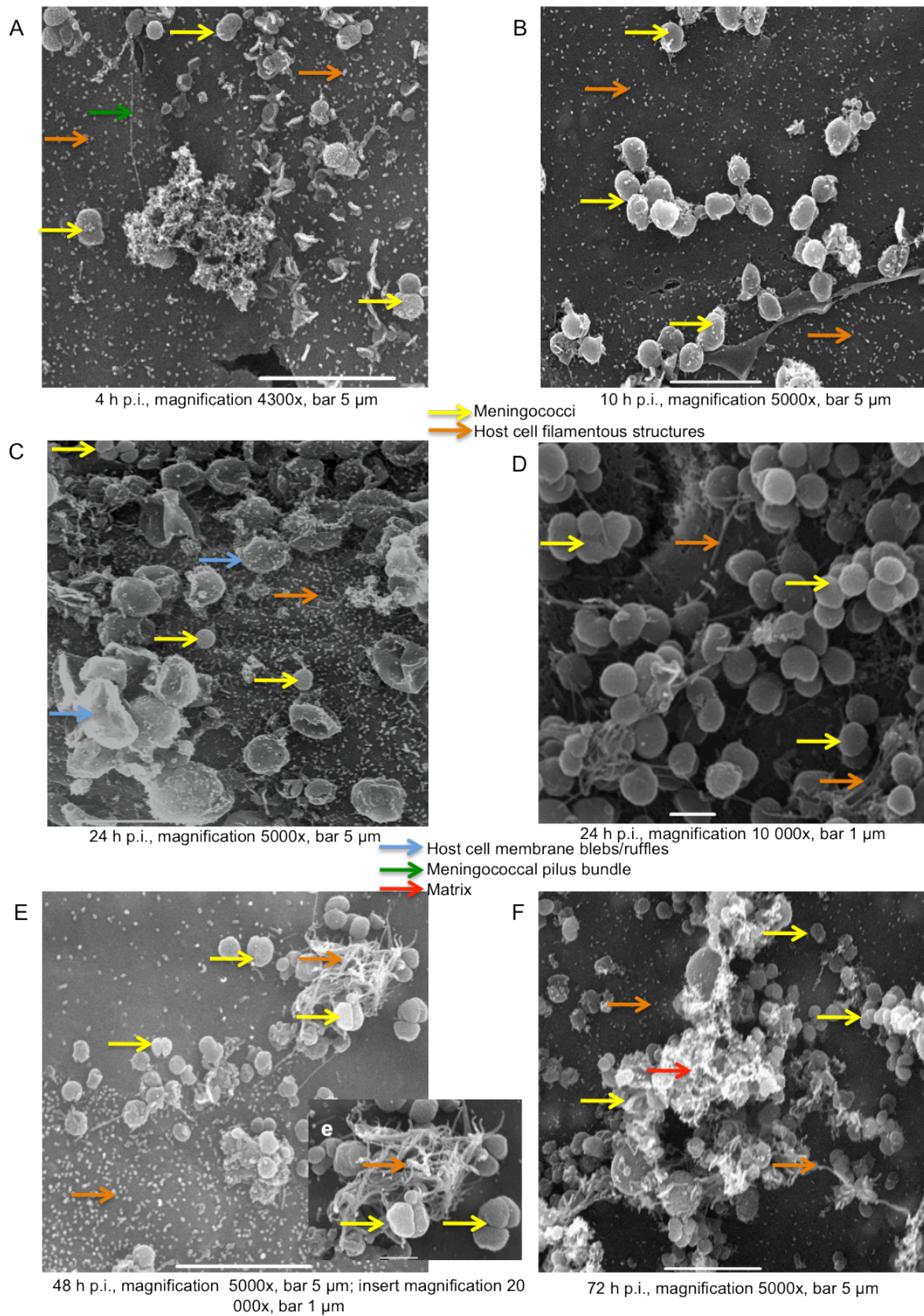
Photographs were regarded as supportive material for this study and I am aware that microscopy can be misleading and is no means for robust statistical analysis. In the next two sections, I present some of the scanning EM images with interpretation and analysis based on previous studies.

3.2.6.1. Comparing infected and non-infected 16HBE14 cells using scanning electron microscopy

Infected and control 16HBE14 monolayers were studied at co-cultivation time points from 4 h up to 21 days p.i. (Figure 3.7). At 4 h p.i. (panel A) scanty meningococci (yellow arrows), some of typical diplococcal appearance were observed on a relatively smooth host cell surface. Occasional short filamentous structures (orange arrow) on the host cell surface appeared, similar to those described as microvilli by Edwards *et al.* (2000). The green arrow points to a possible meningococcal pilus bundle in close contact with a filamentous structure of the host cell.

At 10 h p.i. (panel B), more meningococci (yellow arrows) are visible than at 4 h p.i. and grouped into small bacterial aggregates (microcolonies). The host cell surface shows a few filamentous membrane protrusions (orange arrow), some of them were elongated and in close contact to adherent bacteria. Where no bacteria were present the host cell surface appeared relatively smooth.

At 24 h p.i. (panels C and D) the host cell surface presented further structures including membrane ruffles and blebs; in panel C (blue arrow) similar to the one seen by Edwards *et al.* (2000) co-localized with single or diplococcal meningococci (yellow arrows); in other areas (panel D) with meningococci (yellow arrows) in aggregates (microcolonies,) in close contact with elongated filamentous host cell structures (orange arrows).



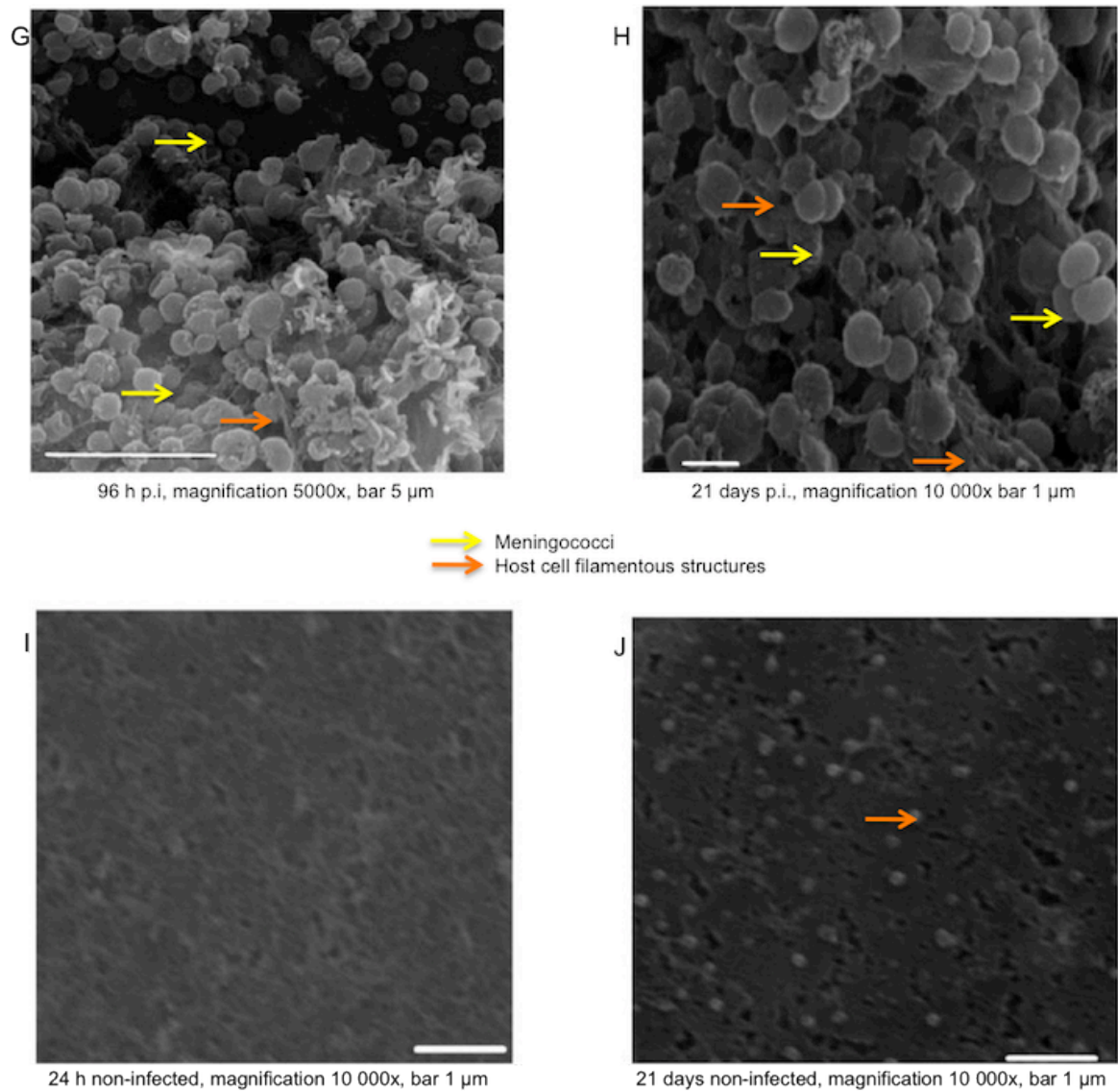


Figure 3.7. Scanning electron microscopy of MC58 infected 16HBE14 monolayers at 4 h (A), 10 h (B) 24 h (C and D), 48 h (E and insert e), 72 h (F), 96 h (G) and 21 days p.i. (H) and of a 24 h non-infected 16HBE14 monolayer (panel I) and 21 day old monolayer (panel J). White bars represent 5 µm in A – E and F- G and 1 µm in insert e and H-J.

At 48 h p.i. (panel E and insert e) meningococci (yellow arrows) were mainly seen as microcolonies. In contrast to the image at 24 h p.i. (panel C), no more membrane ruffles were seen. Host cell filamentous structures or membrane protrusions (orange arrows) appeared elongated in close contact with bacteria (at a higher magnification in the insert panel e) but remained short in areas where no adherent bacteria were present. Similar elongated filamentous structures, thought to be holding the meningococci on the host cell surface to resist shear stress, have been described as actin and/or ezrin filaments by Mikaty *et al.* (2009).

At 72 h p.i. (panel F) an increased number of host cell adherent meningococci (yellow arrows) was observed. Bacteria appeared in large aggregates (converged microcolonies) embedded in a formless mass (red arrow). Whether the origin of this mass is host cell and / or bacteria derived is unknown. Similar structures have been described as the matrix of a biofilm (Costerton *et al.*, 1999), suggested to be composed to a certain extend of bacterial DNA (Lappann *et al.*, 2010; Steichen *et al.*, 2011). Besides the aggregates, which did not cover the entire host cell surface, some individual bacteria were also present. Some short filamentous membrane protrusions (orange arrow) were seen, on a host cell surface, which was generally smoother than at 48 h p.i.

At 96 h p.i. (panel G) large meningococcal aggregates (yellow arrows) cover most of the host cell surface. The photograph in panel H was taken after 21 days p.i. and, compared to that of 96 h p.i., not much difference was seen. Bacterial aggregates composed of a large number of bacteria cover most of the host cell surface. Compared to earlier time points (24 h) the host cell surface is smoother.

Panel I and J show non-infected 16HBE14 cells. The photograph in panel I was taken at 24 h post seeding after cells reached confluence. The photograph in panel J was taken 21 days post seeding. The 24 h old monolayer (panel I) did not show any protruding membrane structures. The image is slightly out of focus, because the cell surface appeared to be smooth and therefore lacking a distinct structure to focus on. Cracks were considered artefacts of sample preparation prior to scanning EM examination. On the 21 day old non-infected monolayer (panel J) some short filamentous surface protrusions were visible

(orange arrow), comparable to the short microvilli seen at 4 h p.i. on infected cells (panel A, orange arrow).

In order to further investigate the nature of the filamentous structures seen especially 48 h p.i. (Figure 3.7, panels E and insert e), i.e. if they are of host cell origin or composed, for example, of meningococcal type IV pilus bundles, 16HBE14 monolayers were infected for 24 h and for 96 h with a MC58 *pilE*-mutant, which lacks expression of type IV pili. Scanning EM photographs are shown in Figure 3.8.

At 24 h p.i. (Figure 3.8, panels i and ii) filamentous membrane protrusions are visible (orange arrow) being elongated where meningococci (yellow arrow) were present and similar to those observed during infection with wild type meningococci (Figure 3.7, panels D, E and insert e). Membrane ruffles (red arrows) were seen at 96 h p.i. with *pilE*- (Figure 3.8. panels iii and iv) similar to those seen at 24 h p.i. with the wild type (Figure 3.7, panel C).

In summary, the overall morphology of the host cell surface appeared to be similar between infection with the wild type or the *pilE*- mutant MC58 strain, although host cell surface structures appeared more prominent when infected with wild type bacteria. A possible explanation may be the difference in the number of host cell associated bacteria, i.e. wild type meningococci adhered to host cells in greater numbers than the *pilE* mutant did.

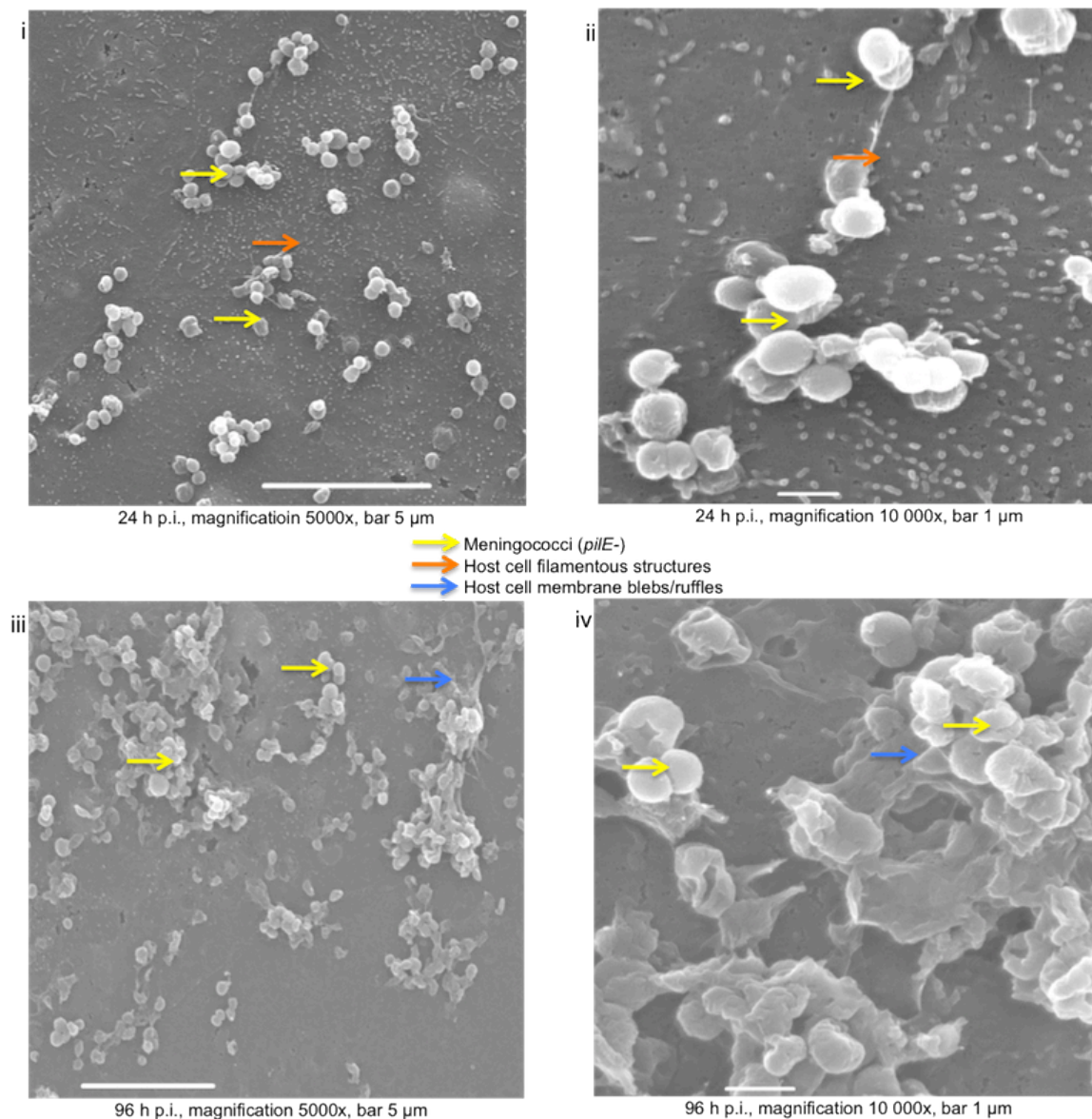


Figure 3.8. 16HBE14 cells infected with MC58 *pilE*- mutant (yellow arrows) for 24 h (panels i and ii) and 96 h (panels iii and iv). White bars in panels i and iii represent 10 μ m, in ii and iv 1 μ m. Short and elongated filamentous surface protrusions of the host cell could be seen at 24 h p.i., indicated by orange arrows (panels i and ii). Elongated filamentous protrusions were present in close contact to meningococci. At 96 h p.i., filamentous protrusions were reduced. Membrane ruffles (blue arrows in panels iii and iv) evolved around meningococci (yellow arrows).

3.2.6.2. Imaging of infected and non-infected MucilAir monolayers

MucilAir monolayers are composed of fully differentiated, cultured primary human nasopharyngeal epithelial cells. Containing ciliated, basal and mucus producing cells, MucilAir monolayers mimic the human nasopharyngeal epithelium (www.epithelix.com). MucilAir monolayers were used to visualize the associative morphology of wild type meningococci at 24 h p.i. and 96 h p.i., respectively, by scanning EM (Figure 3.9) in comparison with non-infected control monolayers. Scanning EM photographs were also compared to those obtained from experiments with 16HBE14 cells.

As shown in Figure 3.9, the non-infected cell surface (panels A and B) was relatively smooth. The three different cell types (ciliated, mucus producing and basal cells) could not be clearly distinguished. In some areas, short membrane protrusions were visible similar to those observed on non-infected 16HBE14 cells (21 days post seeding, Figure 3.7, panel J) and described by Mikaty *et al.*, (2006). In panel A (Figure 3.9), a relatively clear dividing line separating a smooth area of the monolayer surface and an area with short membrane protrusions is indicated by an orange arrow. In panel B (Figure 3.9), the orange arrow indicates a single short membrane protrusion on the cell surface at a higher magnification.

After 24 h p.i. with meningococci (Figure 3.9, panels C – E), the orange arrows indicate elongated membrane protrusions in close proximity to meningococci (yellow arrow), which were mainly present as singular diplococci and small bacterial aggregates. With exception of the prolonged membrane protrusions, mainly present in areas where meningococci adhered (panel E orange arrows) no other surface structures were observed. At 24 h p.i. 16HBE14 cells showed membrane ruffles and blebs (Figure 3.7, panel C). Prolonged membrane protrusions appeared to be more abundant on infected MucilAir than on 16HBE14 cells at 24 h p.i. (Figure 3.9, panels D and E; Figure 3.7, panels E and insert e (48 h p.i.))

On MucilAir monolayers, meningococci appeared to adhere preferentially to one region / type of cells as can be seen in panels C and F (Figure 3.9), unlike the observation made on 16HBE14 cells, in which bacteria were more evenly distributed on the monolayer surface. However, bacterial aggregates (at time

points ≥ 48 h) did not cover the entire 16HBE14 surface indicating a possible preference of meningococci to adhere to certain regions of cells (16HBE14 cells are believed to be composed of one cell type only).

At 96 h p.i., compared to 24 h p.i., bacteria associated with MucilAir cells have increased in number (Figure 3.9, panels F-H), although the numbers appeared to be lower if compared with the ones on 16HBE14 cells. Meningococci (Figure 3.9, yellow arrows) formed occasional small aggregates (panels F-H) although mainly present as individual diplococci (panel F). On 16HBE14 cells, larger bacterial aggregates were observed, covering most of the host cell surface. On MucilAir cells, the prolonged membrane protrusions present at 24 h p.i. were reduced at 96 h p.i. Only a few could be observed in close proximity to adhering bacteria (orange arrow Figure 3.9, panel H).

In summary, visualization of meningococcal association with MucilAir cells showed increasing numbers of adherent bacteria over time (as with 16HBE14 monolayers) but qualitative and possibly quantitative differences were apparent at 96 h p.i. comparing with 16HBE14 monolayers. A tropism of meningococci to adhere to certain cells or regions was observed in particular for MucilAir monolayers (plausibly a preference towards a particular differentiated phenotype).

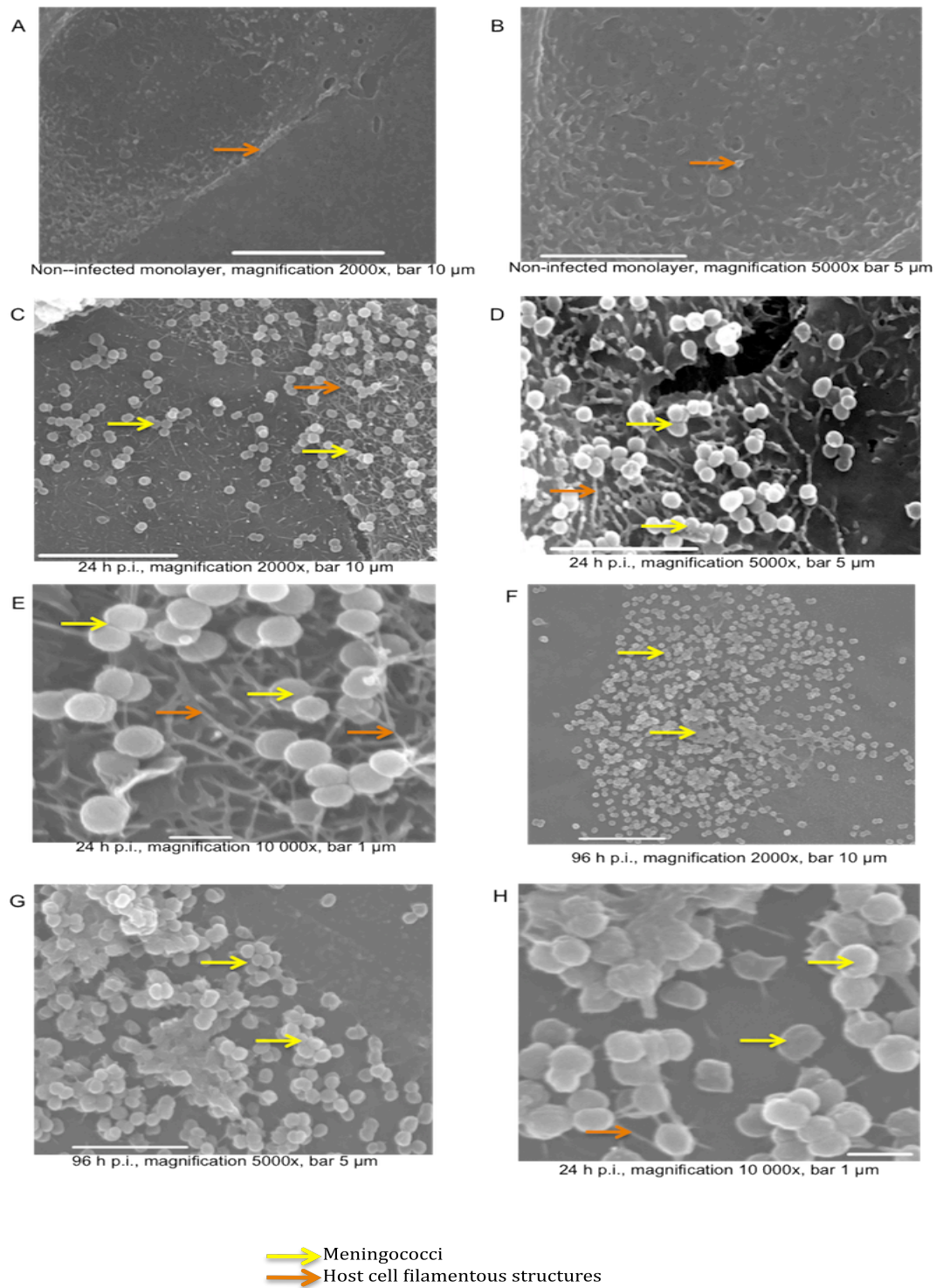


Figure 3.9. Scanning EM photographs of MucilAir cells non-infected (panels A and B) and infected with MC58 wild type at 24 h (panels C-E) and 96 h (panels F-H). White bars in panels A, C and F indicate 10 μm , in panels B, D and G 5 μm and in panels E and H 1 μm .

3.3. Discussion

Based on the results of the experiments described in this chapter, 16HBE14 cells were selected as the optimal cell line from the range considered to model nasopharyngeal colonisation. This decision was made in particular because 16HBE14 cells survived co-cultivation with MC58 for up to 21 days. In addition, meningococcal - host cell association experiments over 21 days indicated an increase of cell associated CFU to 16HBE14 cells, sufficient for future bacterial RNA extractions from time points as early as 4 h p.i. The increase in cell association reflected adherence of MC58 rather than invasion, which was minimal (<0.1% at all time points). A supportive finding of using 16HBE14 cells is the expression of CD46, discussed as neisserial pilus receptor as described in Chapter 1 (Kirchner *et al.*, 2005; Johansson *et al.*, 2003). On probing electrophoretically separated whole cell protein extracts of MC58 infected and non-infected 16HBE14 monolayers with the Cyt-1 antibody, CD46 specific bands were of greater intensity in the former than the latter. These results suggest that MC58 infection of 16HBE14 increased expression of CD46. Taken together with the results of the RT-PCR, showing a band in infected 16HBE14 but not in non-infected 16HBE14 cells, further supports the finding that infection with MC58 up-regulates CD46 expression in 16HBE14 cells.

Two of the tested cell lines, Chang and COR L23, had to be excluded from further consideration as neither survived prolonged co-cultivation with MC58. Whether cell death occurred due to apoptosis or necrosis was not investigated. Cell death due to nutrient competition was unlikely as non-adherent bacteria were washed away and cell culture medium was renewed 4 h p.i., and then twice a day for experiments >4 h. In a study by Deghmane *et al.* (2009), it was shown that many disease causing strains cause apoptosis of human endometrial adenocarcinoma (Hec-1-B) cells. MC58 mediated induction of apoptosis has been shown of HBMEC cells by Schubert-Unkmeir *et al.* (2010). This was considered to be due to breaking of tight junctions by induction of MMP-8 (metalloproteinase 8) resulting in disintegration of the monolayer (Schubert-Unkmeir *et al.*, 2010). Stephens and Farley (1991) described the breakdown of tight junctions in human nasopharyngeal tissue explants after infection with

meningococci. However, using live meningococci (strain H44/76) and purified meningococcal PorB, Massari *et al.* (2000, 2003 and 2010) described prevention of host cell apoptosis (HeLa cells). These findings might suggest a cell type and meningococcal strain dependent induction / prevention of apoptosis. Other possibilities of cell death upon infection with meningococci are the differing number of cell associated CFU when comparing cell lines (for example, Deghmane *et al.* (2009) described induction of apoptosis by adherence of virulent strains to host cells (Hec-1-B cells) but not by invasion) and the cell state i.e. cell cycle of host cells.

A549 and E6/E7 HTE cells were excluded from further experiments because the association of MC58 in each case was impractically low. Sufficient amounts of RNA of host cell adherent bacteria could not be extracted.

In accordance with the quantitative host cell association experiments, increasing numbers of host cell adherent meningococci were observed over time by scanning EM. The formation of bacterial aggregates (Figure 3.7. panels D, E, e and F and Figure 3.9. panels F-H), equivalent to structures in the literature described as microcolonies (Mikaty *et al.*, 2009) were seen. Large bacterial aggregates, covering most of the host cell surface and embedded in a matrix was shown (Figure 3.7. panels F, G and H).

The host cell surface of both, non-infected 16HBE14 and MucilAir monolayers was relatively smooth (Figure 3.7. panel I and Figure 3.9. panel A and B). The formation of other host cell structures, e.g. prolonged filaments, ruffles and microvilli, in particular seen on photographs at 24 h p.i. of 16HBE14 (Figure 3.7. panel C) and on MucilAir cells (Figure 3.9. panels C-E), were hypothesized to have been induced by bacteria. Mikaty *et al.* (2009) described meningococcal association triggered filamentous structures (in Hec-1B cells), similar to those seen in Figure 3.7, panels E and e. These filamentous structures were described to evolve in response to shear stress which occurs especially in the blood stream and to a lesser extent in the human nasopharynx due to mucus flow, sneezing and coughing (Mikaty *et al.*, 2009).

Higashi *et al.* (2009) described induction of filamentous membrane protrusions of A431 cells (human epidermoid carcinoma cell line) by gonococci. The filamentous membrane protrusions (named elongated microvilli) were found in close proximity to gonococcal microcolonies. Induction of microvillous elongation was suggested to be due to gonococcal pilus retraction, exerting a force on host cell structures. Higashi *et al.* (2009) suggested that the increase of the host cell surface area by these filamentous structures was advantageous for bacterial adherence. Pilus retraction was suggested to induce the elongation of the filamentous membrane protrusions as these structures were not formed after infection with a *pilT*- mutant, which is not able to retract type IV pili (Higashi *et al.*, 2009). Agreeing with the observations of Mikaty *et al.* (2009), Higashi *et al.* (2009) observed that bundles of type IV pili were attached to the elongated microvilli. During infection of primary human cervical epithelial cells with gonococci, microvillous structures, membrane ruffles and lamellipodia similar to those observed at 24 h p.i. (Figure 3.7, panel C) of 16HBE14 cells were described (Edwards *et al.*, 2000). These host cell structures were shown to be composed mainly of α -actin, vinculin and ezrin. Membrane ruffles and lamellipodia were not seen on infected MucilAir cells.

Using 16HBE14 cells, filamentous membrane protrusions were also seen upon infection with a *pilE*- mutant although to a lesser extent than observed with wild type meningococci. In the present study investigations into the molecular composition of the filamentous host cell structures were not performed. Therefore, conclusions were only drawn due to the observation of similar morphology of the host cell filamentous structures to those described in the literature. It remains unclear whether the origin and / or composition of the filamentous host cell structures observed in this study were the same as described by Mikaty *et al.* (2009) and Higashi *et al.* (2000).

MC58 association with cells in MucilAir monolayers was observed to be non-uniform, suggesting preferential adherence to one differentiated cell type over another (Figure 3.9) on MucilAir monolayers. Where bacteria adhered, small microvillous structures appeared elongated. A preference of meningococcal binding of non-ciliated nasopharyngeal epithelial cells has been described by Read *et al.*

(1995), who visualized adherence to human nasopharyngeal tissue explants at 4 h p.i. and 24 h p.i. Preference for non-ciliated cells was especially described at 4 h p.i. Loss of cilia and increased discharge of mucus, compared to non-infected explants, was described at 4 h p.i. Membrane blebbing and tissue injury was observed at 24 h p.i. Bacteria were found to adhere to ciliated and mucus-producing cell types at 24 h p.i. (Read *et al.*, 1995). Similar morphological changes, including loss of cilia, altered appearance of microvilli, and cell tropism of non-ciliated cells was described by Rayner *et al.* (1995), in particular for piliated meningococci. Stephens and Farley (1991) described the loss of cilia by infection with meningococci in a nasopharyngeal tissue organ explant. By using scanning EM imaging, a difference between ciliated and non-ciliated MucilAir cells was not observed. Transmission electron microscopy (TEM) would be a suitable approach to resolving the question of which cell type was preferred for association by meningococci. Using TEM, ciliated, goblet and basal cells should be distinguishable. Cell damage or loss of cilia was caused by infection with meningococci would be a plausible explanation for not being able to distinguish the different cell types. However, ciliated cells could not be identified in non-infected MucilAir cells. Some areas of the non-infected monolayer were showing a few short membrane protrusions, which were not present at other areas. If these were ciliated cells, which have, besides cilia, microvillous structures (Mygind and Dahl, 1998), remains unclear. Cell damage and loss of cilia during sample processing could explain these observations.

The clearly demonstrated change in the adherence morphology over time from single bacteria to aggregates covering most of the host cell surface (>72 h p.i.) might indicate a change in the meningococcal transcriptome over this period. Compared to plate or planktonic growing bacteria, a transcriptional change during attachment to an abiotic or host cell surface was previously shown. Transcriptional changes were in particular observed on meningococcal surface structures (Grifantini *et al.*, 2002; Dietrich *et al.*, 2003; O'Dwyer *et al.*, 2009). The meningococcal transcriptome during prolonged host cell association will be considered in the following chapters.

Chapter 4

Optimization of methods to isolate meningococcal RNA from the colonization model

4.1. Introduction

When extracting total RNA from a mixture of bacteria and human cells, it resulted in an RNA mixture predominantly of human origin. Since in the present study the meningococcal transcriptome during co-cultivation with human epithelial cells will be investigated, the presence of large amounts of human RNA should be avoided. Firstly, it will hamper the efficiency of RNA labelling, as large proportions of the fluorescent dye and enzyme (reverse transcriptase), will be consumed by human RNA. Secondly, it will negatively influence the hybridization of labelled meningococcal RNA to cognate sequences on microarray, as increasing amounts of human RNA would promote their non-specific binding with neisserial gene targets (Hinton *et al.*, 2004; Di Cello *et al.*, 2005). Separating meningococci from tightly associated human epithelial cells is the key to achieve relatively pure meningococcal RNA. However, if this separation is taking any considerable length of time to achieve (transcriptional changes of bacteria have been found within 10 min), the meningococcal transcriptome needs to be conserved using cold temperature or chemical reagents (Chuang *et al.*, 1993; McGarvey and Quandt, 2003). In this chapter, the optimization of the procedures to obtain meningococcal RNA with minimum human RNA contamination is described.

4.2. Results

4.2.1. Optimizing procedures to obtain meningococcal RNA from host cell co-cultivation

Three methods of separating meningococci from human cells were tested. The Saponin (Grifantini *et al.*, 2002) and RLT (Di Cello *et al.*, 2005) methods are based on differential lysis of human cells, consequently leaving meningococci intact and collected by centrifugation. The trypsin method (Dietrich *et al.*, 2003) is based on enzymatic separation of meningococci and epithelial cells and followed by differential centrifugation to obtain meningococci. Key procedures and principles are described in following paragraphs, detailed protocols can be found in the Chapter 2.

The key step of the **Saponin** method was to add saponin to infected 16HBE14 monolayers and incubated on ice for 15 - 20 min, until monolayers detached from the bottom of the culture flask and host cells lysed. RNAprotect, a proprietary reagent developed by QIAGEN to conserve the transcriptome, was added and bacteria recovered by centrifugation for 10 min. The bacteria pellet was lysed in Trizol and RNA extracted. The total time before RNA conservation was > 15 - 20 min.

RLT method: RLT buffer, a proprietary reagent developed by QIAGEN to lyse cells, was added to infected 16HBE14 monolayers, which immediately lysed the host cells. The bacteria-cell suspension was vortexed and centrifuged for 10 min. The resulting bacteria pellet was lysed in Trizol and RNA extracted. The total time before RNA conservation was > 10 min.

Trypsin method: 10 X Trypsin-EDTA (0.5% trypsin, 0.2% EDTA) was added to infected 16HBE14 monolayers and incubated at 37°C for 5 - 8 min, compared to the protocol by Dietrich *et al.* (2003) who used 1 x trypsin EDTA, and required 20 – 30 min to achieve cell detachment. The bacteria – host-cell suspension was vortexed and centrifuged for 3 min (instead of 10 min as described by Dietrich *et al.* (2003)), before RNAprotect was added to the supernatant (containing bacteria). Bacteria were recovered by centrifugation and the pellet lysed in Trizol before RNA extraction. The total time before RNA conservation was 10 – 12 min (when performed according to Dietrich *et al.* (2003): 30 – 40 min).

In order to select the best protocol, the resulting RNA was further analysed for quality and quantity.

Bacterial RNA was quantified using the Nanodrop and the quality analyzed using capillary gel electrophoresis (Bioanalyzer). RNA quality was evaluated based on i) by the relative height of peaks and strength of bands corresponding of bacterial RNA (16S and 23S ribosomal RNA bands) in the electropherogram and gel-like images compared to (inevitable) contaminating human RNA (18S and 28S ribosomal RNA bands); ii) by the level of RNA integrity (samples appearing in a smear on the generated gel-like image were excluded); and iii) the yield of >1 µg of total RNA concentration which was the minimal amount required for microarray hybridization experiments.

4.2.2. Comparison of RNA quality and quantity after different methods of RNA preparation

RNA yields were highest when the saponin method was used. However, on analysis most samples were found to retain substantial amounts of human RNA, suggesting that lysing of host cells was not sufficient to remove host cell RNA.

The use of RLT buffer was found to lyse both, host cells and meningococci. In contrast to *E. coli*, on which this method was used (Di Cello *et al.*, 2005), *N. meningitidis* did not survive treatment with RLT and the RNA yield was inadequate (data not shown).

The best bacterial RNA quality with low to non-detectable human RNA contamination was achieved when the trypsin-EDTA method was used to separate host cells from bacteria prior to RNA extraction. However, when this method was performed as described by Dietrich *et al.* (2003) a minimum of 30 min was required to separate meningococci from host cells. This imposed an unacceptable risk of change in the bacterial transcriptome in response to the reagents present, such as the trypsin-EDTA. To shorten the time before the bacterial transcriptome was conserved, the concentration of trypsin-EDTA was increased ten-fold, i.e. 0.5 % trypsin and 0.2% EDTA was used. This protocol accelerated the detachment of 16HBE14 monolayers to ca. 5 min. Together with a reduction in time of the differential spin to 3 min (instead of 10 min), the total time of separating bacteria from host cells was reduced to ca. 10 min. The reduction in time of the differential spin was experimentally tested by quantification of contaminating human RNA in the supernatant (which should be free of human cells after centrifugation; data not shown).

Bioanalyzer images of representative samples of extracted RNA following the saponin trypsin-EDTA protocols are shown in Figure 4.1. In panel A (gel-like image), independently prepared RNA samples extracted after the trypsin-EDTA protocol (lane 1 and 2) show strong bands of bacterial 16S (black arrow) and 23S rRNA (blue arrow) without any signs of RNA degradation. A weak band of human 28S rRNA (orange arrow) was present in lane 2.

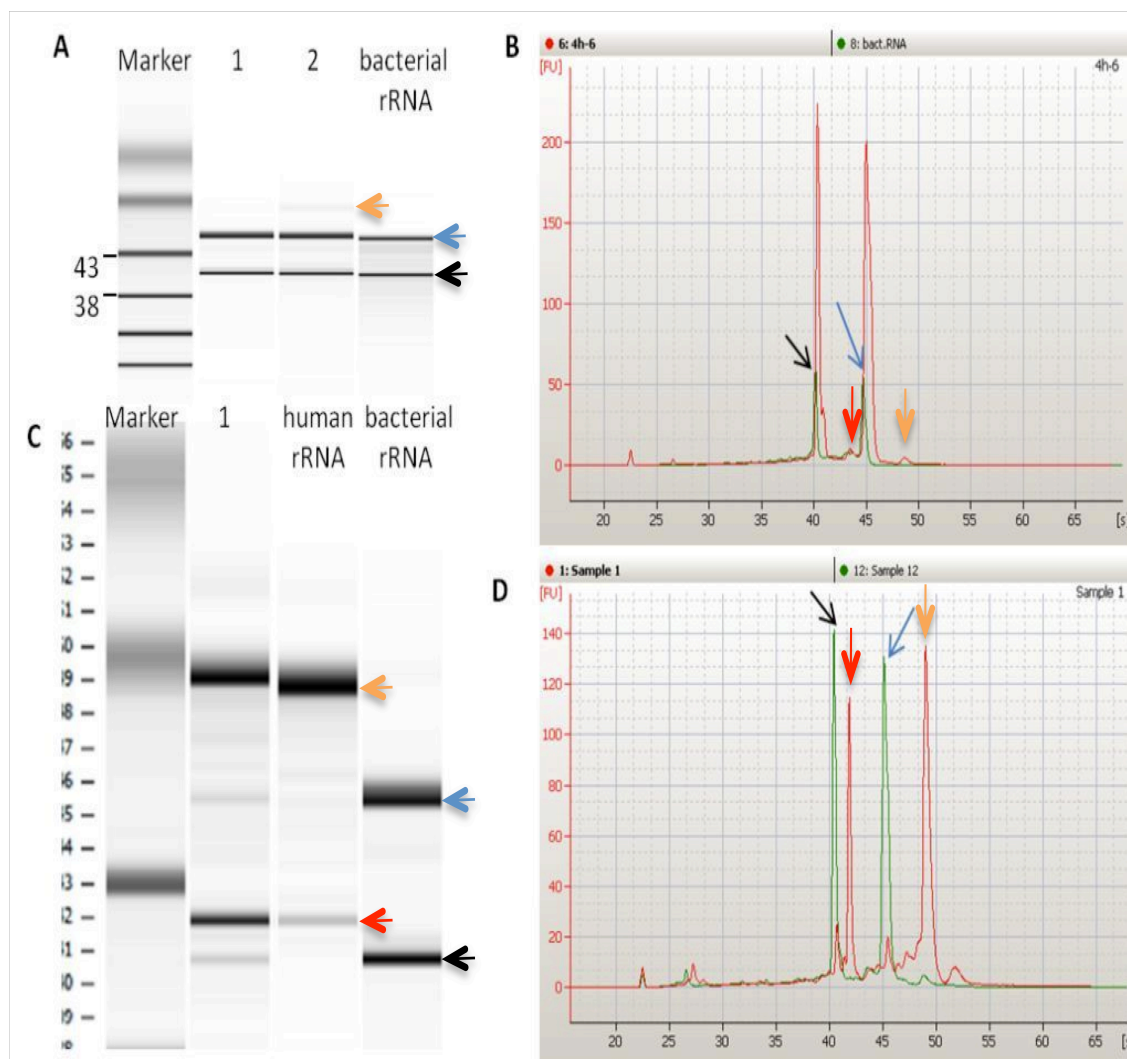


Figure 4.1. RNA extractions analyzed using a Bioanalyzer 2100 (Agilent). Gel-like images are shown in panels A and C; panels B and D show electropherograms. Bacterial 16S and 23S rRNA are indicated by black and blue arrows, respectively. Human 18S and 28S are indicated by red and orange arrows, respectively.

In panel A two bacterial RNA samples extracted using the trypsin-EDTA method are shown (lane 1 and 2) besides a bacterial rRNA control. In panel B an RNA sample extracted using the saponin method is shown besides a human rRNA and bacterial rRNA control. Panel B shows in an electropherogram the overlay of the bacterial RNA control (green) and one of the samples (red) extracted after the use of the trypsin-EDTA method. Peaks of 16S RNA (black arrow) and 23S RNA (blue arrow) in the control and sample preparations overlapped. The RNA concentration of the sample was higher than that of the control RNA

resulting in higher peaks of the sample than the marker. The sample shows two additional very small peaks (red and orange arrows) of human 18S and 28S rRNA. No further signals, indicating RNA degradation or significant amounts of human RNA are visible.

Panel C shows a gel-like image of a representative RNA sample (lane 1) extracted after the use of the saponin method. As controls, human RNA and bacterial RNA were analyzed alongside. Although the sample shows faint bands representing bacterial RNA (16S and 23S rRNA marked with black arrow and blue arrows, respectively), the sample shows bands of much greater intensity representing human RNA (18S and 28S rRNA shown by red and orange arrows).

In panel D the same sample is shown as in panel C lane 1 in an electropherogram. The bacterial control RNA (green) is overlaid with the sample (red). Of the bacterial control RNA, 16S and 23S rRNA peaks are indicated by black and blue arrows. In the saponin derived sample, peaks at these levels are visible but much lower compared to the high peaks representing human 18S (orange arrow) and 28S (red arrow) rRNA.

4.3. Discussion

The saponin protocol, previously used in meningococcal host cell transcriptome studies (Grifantini *et al.*, 2002), has some advantages, e. g. that the time before the bacterial transcriptome can be conserved is relatively short (incubation with saponin was for 10 min before RNAprotect was added to conserve the transcriptome) and that saponin works on ice which decreases metabolic activity and therefore may stabilize the meningococcal transcriptome changes during the protocol (cold-shock genes might show a change). However, the extracted RNA was highly contaminated with human RNA and most samples showed signs of severe degradation. Therefore, the saponin method was not used in the present study. None of the RNA extractions using the RLT protocol yielded sufficient material to be carried forward for

analysis on the Bioanalyzer as concentrations were extremely low (ca. 3 ng / μ l compared to ca. 50 – 1000 ng / μ l using the trypsin-EDTA method).

The results clearly indicate that the optimized trypsin-EDTA method gave the best RNA quality among the methods tested. Further RNA extractions of host cell adherent bacteria were performed using the optimized trypsin-EDTA method. For microarray hybridisation 1 μ g of RNA was required (recommended by the BμG@S Group). The total RNA yield was depending on the time point taken, between 2 μ g to 40 μ g per extraction. Further RNA extractions of host cell adherent bacteria were performed using the optimized trypsin-EDTA method.

Evaluating the quality of extracted RNA is crucial prior to gene-expression experiments such as microarrays and q-RT-PCR (Hinton *et al.*, 2004; Fleige and Pfaffl, 2006) which are subsequently performed in this study. Of different approaches, using a Bioanalyzer to examine RNA quality has several advantages. A volume of 1 μ l containing 2 – 500 ng RNA can be analyzed, and in greater detail than is, for example, by using gel-electrophoresis. A computer generated gel-like image is created, as well as an electropherogram displaying peaks proportional to RNA concentration and positioned according to RNA fragment size. Beyond these two visual outputs, the bioanalyzer program offers calculations such as the 23S to 16S ratios. The ratio of 23S to 16S indicates the degradation of the sample. A widely accepted number of 1.5 – 2.0 shows good quality rRNA. In this study, samples with an A260/A280 ratio of 1.9 – 2.2, 23S to 16S ratio of 1.5 – 2.0 were used in further experiments.

Chapter 5

Genome wide transcriptional profiling of
meningococci colonizing human epithelial cells

5.1. Introduction

In the previous two chapters, a meningococcal colonisation model using cultured human 16HEB14 cells was established and the methods for preparing meningococcal RNA from the host cell model were optimized. These have laid necessary foundations for obtaining and analysing the meningococcal transcriptome upon interaction with human epithelial cells.

DNA microarray technology was initially developed by Patrick Brown's research group at Stanford University and in 1995, the first report using microarray technologies to study gene expression patterns in *Arabidopsis* was published (Schena *et al.*, 1995). Since then DNA microarrays have developed into a reliable high-throughput technology in analysing transcriptomes and genome comparison of a vast variety of bacterial pathogens (reviewed in La *et al.*, 2008). In our laboratory (and in collaboration with others) the experiences in using DNA microarray have to a certain extent mirrored the general progress of microarray technologies: from using an early version macroarray (DNA printed on nylon membranes) (Li *et al.*, 2001), moving on to glass slides printed with PCR products (Redfield *et al.*, 2005; Li *et al.*, 2009; O'Dwyer *et al.*, 2009; Beddek *et al.*, 2009; Bossé *et al.*, 2010), and recently using high density arrays printed with oligonucleotide DNA probes (on mammalian genomes) (Ribas *et al.*, 2010; Wong *et al.*, 2011). Compared to PCR arrays, the DNA probes on oligonucleotide arrays have increased gene-sequence specificity, unlike long PCR products carrying ambiguous sequences homologous to other genes. At the beginning of the present study, the oligonucleotide based pan-Meningitidis array became available through collaboration with the Bacterial Microarray Group at St George's (BμG@S) (<http://bugs.sgul.ac.uk>) (described in Section 2.10.1, Chapter 2). In this chapter I will present the transcriptional profiling of meningococci during interaction with human epithelial cells in a colonisation model using the Pan-Meningitis array.

5.2. Results

5.2.1. Samples and hybridization of Pan-Meningitis microarrays

Each microarray slide is composed of eight arrays. For the present study, three slides were used, each hybridized with four replicates of one experimental condition or time point (Figure 5.1).

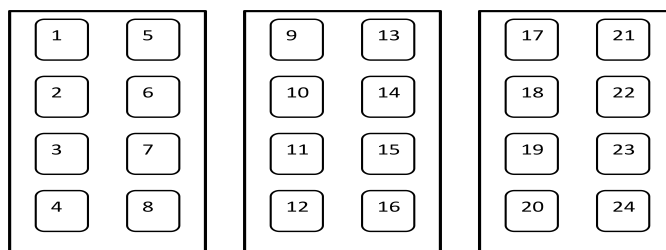


Figure 5.1. Schematic diagram of three microarray slides each carrying eight microarrays. Samples analysed in this study were as follows: arrays 1 – 4: control samples incubated for 3 h in DMEM-10% hi-fbs without subsequent trypsin-EDTA treatment; arrays 5 – 8: control samples incubated for 3 h in DMEM-10% fbs with subsequent trypsin-EDTA treatment for 10 min; arrays 9 – 12: samples extracted at 4h p.i.; arrays 13-16: samples extracted at 24 h p.i.; arrays 17 – 20 samples extracted at 96 h p.i.; and arrays 21-24 samples extracted 21 days p.i.

The first microarray slide (arrays 1 - 8), served as a control to investigate potential changes in the meningococcal transcriptome during the process prior to conservation of RNA. Bacteria were grown overnight on a sGC plate and harvested into 8 flasks containing 10 ml DMEM-10% hi-fbs at an OD₆₀₀ of 0.2. Flasks were incubated on a horizontal shaker at 37 °C with 5 % CO₂ for 3 h before harvesting of the bacteria by centrifugation at 3000 g for 5 min. The pellets were resuspended in 0.1 ml PBS. Of the eight resuspended pellets, four were incubated for 10 min at 37 °C in 5 ml 10 x concentrated trypsin-EDTA and four were incubated for 10 min at 37 °C in 5 ml DMEM-10% hi-fbs. RNA was extracted as described in Section 2.9.1.1 (Chapter 2) and prepared for microarray analysis as described in Section 2.10.2 (Chapter 2).

No significant changes were observed for data obtained from arrays 1 - 4 and 5 - 8 indicating that the protocol for separation of bacteria from host cells had no significant influence on the meningococcal transcriptome. Data of the 3 h samples which were trypsin-EDTA treated (arrays 5 – 8) were used for normalization of data obtained from the host cell association samples (arrays 9 – 24).

5.2.2. Quality control measurements of hybridized microarrays

Microarray data were generated from four independent biological replicates in each case except at the 21 days time point (when one replicate had to be discarded due to a bubble forming during hybridization making interpretation of data impossible, and leaving only three satisfactory samples).

Before microarrays were analysed for the transcriptional profile, data were pre-analyzed in correlation maps and heat maps as quality control measurements for experimental reproducibility. For a general overview of the replicates of each time point, arrays were aligned in a heatmap and for more detailed analysis in a correlation plot (Figure 5.2). In the heatmap, every gene is shown as a bar, coloured depending on the level of expression. All genes of an array are aligned in one column. Correlation mapping has been described as an alternative method of principal component analysis (PCA) and has the advantage that correlation coefficients for each array in comparison to any other array can be obtained (King *et al.*, 2005; Lee and Park, 2007). Correlation mapping of microarrays was described by Flight and Wentzell (2010) as a quality control mean prior to data analysis especially for time course experiments. Advantages include visualization of large data sets in easy-to-view plots and the capability to view dynamic gene expression profiles over time from multiple arrays, allowing exclusion of those clearly revealed to be of poor quality. Microarray data from time points close to each other had a better correlation than those further apart (Flight and Wentzell, 2010).

Differences between time points and similarities between replicates of the same time point were observed.

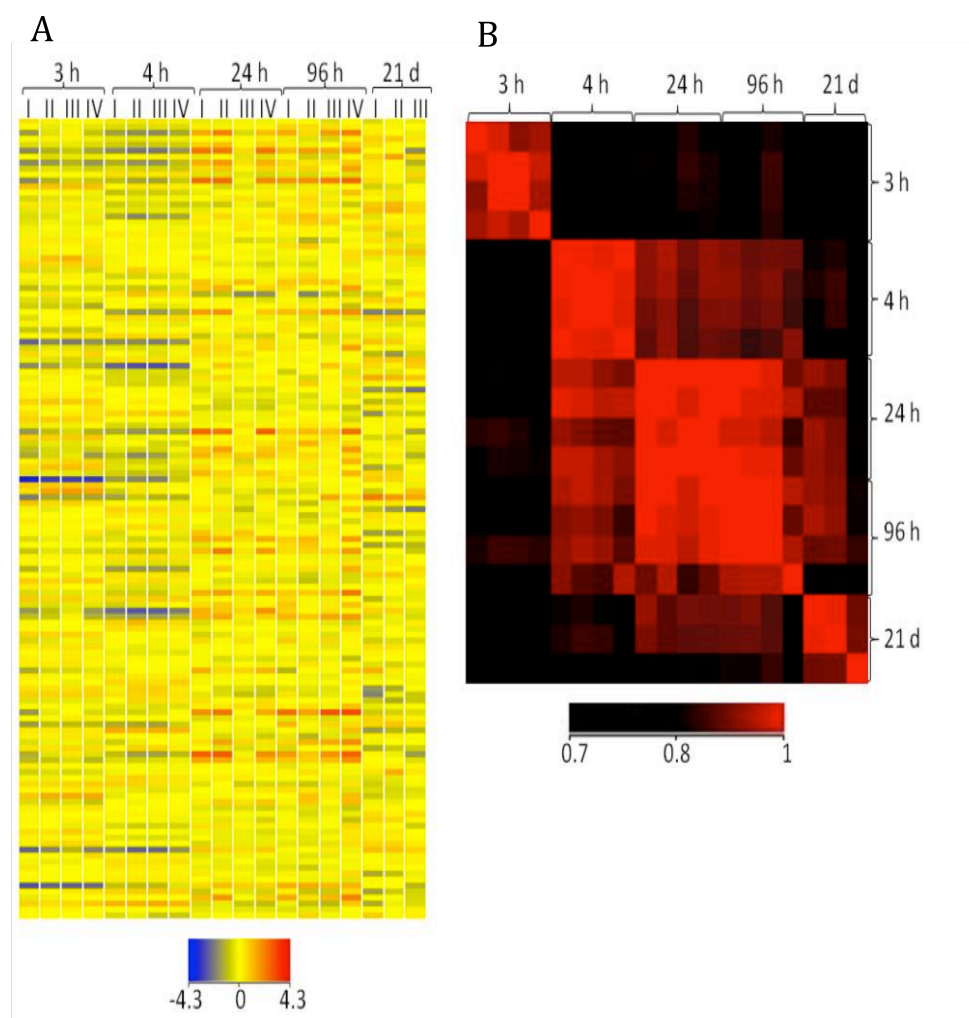


Figure 5.2. Heat map (panel A) and correlation plot (panel B) of replicate arrays of each time point were created in Genespring GX11. Replicate arrays (labelled I-IV for each time point in panel A) of each time point were aligned in a heat map to obtain an overview of reproducibility of the replicates and to estimate similarities / differences between time points. Bars for each single gene were coloured according to the level of expression. A legend showing colour and expression level is shown. Correlation coefficients between replicates of each and between time points were calculated by subjecting data of all arrays to correlation mapping. The correlation plot is shown in panel B. Each replicate (of each time point) is shown in one row and one column. The intensity of red colour represents the level of correlation. Average correlation coefficients of each timepoint are shown in Table 5.1 and a detailed list of calculated values in Table A in the Appendix.

Table 5.1. Average correlation coefficients between replicates of the same (bold) and between different time points.

Sample	3 h	4 h	24 h	96 h	21 days
3 h	0.93	0.76	0.81	0.78	0.77
4 h	0.76	0.96	0.91	0.89	0.80
24 h	0.81	0.91	0.97	0.93	0.86
96 h	0.78	0.89	0.93	0.91	0.83
21 d	0.77	0.80	0.86	0.83	0.92

The correlation plot shows the relationship between the replicates of each time point and between the different time points: the more intense the red colour is the higher the correlation (Figure 5.2).

Additionally, the correlation for every array in comparison with all other arrays was calculated (a complete table is shown in Table A in the Appendix). Table 5.1 shows the average correlation values between replicates of each, as well as between different time points. Correlation coefficients between the replicates of each and different time points were >91 % and >76 %, respectively. Replicates of different time points obtained from host cell association experiments (4 h, 24 h and 96 h) had a greater correlation than compared to the 3 h experiments without host cells, indicating the influence of the presence of host cells on the meningococcal transcriptome. For example, 96 h and 24 h samples had a correlation of 93% whereas 24 h and 3 h (host cells absent) samples had a correlation of 81%.

5.2.3. Overview of the transcriptional profile of *N. meningitidis* during host cell interaction over 21 days

In the following Sections, the transcription profile of MC58 during prolonged host cell association (4 h, 24 h, 96 h and 21 days) is compared to the transcriptome during growth in DMEM-10 % hi-fbs for 3 h (without the presence of host cells, hereafter termed 3 h-DMEM). The normalization of host cell co-cultivation transcriptional data to 3 h-DMEM was chosen to identify genes potentially involved in host cell association. Changes in the transcriptional profile are for example, influenced by the culture medium. To minimize variations in the meningococcal transcriptome due to different culture medium,

DMEM-10 % hi-fbs was chosen for bacterial culture without host cells (3 h-DMEM samples) over bacterial broth (such as sBHI) or sGC agar plates. Normalized data were paired (4 h vs 3 h-DMEM, 24 h vs 3 h-DMEM, 96 h vs 3 h-DMEM and 21 days vs 3 h-DMEM). Hereafter, if not stated otherwise, data compared to 3 h-DMEM are referred to as 4 h, 24 h, 96 h and 21 days. Paired data were subjected to ANOVA, filtered on a p-value ≤ 0.01 (in all pairs) and a fold change of ≥ 2.0 (in at least one pair of comparison) resulting in a list of 445 differentially expressed genes (the complete list can be viewed in Table B of the Appendix). Figure 5.3 shows the numbers of up- and down-regulated genes for each time point. At 4 h, more genes were down-regulated (254) than up-regulated (191) in contrast to later time points of host cell interaction (24 h, 96 h and 21 d) when more than half of the 445 differentially expressed genes were up-regulated (273, 280 and 236, respectively). When assigned into COGs (Table 5.2), the largest number of down-regulated genes at 4 h with a known function was within the 'translation' group. Of the 38 genes down-regulated at 4 h, 21 are classified as ribosomal. That the majority of genes were down-regulated upon host cell contact (4 h vs 3 h-DMEM) followed by up-regulation at later time points, indicates a change in the meningococcal transcriptome during host cell interaction.

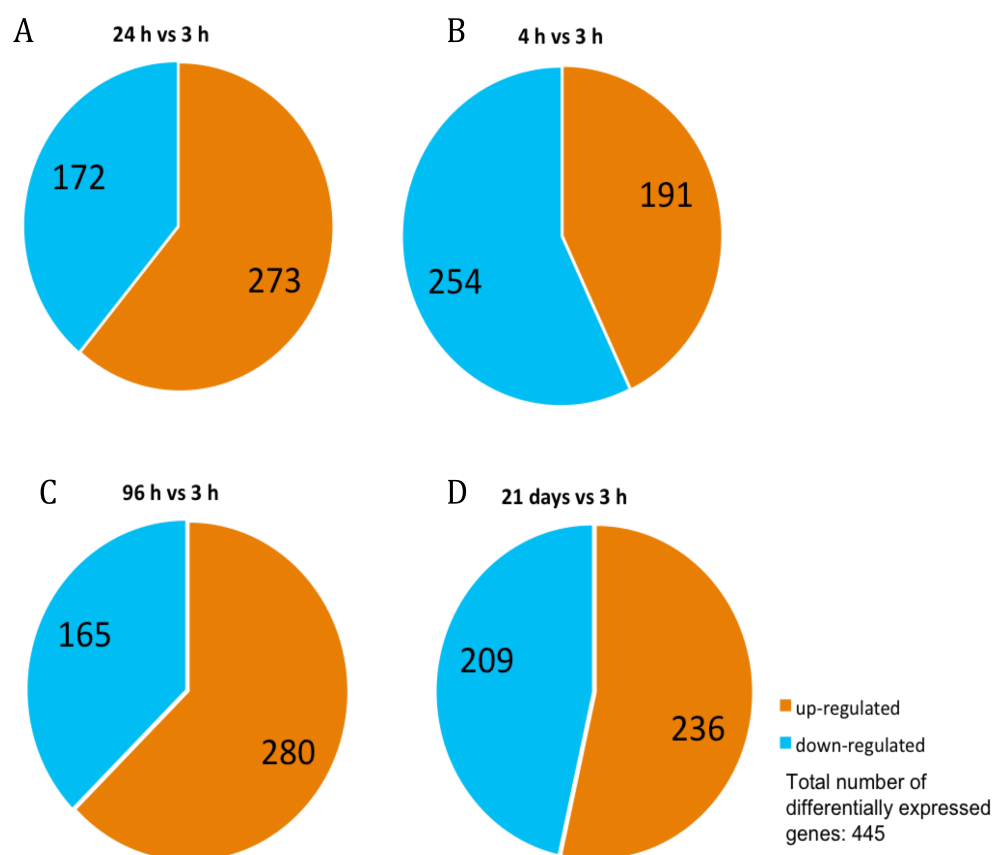


Figure 5.3. Numbers of up- and down-regulated genes of each time point compared to 3 h-DMEM samples. Data were filtered on a p-value ≤ 0.01 for each pair compared and a fold change of ≥ 2.0 in at least one pair of comparison. The total number of differentially expressed genes at each time point was 445. Complete lists for each time point are shown in Table B of the Appendix.

Table 5.2. Numbers of differentially expressed genes at 4 h, 24 h, 96 h and 21 days normalized to 3 h-DMEM data ($p \leq 0.01$, fold change ≥ 2.0) grouped into COGs gene groups.

COG group	n	4 h vs 3 h- DMEM up / down	24 h vs 3 h- DMEM up / down	96 h vs 3 h- DMEM up / down	21 days vs 3 h-DMEM up / down
Amino acid transport and metabolism genes	16	10 / 6	9 / 7	9 / 7	8 / 8
Carbohydrate transport and metabolism genes	6	2 / 4	3 / 3	3 / 3	3 / 3
Cell cycle control, mitosis and meiosis genes	1	1 / -	1 / -	1 / -	- / 1
Cell wall/membrane biogenesis genes	15	5 / 10	7 / 8	6 / 9	8 / 7
Coenzyme transport and metabolism genes	15	7 / 8	8 / 7	10 / 5	8 / 7
Defense mechanism genes	1	- / 1	1 / -	1 / -	- / 1
Energy production and conversion genes	12	5 / 7	9 / 3	9 / 3	9 / 3
Function unknown genes	27	12 / 15	15 / 12	17 / 10	14 / 13
General function prediction only genes	29	10 / 19	14 / 15	15 / 14	17 / 12
Inorganic ion transport and metabolism genes	17	5 / 12	7 / 10	7 / 10	8 / 9
Intracellular trafficking and secretion genes	8	4 / 4	5 / 3	5 / 3	4 / 4
Lipid transport and metabolism genes	4	1 / 3	2 / 2	2 / 2	1 / 3
Not in COGs genes	153	72 / 81	97 / 56	101 / 52	86 / 67
Nucleotide transport and metabolism genes	2	- / 2	- / 2	- / 2	- / 2
Post-translational modification, protein turnover, chaperones genes	20	8 / 12	13 / 7	12 / 8	9 / 11
Replication recombination and repair	13	5 / 8	9 / 4	7 / 6	5 / 8
Secondary metabolites biosynthesis, transport and catabolism genes	3	1 / 2	1 / 2	1 / 2	2 / 1
Signal transduction mechanisms genes	4	1 / 3	3 / 1	3 / 1	2 / 2
Transcription	13	5 / 8	9 / 4	11 / 2	9 / 4
Translation	51	13 / 38	39 / 12	40 / 11	30 / 21
Non-groupable	35	25 / 10	18 / 17	21 / 14	13 / 22

n: total numbers of differentially expressed genes

The function of the products of most of the genes identified as differentially expressed during epithelial cell association (244 of 445 or 54.8 %) remained unclear, being grouped into 'not in COGs genes' (153), 'non groupable' (35), 'general function prediction only' (29) or 'function unknown' (27). Of the remaining differentially expressed genes of known function (201), 51 were grouped into 'translation'. Of these 51 genes, 39, 40 and 30 were up-regulated at 24 h, 96 h and 21 days respectively, indicating increased translation activity at these time points. In comparison, at 4 h, only 13 of 51 genes were up-regulated.

The group, 'post-translational modification, protein turnover, chaperones genes' contained the 2nd largest number (20 of 445 or 4.49 %) of differentially expressed genes after 'translation'. At 24 h and 96 h, more than half of the 20 genes were up-regulated (13 and 12, respectively). At 4 h and 21 days more than half of the genes were down-regulated (12 and 11, respectively). An increased activity in translation, post-translational modification and protein turnover was suggested by up-regulation of genes in these groups at 24 h and 96 h compared to 4 h and 21 days.

5.2.4. Comparison of the different time points normalized to 3 h-DMEM using Venn diagrams

Normalized expression profiles of 4 h, 24 h, 96 h and 21 days compared to 3 h-DMEM were grouped into up- and down-regulated genes ($p \leq 0.01$, fold change ≥ 2.0). The numbers of up- and down-regulated gene lists of each time point were compared using Venn diagrams to identify genes potentially involved in host cell interaction (genes up-regulated at late or all time points).

In Figure 5.4, 24 h and 96 h data were compared. Between the two time points major similarities were found. Of the 267 up-regulated genes at 24 h, 257 were also up-regulated at 96 h. Only ten and 17 genes were uniquely up-regulated at 24 h and 96 h, respectively. Of the 162 down-regulated genes at 24 h, 145 were also found to be down-regulated at 96 h. Only 17 and ten genes were uniquely down-regulated at 24 h and 96 h. Detailed lists are shown in Table C of the Appendix.

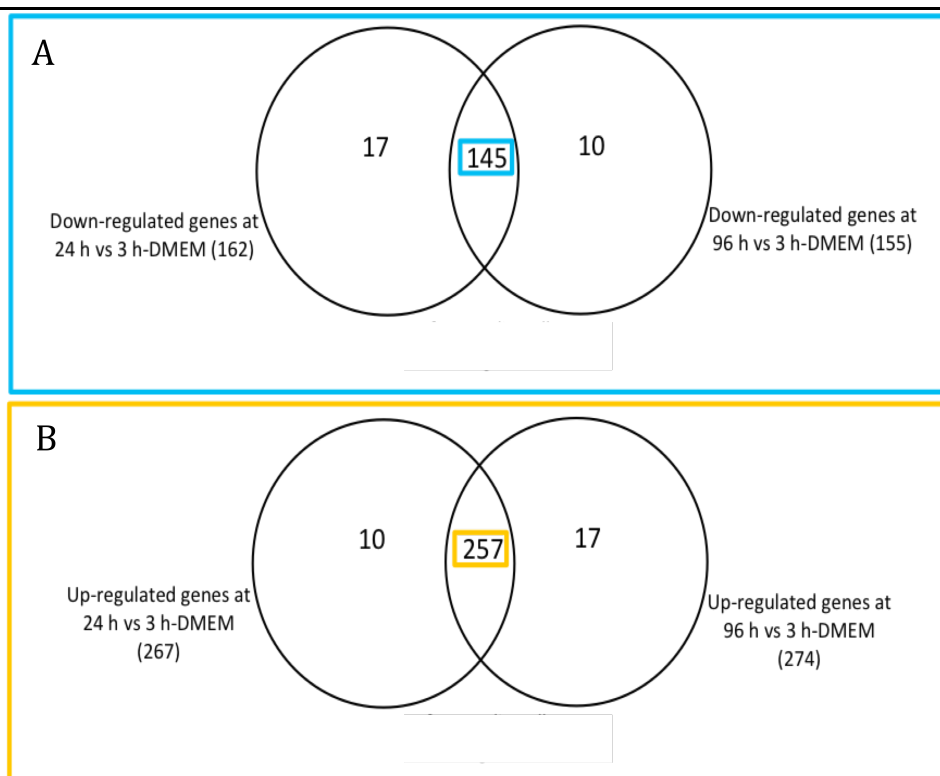


Figure 5.4. Venn diagrams comparing down-regulated (panel A) and up-regulated (panel B) genes of 24 h and 96 h. The total number of genes of each time point is shown in brackets. Common genes of both time points are shown in the central regions. For further comparisons, lists of the central regions were used. Lists of each Section are shown in Table C of the Appendix.

In Venn diagrams, three lists can be compared. For comparisons with 4 h and 21 days in Venn diagrams, 24 h and 96 h data were combined due to their similarities (and hereafter termed 24-96 h). Obtained from Figure 5.4, the up-regulated list of 257 and the down-regulated of 145 genes were used.

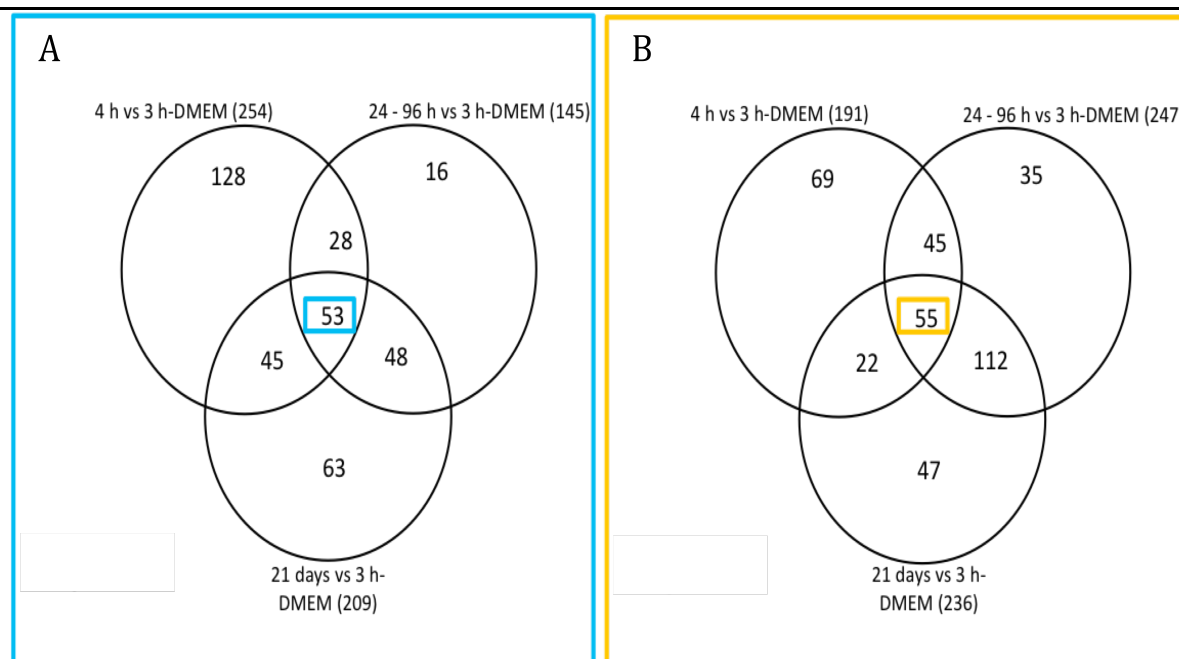


Figure 5.5. Venn diagrams comparing down-regulated (panel A) and up-regulated (panel B) genes at 4 h, 24-96 h and 21 days. The total number of genes at each time point is shown in brackets. Detailed gene lists at each time point can be viewed in Table D of the Appendix.

Venn diagrams (Figure 5.5) of genes down-and up-regulated at 4 h, 24 - 96 h and 21 days suggested similarities of the transcription profile between all time points. As shown in the central Sections of the Venn diagrams (Figure 5.5), 53 and 55 genes were commonly down- and up-regulated at all time points (of 445 differentially expressed genes).

At 4 h, 128 genes (of 247) were uniquely down-regulated, including *pilE*, *porA*, *porB* and *hfq*. Of 191 up-regulated genes at 4 h, 69 were uniquely up-regulated. At 21 days, 63 (of 209) genes were found to be uniquely down-regulated and of the 236 up-regulated genes, 47 were uniquely up-regulated at 21 days.

Only 16 genes were found to be down-regulated solely at 24 – 96 h. Of the 247 up-regulated genes at 24 – 96 h, 167 (55+112) were also up-regulated at 21 days, including, for example, genes encoding (surface) structures such as *pilE*, *porA*, *porB* and *opc*. At 24 – 96 h and at 4 h, 100 (55+45) genes were up-regulated. Of the 236 genes up-regulated at 21 days, 77 (55+22) were also found to be up-regulated at 4

h. The numbers shown in the Venn diagram indicated a greater similarity of 21 days to 24 – 96 h than to 4 h. Complete lists of each Section of the Venn diagrams are shown in Table D of the Appendix.

5.2.5. The meningococcal transcription profile during host cell interaction over 21 days compared to 3

h-DMEM: a detailed analysis

Following the general overview of the gene expression profiling during 21 days of host cell interaction described in the previous Section, data were analyzed in more detail with regard to genes involved in expression of specific meningococcal surface structures such as i) capsule; ii) type IV pili; iii) LOS; and iv) OMPs, putative and known adhesins, virulence factors and vaccine candidates and genes involved in biofilm formation. Besides the meningococcal surface structures, the expression of genes involved in oxygen and nitrate respiration, of *hfq* and its regulon, iron metabolism and tolerance to antibacterial host cell defence was investigated. Genes potentially involved in prolonged host cell association were identified such as the *NMB0342-NMB0348* locus. Data were filtered on $p \leq 0.01$, fold change ≥ 2.0 , however, because of their known biological importance, the expression profile of some genes was investigated regardless their statistical significance.

5.2.5.1. The expression profile over 21 days of meningococcal genes involved in capsule biosynthesis

Upon contact with a surface (such as of host cells), it has been described that meningococci down-regulate their capsule biosynthesis genes including *siaC* (*NMB0068*), *siaB* (*NMB0069*), *synX* (*NMB0070*) and the polysaccharide export genes *ctrA* (*NMB0071*), *ctrB* (*NMB0072*), *ctrC* (*NMB0073*) and *ctrD* (*NMB0074*) (Deghmane *et al.*, 2002; Lappann *et al.*, 2006; O'Dwyer *et al.*, 2009).

Shown in Table 5.3 and Figure 5.6, *siaC*, *siaB* and *synX* were found to be down-regulated at all time points. However, *siaD* (*NMB0067*) was up-regulated at 24 h and 96 h but down-regulated at 4 h and 21 days. The genes involved in polysaccharide export were not to be found differentially expressed even

when data were filtered solely on a p value of ≤ 0.05 (rather than the usual < 0.01) without filtering on expression levels.

Table 5.3. Expression profiles of genes involved in capsule biosynthesis.

Gene ID	NMB0067	NMB0068	NMB0069	NMB0070
COG	Not in COGs genes	Replication recombination and repair	General function prediction only genes	Cell wall / membrane biogenesis genes
Description (KEGG)	Polysialic acid capsule biosynthesis protein SiaD	Polysialic acid capsule biosynthesis protein SiaC	Polysialic acid capsule biosynthesis protein SiaB	Polysialic acid capsule biosynthesis protein SynX epimerase
4h vs 3 h-DMEM	-1.08	-1.38	-1.93	-1.69
24 h vs 3 h-DMEM	1.68	-1.04	-1.11	-1.12
96 h vs 3 h-DMEM	1.21	-1.10	-1.24	-1.15
21 d vs 3 h-DMEM	-2.36	-2.29	-2.62	-3.00
24 h vs 4 h	1.82	1.32	1.74	1.51
96 h vs 4 h	1.31	1.25	1.56	1.47
21 d vs 4 h	-2.18	-1.66	-1.36	-1.78
p-value	$3.07*10^{-3}$	$4.46*10^{-3}$	$8.70*10^{-4}$	$3.05*10^{-3}$
References	Tzeng <i>et al.</i> , 2005a	Tzeng <i>et al.</i> , 2005a; O'Dwyer <i>et al.</i> , 2009	Tzeng <i>et al.</i> , 2005a; O'Dwyer <i>et al.</i> , 2009	Swartley and Stephens, 1994; O'Dwyer <i>et al.</i> , 2009

Expression of later time points (fold change) of host cell interaction (4 h, 24 h, 96 h and 21 days) was compared to earlier time points (3 h-DMEM and 4 h) and filtered on a p-value ≤ 0.01 (in all pairs of comparison) and a fold change ≥ 2.0 in at least one pair of comparison. Genes highlighted in blue are selected down-regulated genes at 4 h, 24 - 96 h and 21 days (obtained from the Venn diagram in Figure 5.5).

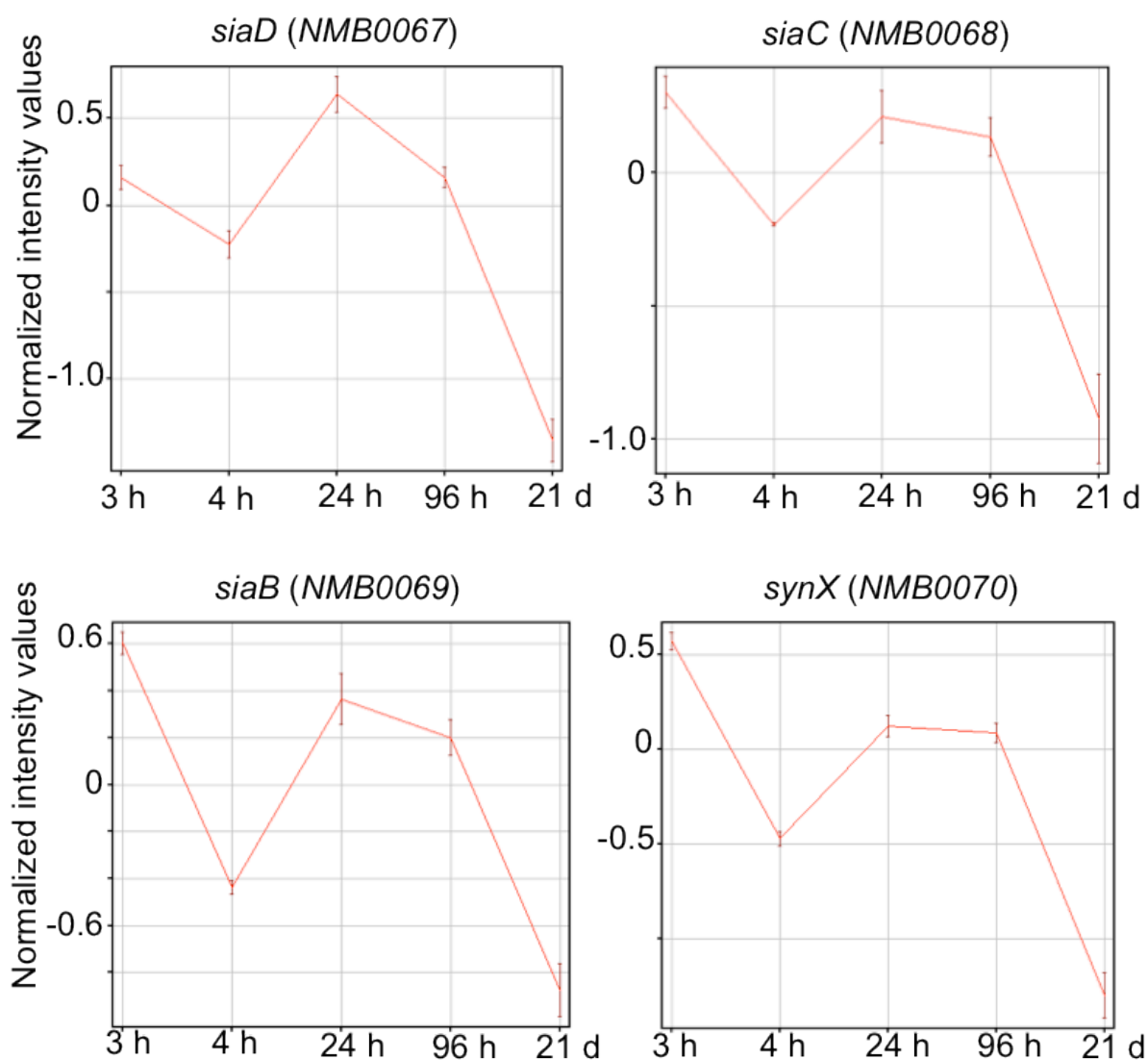


Figure 5.6. Normalized intensity values of *siaD*, *C*, *B* and *synX* over 21 days obtained from GX11 software. Error bars indicate the SD. The 3 h samples are those obtained from culture in DMEM in absence of host cells (3 h-DMEM). Replicates of each time point were averaged and normalized to the mean intensity values.

5.2.5.2. The expression profile over 21 days of meningococcal genes involved in type IV pilus

biosynthesis and function

Type IV pili have been described to be down-regulated after the initial attachment to epithelial cells (Deghmane *et al.*, 2002). In the present study, the major pilus subunit *pilE* (NMB0018) was down-regulated at 4 h, as shown in Table 5.4 and Figure 5.7. However, at time points of 24 h, 96 h and 21 days *pilE* was significantly up-regulated and likewise when compared to 4 h (Figure 5.4.2).

The prepilin peptidase *pilD* (NMB0332), involved in pilus assembly (Parge *et al.*, 1995, Merz and So 2000), presented a fluctuating expression over time (Table 5.4 and Figure 5.7). At 4 h *pilD* was up-regulated, followed by down-regulation at 24 h and up-regulation at 96 h before a down-regulation at 21 days.

In the present study, *pilG* was down-regulated at 4 h, 24 h, 96 h and 21 days compared to 3 h-DMEM. When compared to 4 h, *pilG* expression was up-regulated at 24 h, 96 h and 21 days. These results indicate a peak down-regulation at 4 h and although being up-regulated at other time points compared to 4 h, expression of *pilG* remained below the level at 3 h-DMEM (Figure 5.7).

pilT1 (NMB0052) and *pilT2* (NMB0768) were down-regulated at 4 h followed by up-regulation at 24 h and 96 h before *pilT1* was down-regulated at 21 days and *pilT2* was up-regulated. *PilG*, *pilT1* and *pilT2* (as described in the introduction to be involved in twitching motility) were down-regulated at 4 h suggesting that, compared to the situation in planktonic growth at 3 h-DMEM, a possible twitching motility might be reduced upon initial host cell contact. Later up-regulation of *pilG*, *pilT1* and *pilT2* suggest the possibility of a later increase in twitching motility activity (though reduced in comparison to 3 h-DMEM), as meningococci might redistribute (spreading out) on the colonized host cell surface.

Glycosylation of type IV pili has been described to be mediated by products of the pilus glycosylation gene *pglA* (NMB0208) and a cluster of genes *pglB*, *pglC* and *pglD* (NMB1820 - NMB1822) organised in an operon (Power *et al.*, 2000). Although the biological importance of pilus glycosylation remains unclear, involvement in resistance to host cell defence mechanisms has been suggested (Parge *et al.*, 1995).

Table 5.4. Expression profiles of genes involved in type-IV pilus biosynthesis and function.

Gene ID	NMB0018	NMB0052	NMB0768	NMB0332	NMB0333	NMB1811	NMB1822
COG	Inorganic ion transport and metabolism genes	Intracellular trafficking and secretion genes;Cell motility genes	Intracellular trafficking and secretion genes;Cell motility genes	Post-translational modification, protein turnover, chaperones genes	Carbohydrate transport and metabolism genes	Cell wall / membrane biogenesis genes	Carbohydrate transport and metabolism genes; Cell wall/membrane
Description (KEGG)	Pilin PilE fimbrial protein precursor (pilin)	Twitching motility protein PilT1	Twitching motility protein PilT2	Type IV prepilin peptidase PilD	Glucose-6-phosphate isomerase PilG	Type IV pilus assembly protein PilP	Pilin glycosylation protein PilD
4h vs 3 h-DMEM	-1.02	-1.20	-1.18	1.30	-1.87	-2.21	1.36
24 h vs 3 h-DMEM	6.66	1.74	1.07	-1.13	-1.58	-1.31	1.81
96 h vs 3 h-DMEM	3.65	1.46	1.19	1.08	-1.51	-1.51	1.46
21 d vs 3 h-DMEM	6.64	-1.14	1.53	-1.05	-1.63	-1.02	1.21
24 h vs 4 h	6.82	2.10	1.26	-1.47	1.18	1.69	1.33
96 h vs 4 h	3.74	1.75	1.40	-1.19	1.23	1.47	1.08
21 d vs 4 h	6.8	1.06	1.80	-1.36	1.14	2.17	-1.12
p-value	$2.87*10^{-3}$	0.0316	0.0126	$9.99*10^{-3}$	0.0145	$2.72*10^{-3}$	0.0368
References	Carbonnelle <i>et al.</i> , 2005	Carbonnelle <i>et al.</i> , 2006	Carbonnelle <i>et al.</i> , 2006	Lory <i>et al.</i> , 1997, Carbonnelle <i>et al.</i> , 2006	Tønnum <i>et al.</i> , 1995; Carbonnelle <i>et al.</i> , 2006; Collins <i>et al.</i> , 2007	Carbonnelle <i>et al.</i> , 2005	Power <i>et al.</i> , 2000

Expression (fold change) of later time points of host cell interaction (4 h, 24 h, 96 h and 21 days) was compared to earlier time points (3 h-DMEM and 4 h) and filtered on a p-value ≤ 0.01 (in all pairs of comparison) and a fold change ≥ 2.0 in at least at one pair of comparison (black) or

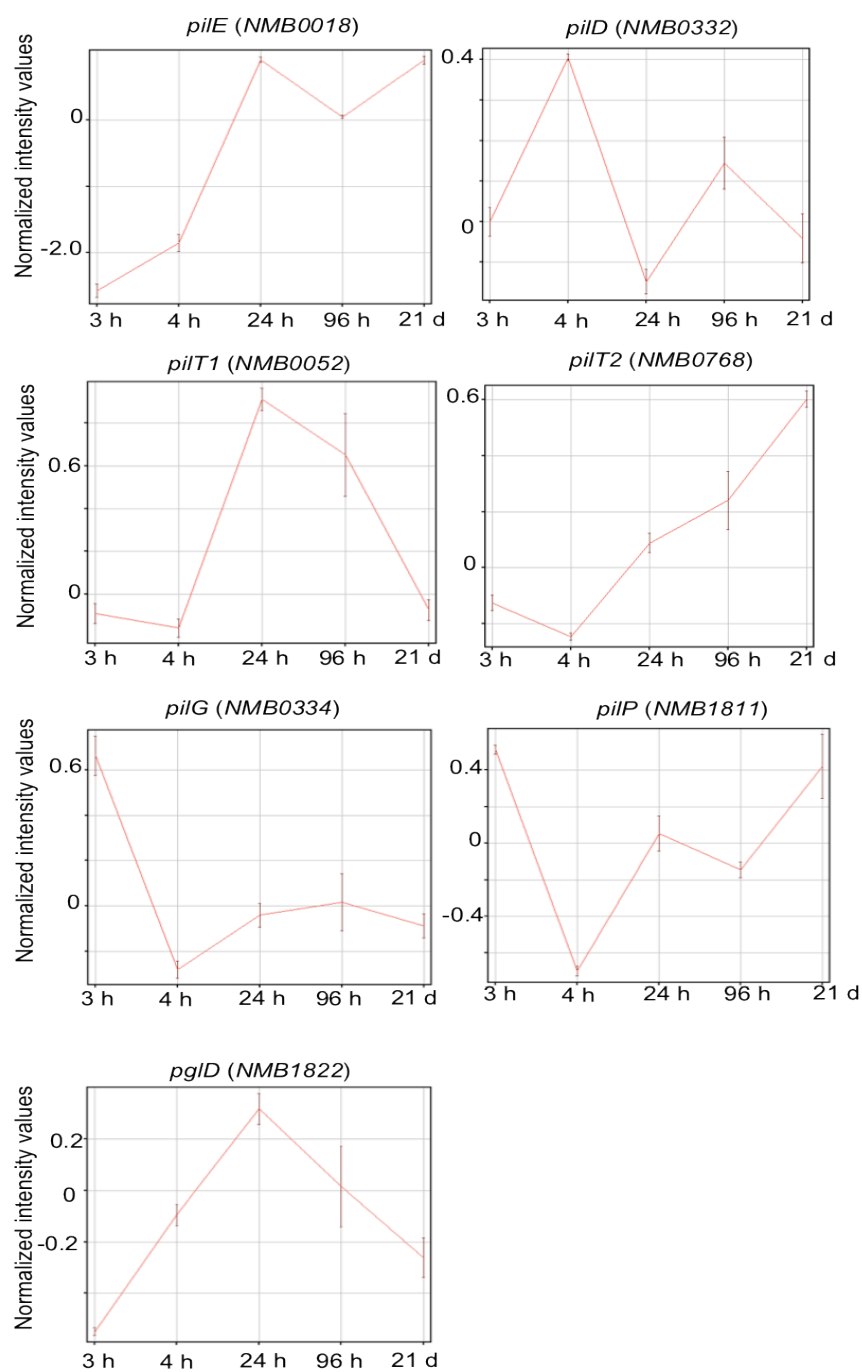


Figure 5.7. Normalized intensity values of *pilE*, *T1*, *T2*, *D*, *G*, *P* and *pglD* over 21 days obtained from GX11 software. Error bars indicate the SD. The 3 h samples are those obtained from culture in DMEM in absence of host cells (3 h-DMEM). Replicates of each time point were averaged and normalized to the mean intensity values.

In the present study, *pglD* was found to be up-regulated at 4 h, 24 h, 96 h and 21 days (Table 5.4).

And this up-regulation peaked at 24 h (Figure 5.7). However, the other genes involved in pilus glycosylation, *pglA*, *pglB* and *pglC* did not pass the applied filter settings ($p \leq 0.05$) indicating they were not significantly differentially expressed.

Pilus modification with phosphoglycerol mediated by *NMB1508* (*pptB*) has recently been described to be involved in bacterial aggregation (Chamot-Rooke *et al.*, 2011). These investigators have found that while a *pptB*-deletion mutant had increased bacterial aggregation in liquid culture, aggregate formation was abrogated when *pptB* was over-expressed. Upon epithelial infection (Hec-1B cells), levels of *pptB* expression did not influence bacterial adherence or morphology. However, bacterial detachment from microcolonies during epithelial infection was shown to be decreased in the *pptB*-deletion mutant compared to wild-type, and the *pptB*-deletion mutant showed decreased ability to cross the epithelium. Relevance to virulent behaviour might be that detached bacteria have an increased propensity to invade the epithelial surface and cause invasive disease and / or to pass via droplet transmission to a new host than bacteria in a biofilm (Chamot-Rooke *et al.*, 2011). However, in the present study, *pptB* did not pass the filter settings of $p \leq 0.05$ indicating that *pptB* is not differentially expressed during host cell interaction. Future work might include experiments such as western blots or quantitative immunoprecipitation to confirm expression of PptB at a similar level over time during host cell interaction.

5.2.5.3. The expression profile over 21 days of meningococcal LOS biosynthesis genes

The gene products of *lgtA*, *lgtB* and *lgtE* (*NMB1929*, *NMB1928* and *NMB1926*, respectively) are involved in LOS immunotype determination as through catalyzing the addition of terminal sugars to the LOS core structure (Scholten *et al.*, 1994; Berrington *et al.*, 2002) as described in the Introduction (Chapter 1).

Shown in Table 5.5 and Figure 5.8, expression of *lgtA*, *lgtB* and *lgtE* was up-regulated at 4 h. *lgtE* remained up-regulated at 24 h and 96 h before down-regulation at 21 days. The genes *lgtA* and *lgtB* were down-regulated at 24 h, 96 h and 21 days.

lgtA is involved in the switch from immunotype L8 to L3 by adding the L3 specific β -N-acetylglucosamid to the terminal β -galactose of L8. *lgtB* links the L3 specific β -galactose to the β -N-acetylglucosamid (added by *lgtA* to the L8 backbone, see Figure 1.1 in Chapter 1) (Scholten *et al.*, 1994; Berrington *et al.*, 2002). *lgtE* is involved in processing of L8 by adding the terminal β -galactose to the inner core of LOS. *lgtA* and *lgtB* up-regulation at 4 h followed by a down-regulation at 24 h, 96 h and 21 days in parallel with an up-regulation of *lgtE* at 4 h, 24 h and 96 h might indicate a possible switch from the L3 immunotype to L8.

A switch from L3,7,9 to L8 has been described for MC58 at 2 h p.i. with A549 cells (Milhau *et al.*, 2010). Expressing solely immunotype L3, 7, 9 during growth in broth, a switch to L8 has been described for parts of the bacterial population after host cell association. However, only one immunotype per bacteria was detected at any time (Milhau *et al.*, 2010).

Table 5.5. Expression profiles of genes involved LOS biosynthesis.

Gene ID	NMB1926	NMB1928	NMB1929
COG	Cell wall/membrane biogenesis genes	Cell wall/membrane biogenesis genes	Cell wall/membrane biogenesis genes
Description (KEGG)	Lacto-N-neotetraose biosynthesis glycosyl transferase LgtE	Lacto-N-neotetraose biosynthesis glycosyl transferase LgtB	Lacto-N-neotetraose biosynthesis glycosyl transferase LgtA
4h vs 3 h-DMEM	1.13	1.15	1.05
24 h vs 3 h-DMEM	1.04	-1.20	-1.37
96 h vs 3 h-DMEM	1.03	-1.22	-1.42
21 d vs 3 h-DMEM	-1.91	-1.29	-1.14
24 h vs 4 h	-1.08	-1.38	-1.43
96 h vs 4 h	-1.1	-1.40	-1.49
21 d vs 4 h	-2.15	-1.48	-1.20
p-value	$1.11 * 10^{-3}$	$4.91 * 10^{-4}$	$4.65 * 10^{-4}$
References	Gotschlich, 1994; Scholten et al., 1994; Berrington et al., 2002; Braun and Stein, 2004	Scholten et al., 1994; Berrington et al., 2002	Scholten et al., 1994; Berrington et al., 2002

Expression (fold change) of later time points of host cell interaction (4 h, 24 h, 96 h and 21 days) was compared to earlier time points (3 h-DMEM and 4 h) and filtered on a p-value ≤ 0.01 (in all pairs of comparison) and a fold change ≥ 2.0 in at least at one pair of comparison.

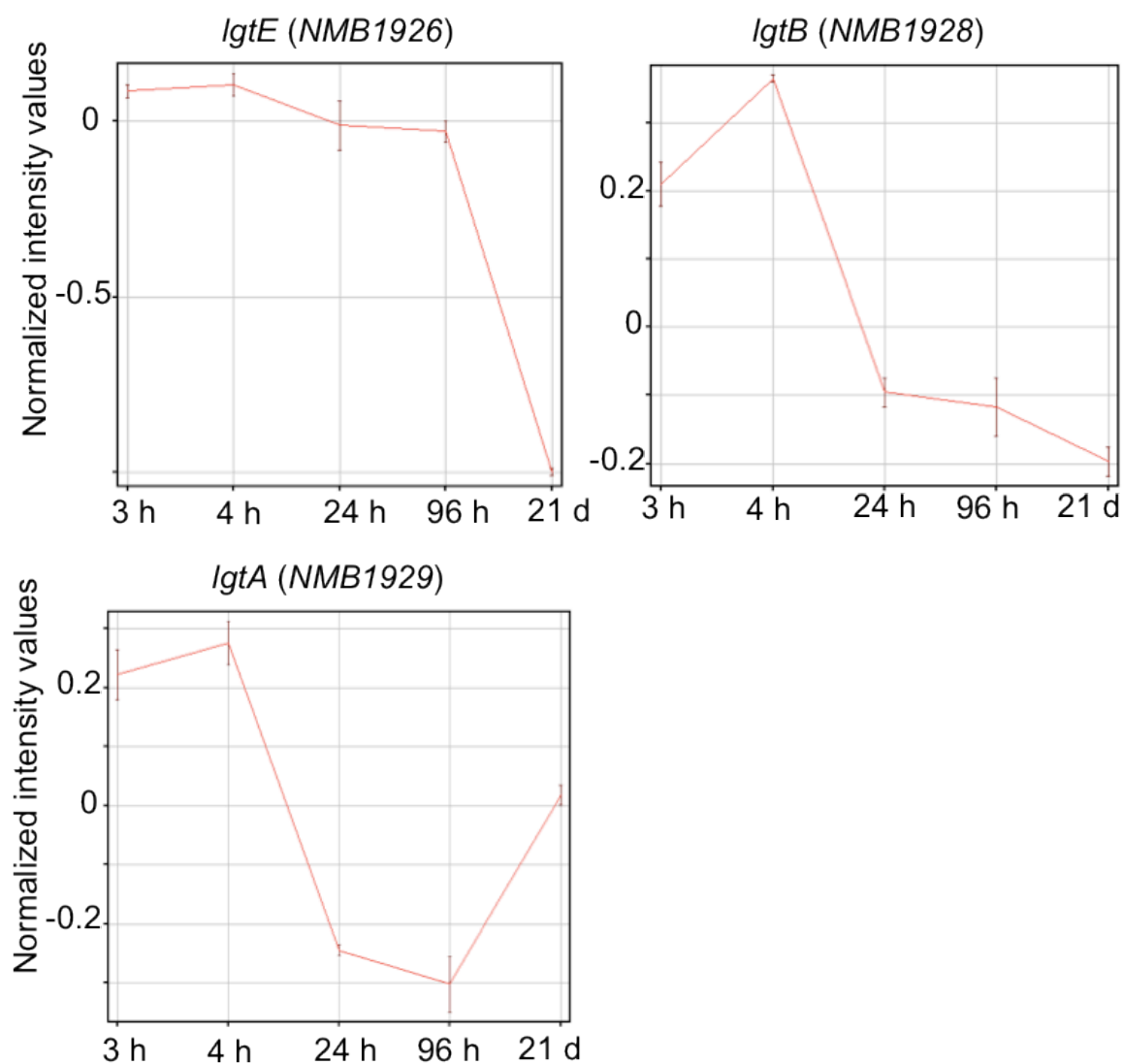


Figure 5.8. Normalized intensity values of *lgtE*, *lgtB* and *lgtA* over 21 days obtained from GX11 software. Error bars indicate the SD. The 3 h samples are those obtained from culture in DMEM in absence of host cells (3 h-DMEM). Replicates of each time point were averaged and normalized to the mean intensity values.

5.2.5.4. The expression profile over 21 days of meningococcal genes encoding OMPs, adhesins, virulence factors, vaccine candidates and of a gene involved in biofilm formation

In previous meningococcal transcriptome studies, profound changes in surface structures were found upon host cell contact (Grifantini *et al.*, 2002; Dietrich *et al.*, 2003). These changes included altered expression of meningococcal OMPs, putative and known adhesins, virulence factors, vaccine candidates and genes involved in biofilm formation. The transcriptional profile of these genes were duly followed over 21 days in the data obtained in the present study.

5.2.5.4.1. The expression profile over 21 days of genes encoding meningococcal OMPs, adhesins and a gene involved in biofilm formation

The two major porins *porA* (*NMB1429*) and *porB* (*NMB2039*) described in more detail in the Chapter 1, had a similar expression pattern in the present study (Table 5.6 and Figure 5.9). At 4 h, both were initially down-regulated followed by up-regulation at the later time points indicating a potential role in prolonged host cell association.

A similar transcription profile, i.e. down-regulation at 4 h followed by up-regulation at 24 h, 96 h and 21 days, was observed for several other meningococcal genes encoding surface structures such as *NMB0382* (OMP class 4), *NMB0995* (macrophage infectivity potentiator-related protein), *NMB1946* (outer membrane lipoprotein) and *NMB2095* (putative adhesion complex protein), described below. Expression levels for each time point are shown in Table 5.6.

As described in the Introduction (Section 1.6.4), MC58 has four *opa* gene loci, namely *NMB0442*, *NMB0926*, *NMB1465* and *NMB1636* of which all but *NMB0926* contain a frame shift mutation apparently abolishing their expression (Tettelin *et al.*, 2000). *NMB0926* did not pass the filter setting of $p \leq 0.05$ indicating not to be differentially expressed during prolonged host cell interaction.

The class 4 OMP (NMB0382) has been shown to be immunogenic and is a component of the Norwegian OMV vaccine (Klugman *et al.*, 1989; Rosenqvist *et al.*, 1999). In the present study, *NMB0382* was down-regulated at 4 h followed by up-regulation at 24 h, 96 h and 21 days. The down-regulation at 4 h followed by up-regulation of 24 h, 96 h and 21 days suggests that this gene may be involved in prolonged host cell association.

FHBP is part of the four component serogroup B vaccine (Men4B) currently undergoing clinical trials as described in the introduction. Regulation of the gene *NMB1870 (fHbp)*, encoding FHBP, was accordingly of interest, the expression profile was investigated in the present study. Compared to 3 h-DMEM, *fHbp* was found to be up-regulated at 4 h, 24 h, 96 h and 21 days (Table 5.6, Figure 5.9). Although being statistically significant ($p < 0.05$), *fHbp* did not pass the filter settings of a fold change of ≥ 2.0 at any time point. However, the < 2.0 fold up-regulation might still indicate involvement of FHBP in host cell interaction. The expression profiles of the other components of the four component vaccine (4CMenB) NHBP and the fusion proteins NMB1030 and NMB2091, but not *NadA*, were found to be differentially expressed (Table 5.6). In contrast to *NMB2091* which was up-regulated, *NMB1030* and *nhbp (NMB2132)* were down-regulated at all time points compared to 3 h-DMEM. The down-regulation of *NMB1030* and *nhbp* indicate they might not be exposed to the selective pressure occurring in the nasopharynx in contrast to the up-regulated *NMB2091* and, to a lesser extent to the non-differentially expressed *nadA*.

NspA (NMB0663) has a potential role in invasion of host cells (Vandeputte-Rutten *et al.*, 2003) and recently fH binding capability has been demonstrated (Lewis *et al.*, 2010). Because of its location on the meningococcal surface (Vandeputte-Rutten *et al.*, 2003), the expression profile of *nspA* was followed in this study. However, the fluctuating expression of *nspA* over 21 days (Table 5.6 and Figure 5.9) is difficult to interpret and the role of *nspA* in prolonged host cell association remains unclear.

NMB0995, the surface expressed macrophage infectivity potentiator related protein, has been described to be up-regulated in previous meningococcal – host cell association transcriptome studies (Grifantini *et al.*, 2002, Dietrich *et al.*, 2003). Its expression during a model of septicaemia remains unclear. Up-regulation was observed by Hedman (2009), but down-regulation by Echenique-Rivera *et al.*, (2011). In the present study, *NMB0995* was down-regulated at 4 h followed by up-regulation at the later time points indicating a potential role in prolonged host cell association (Table 5.6). A similar result was obtained by Kuwae *et al.*, (2011) who found up-regulation of *NMB0995* at 6 h p.i. of a human pharyngeal carcinoma cell line (FaDu).

NMB0419, a surface expressed conserved hypothetical protein has recently been shown to be involved in meningococcal invasion of COR L23 cells. A *NMB0419*-deletion mutant showed impaired invasion of host cells (Li *et al.*, 2003). In the present study, *NMB0419* was up-regulated at 4 h but down-regulated at 24 h, 96 h and 21 days suggesting a possible role in early host cell association (Table 5.6).

Involvement of the lactate permease encoded by *NMB0543* in meningococcal - host Pan-Meningitis has been explored by Exley *et al.* (2005). An *NMB0543*-deletion mutant associated less than wild-type bacteria with host cells in an organ culture model. However, the authors also found that the association of the deletion mutant with Chang epithelial cells in culture was significantly higher at 3 h p.i. than that of wild-type bacteria (Exley *et al.*, 2005). In the present study, *NMB0543* was down-regulated at all time points (Table 5.6) suggesting a negative influence of *NMB0543* expression on association to host cell lines as was described by Exley *et al.*, (2005).

NMB1946 is an outer membrane lipoprotein that has been found to be involved in meningococcal biofilm formation in a Sorbarod model (O'Dwyer *et al.*, 2009). In comparison to plate-grown bacteria, *NMB1946* was in the top-50 list of up-regulated genes in these authors' biofilm model. In the present study, *NMB1946* was down-regulated at 4 h and up-regulated at 24 h, 96 h and 21 days, suggesting a potential role during prolonged host cell interaction (Table 5.6). However, up-regulation

was not shown to be as dramatic as in the Sorbarod model perhaps suggesting a potential role rather in bacterial adherence to an abiotic surface than in bacteria – host cell interaction.

mspA (*NMB0992*) was found to be down-regulated at all time points (Table 5.6). Although being reported to be involved in meningococcal host cell association (Turner *et al.*, 2006), no evidence for a similar role was found in the present study.

The transcriptional profile of *NMB0345*, was followed in the present study as it was investigated as vaccine candidate (Bakshi, 2004; Hedman, 2009) and was shown to have a role in virulence (Sun *et al.*, 2000). *NMB0345* was shown to be up-regulated during a model of septicaemia (Hedman, 2009) as it was also named as putative cell-binding factor (KEGG), indicating a role in host cell interaction, the expression of *NMB0345* was followed in the present study. *NMB0345* was up-regulated at 24 h, 96 h and 21 days, indicating a potential role in prolonged host cell association.

Little is known about *NMB2095*, in the KEGGs database described as a putative adhesion complex protein. In an *NMB0033*-deletion mutant, *NMB2095* was one of five proteins released into the supernatant (Adu-Bobie *et al.*, 2004). In the present study, *NMB2095* was significantly down-regulated at 4 h and up-regulated at 24 h, 96 h and 21 days suggesting a potential role of this gene in prolonged host cell interaction (Table 5.6).

Expression data of the other genes encoding for meningococcal surface structures, such as *opc* (*NMB1053*), *nhhA* (*NMB0992*) and *app* (*NMB1985*), described in detail in Chapter 1 (Sections 1.7.4; 1.7.6; 1.7.12 and 1.7.10), did not pass the filter settings of $p \leq 0.05$ indicating they were not significantly differentially expressed.

After initial adherence meningococci start to colonize the host cell surface which eventually leads to biofilm formation. The transcriptional profile of *cidA* (*NMB2003*), a gene involved in meningococcal biofilm formation and DNA release for biofilm matrix synthesis (Lappann *et al.*, 2010) was analysed. In the present study, *NMB2003* was up-regulated at 4 h and down-regulated at the later time points, indicating a role in early host cell association (Table 5.6).

5.2.5.4.2. The expression profile over 21 days of genes involved in meningococcal virulence

The glutamate dehydrogenase gene *gdhA* (NMB1710) is highly conserved in *N. meningitidis* strains (Pagliarulo *et al.*, 2004). In an infant rat model, *gdhA* was a gene shown to be essential for causing invasive disease (Sun *et al.*, 2000). Expression of *gdhA* was shown to be positively regulated by *gdhR* (NMB1711), a gene adjacent to but orientated in the opposite direction of NMB1710 (Pagliarulo *et al.*, 2004). *GdhA* has been shown to be down-regulated in a *hfq*-deletion mutant suggesting the potential transcriptional control of *gdhA* by Hfq (Pannekoek *et al.*, 2009). In the present study, after down-regulation at 4 h, *gdhA* was up-regulated at 24 h, 96 h and 21 days (Table 5.6). As up-regulation of *gdhA* has, in particular, been observed at later time points of host cell interaction (24 h, 96 h and 21 days compared to either 3 h-DMEM or 4 h data), a potential involvement of the gene during host cell interaction is indicated.

In the present study, *gdhR*, described as a positive regulator of *gdhA* (Pagliarulo *et al.*, 2004) was down-regulated at 4 h, 24 h and 96 h and up-regulated at 21 days (Table 5.6) not indicating a role as positive regulator of *gdhA*.

Table 5.6. Expression profiles of genes coding for meningococcal OMPs, genes involved in biofilm formation and virulence.

Gene ID	NMB0345	NMB0382	NMB0419	NMB0442	NMB0543	NMB0663	NMB0995
COG	Carbohydrate transport and metabolism genes	Not in COGs genes	Cell wall/membrane biogenesis genes	Not in COGs genes	Intracellular trafficking and secretion genes	Cell wall/membrane biogenesis genes	Inorganic ion transport and metabolism genes
Description (KEGG)	Putative cell-binding factor	OMP class 4	Conserved hypothetical protein	Opacity protein Opa95	L-lactate permease, putative	Outer membrane protein NspA	Macrophage infectivity potentiator-related protein
4h vs 3 h-DMEM	-1.22	-2.96	1.46	1.67	-1.61	-1.16	-1.30
24 h vs 3 h-DMEM	3.71	4.47	-1.61	13.82	-2.26	1.45	3.58
96 h vs 3 h-DMEM	2.70	2.40	-1.44	7.25	-2.22	-1.02	4.61
21 d vs 3 h-DMEM	1.15	2.47	-1.41	6.27	-1.54	1.14	2.20
24 h vs 4 h	4.53	13.21	-2.35	8.28	-1.41	1.68	4.65
96 h vs 4 h	3.3	7.09	-2.1	4.34	-1.38	1.14	5.99
21 d vs 4 h	1.41	7.3	-2.06	3.75	1.05	1.33	2.86
p-value	7.19×10^{-4}	1.00×10^{-4}	6.25×10^{-4}	1.27×10^{-3}	3.18×10^{-3}	0.0154	1.08×10^{-4}
References	Bakshi 2004	Adu-Bobie <i>et al.</i> , 2004	Li <i>et al.</i> , 2000	Tettelin <i>et al.</i> , 2005	Exley <i>et al.</i> , 2005	Vandeputte-Rutten <i>et al.</i> , 2003	Grifantini <i>et al.</i> , 2002; Kuwaeet <i>et al.</i> , 2011

Expression (fold change) of later time points of host cell interaction (4 h, 24 h, 96 h and 21 days) was compared to earlier time points (3 h-DMEM and 4 h) and filtered on a p-value ≤ 0.01 (in all pairs of comparison) and a fold change ≥ 2.0 in at least at one pair of comparison (black) or on a p-value ≤ 0.05 (grey). Highlighted in blue are selected down-regulated genes at 4 h, 24 - 96 h and 21 days (obtained from the Venn diagram in Figure 5.5).

Table 5.6. – continued. Expression profiles of genes coding for meningococcal OMPs, genes involved in biofilm formation and virulence.

Gene ID	NMB1030	NMB1429	NMB1465	NMB1710	NMB1711	NMB1729	NMB1870
COG	Not in COGs genes	Not in COGs genes	Not in COGs genes	Not in COGs genes	Transcription	General function prediction only genes	Not in COGs genes
Description (KEGG)	Conserved hypothetical protein	Outer membrane protein PorA	Opacity outer membrane protein	Glutamate dehydrogenaseGdhA	Transcriptional regulator, GntR family GdhR	Biopolymer transport protein ExbB	Hypothetical protein, FHBP
4h vs 3 h-DMEM	-1.57	-3.54	1.24	-1.33	-1.15	-2.27	1.26
24 h vs 3 h-DMEM	-1.85	3.99	6.91	3.32	-1.44	-1.78	1.19
96 h vs 3 h-DMEM	-1.63	3.31	3.40	2.78	-1.49	-1.10	1.15
21 d vs 3 h-DMEM	-1.26	1.81	5.63	1.45	1.02	-1.78	1.35
24 h vs 4 h	-1.18	14.12	5.56	4.40	-1.25	1.28	-1.06
96 h vs 4 h	-1.04	11.69	2.74	3.69	-1.23	2.06	-1.10
21 d vs 4 h	1.24	6.4	4.53	1.92	1.18	1.28	1.07
p-value	0.0029	7.39*10 ⁻⁵	0.0045	1.06*10 ⁻³	1.93*10 ⁻⁴	2.88*10 ⁻³	0.0278
References	Guilliani <i>et al.</i> , 2006	Van der Ley <i>et al.</i> , 1991	Tettelin <i>et al.</i> , 2000	Pagliarulo <i>et al.</i> , 2004	Pagliarulo <i>et al.</i> , 2004	Sun <i>et al.</i> , 2000	Fletcher <i>et al.</i> , 2004; Jacobsson <i>et al.</i> , 2006; Bambini <i>et al.</i> , 2009

Table 5.6. – continued. Expression profiles of genes coding for meningococcal OMPs, genes involved in biofilm formation and virulence.

Gene ID	NMB1946	NMB1998	NMB2003	NMB2039	NMB2095	NMB2132
COG						
Description (KEGG)	Inorganic ion and metabolism genes	Intracellular trafficking and secretion genes	Inorganic ion transport and metabolism genes	Not in COGs genes	Not in COGs genes	Not in COGs genes
	Outer membrane lipoprotein	MspA serine-type peptidase	Hypothetical inner membrane protein	Major outer membrane protein PorB ein	Putative phosphor complex prot	Transferrin-binding protein-related protein; NHBP
4h vs 3 h-DMEM	-1.38	-1.58	1.25	-4.61	-2.94	-1.28
24 h vs 3 h-DMEM	1.05	-1.39	-1.91	3.15	5.16	-1.36
96 h vs 3 h-DMEM	1.29	-1.35	-1.63	2.05	2.54	-1.5
21 d vs 3 h-DMEM	1.20	-1.47	-1.41	3.63	5.12	-1.33
24 h vs 4 h	1.45	1.13	-2.38	14.52	15.18	-1.06
96 h vs 4 h	1.78	1.17	-2.03	9.45	7.46	-1.17
21 dvs 4 h	1.66	1.07	-1.76	16.75	15.07	-1.04
p-value	0.0115	0.0166	7.91×10^{-4}	7.52×10^{-5}	3.98×10^{-4}	0.0386
References	O'Dwyer <i>et al.</i> , 2009;	Turner <i>et al.</i> , 2006	Lappann <i>et al.</i> , 2010	Massari <i>et al.</i> , 2000	Adu-Bobie <i>et al.</i> , 2004	Serruto <i>et al.</i> , 2010

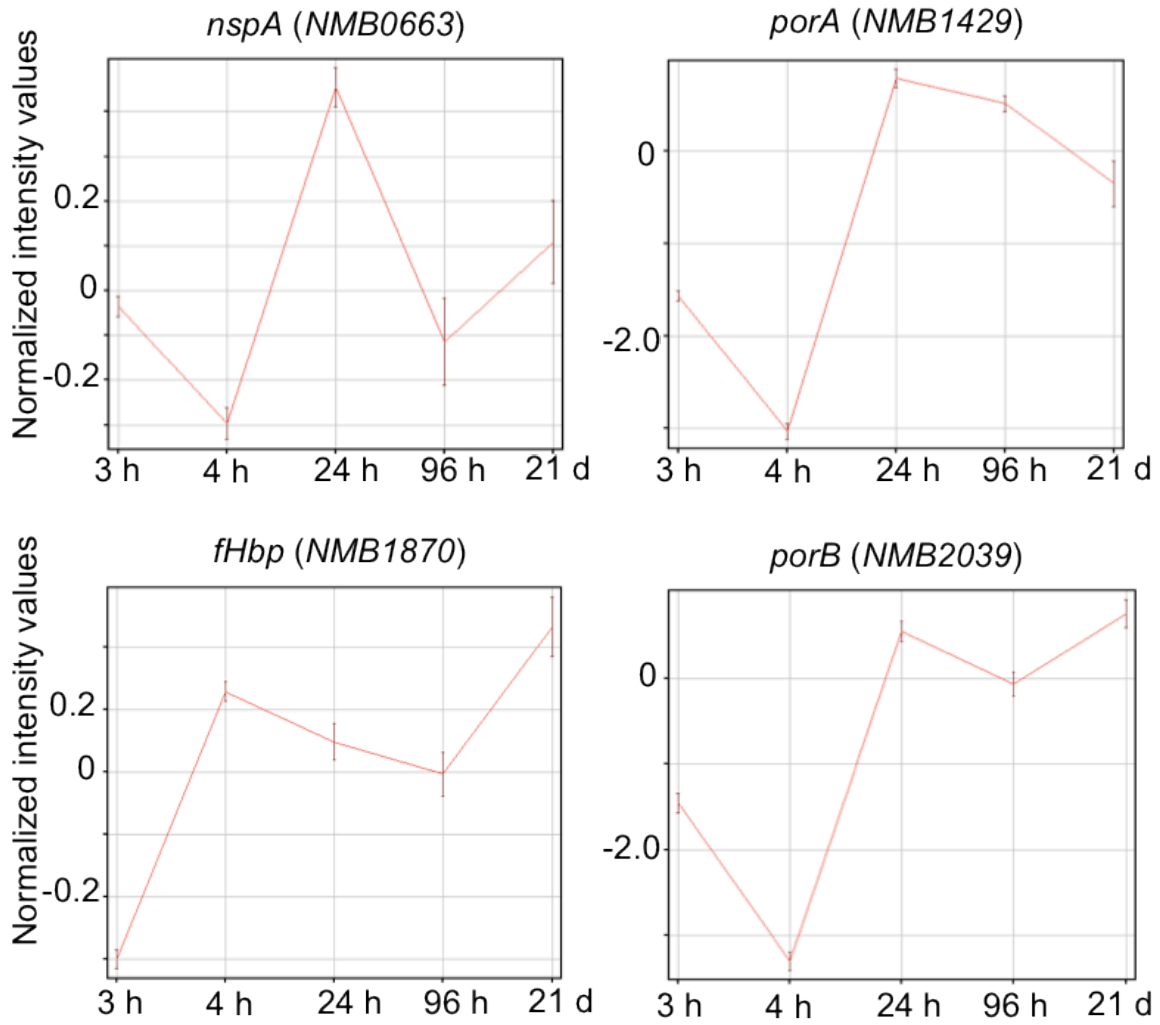


Figure 5.9. Normalized intensity values of *nspA*, *porA*, *fHbp* and *porB* over 21 days obtained from GX11 software. Error bars indicate the SD. The 3 h samples are those obtained from culture in DMEM in absence of host cells (3 h-DMEM). Replicates of each time point were averaged and normalized to the mean intensity values.

5.2.5.5. The expression profile over 21 days of meningococcal genes involved in oxygen

metabolism and nitrate reduction

During growth in biofilms bacteria may be expected to encounter oxygen limited conditions (Santos *et al.*, 1999). *N. meningitidis* usually grows in aerobic conditions, using oxygen as an electron acceptor. In *N. meningitidis* the cytochrome cbb_3 oxidase, encoded by *fixN*, *O*, *Q* and *P* (*NMB1725*, *NMB1724* and *NMB1723*, respectively), mediates reduction of oxygen to water (Anjum *et al.*, 2002). The gene *fixQ* is located downstream of *fixO* but was not yet assigned to a NMB number (Deeudom, 2007; TIGR) and is therefore not present on the array. Genes potentially supporting oxygen reduction are, for example, the putative cytochrome (*NMB0717*) named *cytochrome cx* by Deeudom *et al.* (2008) and cytochrome *c4* encoded by *NMB1805* (Deeudom *et al.*, 2008). Additionally, for respiration in oxygen limited (microaerophilic) conditions, *N. meningitidis* harbours several genes involved in reduction of nitrite (NO_2^-) to nitrous oxide (N_2O), which could be relevant for respiration in oxygen limited conditions (Anjum *et al.*, 2002). The gene *aniA* (*NMB1623*) encodes a nitrite reductase, catalysing the reduction of NO_2^- to NO . The gene *norB* (*NMB1622*) encodes a nitric oxide (NO) reductase, catalysing the reduction of NO to N_2O . NO is an intermediate in denitrification. Cytochrome *c'* (*cycP*, *NMB0923*) potentially binds NO on the meningococcal surface. The cytochrome *c5* (*NMB1677*) is another gene potentially involved in nitrite reduction. The gene *norB* has been shown to be essential for microaerobic growth of meningococci through reduction of nitrite to NO (Anjum *et al.*, 2002; Deeudom *et al.*, 2008).

The increasing expression level of *aniA*, *norB* and *cytochrome c'* over 21 days (Table 5.7 and Figure 5.10) at all time points suggests increased nitrite reduction. The down-regulation of *NMB1724* (*fixO*) and *NMB1725* (*fixN*) at 24 h and 96 h and of *NMB1805* (involved in oxygen respiration) at all time points might indicate oxygen limited conditions and a potential switch from oxygen respiration to nitrite reduction. The expression profile of *fixP* (*NMB1723*) did not pass the filter of $p \leq 0.05$, indicating not to be differentially expressed at all time points.

The down-regulation of *NMB0717*, potentially involved in oxygen respiration, plausibly supports the switch to nitrite reduction at later time points. *NMB0717* was up-regulated at 4 h and down-regulated at 24 h, 96 h and 21 days. However, the expression levels of *NMB1677*, potentially involved in nitric oxide metabolism, did not indicate a switch from oxygen respiration to nitrite reduction before 21 days. *NMB1677* was down-regulated at 4 h, 24 h, 96 h and slightly up-regulated at 21 days. Therefore, the switch from oxygen respiration to nitrite reduction remains inconclusive but potentially both pathways were used.

As a biofilm is composed of multiple layers of aggregated bacteria, a difference in respiration between bacteria in deep layers and more surface located bacteria might be a plausible explanation of the results obtained for the different genes. The data described above might indicate that at later time points of host cell association more bacteria grew in oxygen limited conditions.

Table 5.7. Expression profiles of genes involved in oxygen and nitric oxide metabolism.

Gene ID	NMB0717	NMB0923	NMB1622	NMB1623	NMB1677	NMB1724	NMB1725	NMB1805
COG	General function prediction only genes	Not in COGs genes	Post-translational modification, protein turnover, chaperones genes	Function unknown genes	Energy production and conversion genes	Energy production and conversion genes	Posttranslational modification, protein turnover, chaperones genes	Not in COGs genes
Description (KEGG)	Cytochrome, putative	Cytochrome c'	Nitric oxide reductase NorB	Anaerobically induced OMP; nitrite reductase, AniA	Cytochrome c5	Cytochrome C oxidase, subunit II	Cytochrome C oxidase, subunit I	Cytochrome c4
4h vs 3 h-DMEM	1.37	-1.18	2.29	-1.17	-1.26	-1.23	1.29	-1.30
24 h vs 3 h-DMEM	-1.99	1.16	1.53	-1.06	-1.53	-1.39	-1.28	-2.07
96 h vs 3 h-DMEM	-1.92	1.11	2.04	1.07	-1.18	-1.42	-1.16	-2.05
21 d vs 3 h-DMEM	-3.30	1.37	3.16	1.72	1.05	1.10	1.50	-1.34
24 h vs 4 h	-2.72	1.38	-1.5	1.1	-1.22	-1.12	-1.65	-1.6
96 h vs 4 h	-2.62	1.31	-1.12	1.25	1.07	-1.15	-1.50	-1.58
21 d vs 4 h	-4.55	1.63	1.38	2.02	1.31	1.35	1.16	-1.03
p-value	1.17×10^{-3}	0.023	1.68×10^{-6}	1.99×10^{-3}	0.0358	8.52×10^{-4}	7.04×10^{-4}	2.89×10^{-3}
References	Deeudom <i>et al.</i> , 2008	Deeudom <i>et al.</i> , 2002	Anjum <i>et al.</i> , 2002	Anjum <i>et al.</i> , 2002	Deeudom <i>et al.</i> , 2008	Deeudom <i>et al.</i> , 2007	Deeudom <i>et al.</i> , 2007	Bartolini <i>et al.</i> , 2006

Expression (fold change) of later time points of host cell interaction (4 h, 24 h, 96 h and 21 days) was compared to earlier time points (3 h-DMEM and 4 h) and filtered on a p-value ≤ 0.01 (in all pairs of comparison) and a fold change ≥ 2.0 in at least at one pair of comparison (black) or on a p-value ≤ 0.05 (grey). Genes highlighted in orange are selected common up-regulated at 4 h, 24 - 96 h and 21 days; highlighted in blue are selected down-regulated genes at 4 h, 24 - 96 h and 21 days (obtained from the Venn diagram in Figure 5.5).

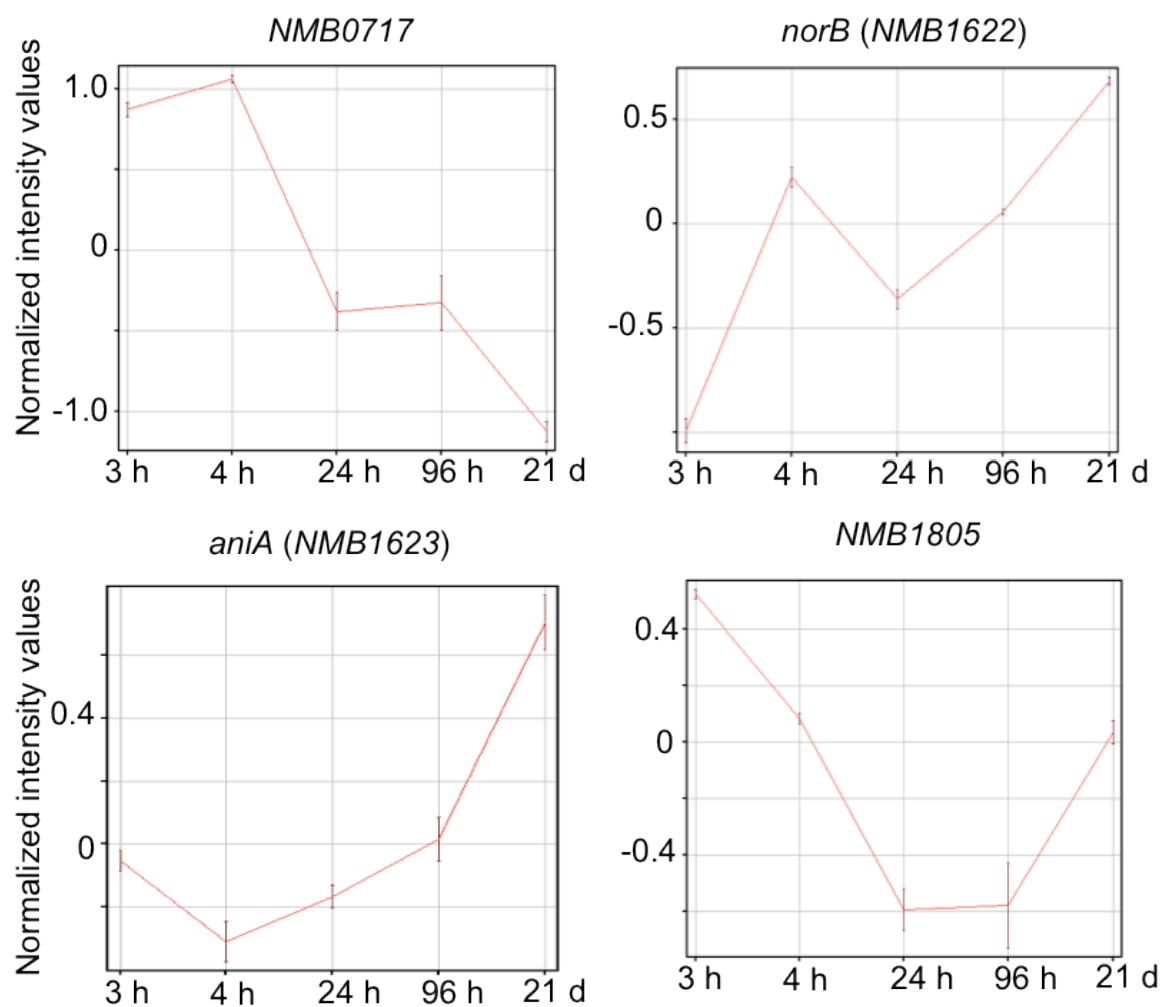


Figure 5.10. Normalized intensity values of *NMB0717*, *norB*, *aniA* and *NMB1805* over 21 days obtained from GX11 software. Error bars indicate the SD. The 3 h samples are those obtained from culture in DMEM in absence of host cells (3 h-DMEM). Replicates of each time point were averaged and normalized to the mean intensity values.

5.2.5.6. The expression profile over 21 days of host factor-q (*hfq*) and genes regulated by Hfq

Hfq (NMB0748) is a regulator and RNA chaperone which is involved in stress response and which negatively regulates transcription of 28 proteins including virulence factors such as *pilE* and *gdhA*. Compared to wild-type meningococci, an *hfq*-deletion mutant showed decreased fitness in culture medium, virulence in an infant rat model and survival in serum (Sun *et al.*, 2000; Pannekoek *et al.*, 2009; Fantappiè *et al.*, 2009).

In a meningococcal gene expression study modelling infection of blood, *hfq* was found to be up-regulated (Fantappiè *et al.*, 2009; Echenique-Rivera *et al.*, 2011), indicating involvement in blood infection. In the present study, although being down-regulated at 4 h, *hfq* was found to be increasingly up-regulated at later time points of host cell interactions (24 h, 96 h and 21 days) indicating a potential role in prolonged host cell interaction (Table 5.8). The genes described to be negatively regulated by Hfq (Pannekoek *et al.*, 2009; Fantappiè *et al.*, 2009) might reasonably be expected to be up-regulated at time points where *hfq* was down-regulated and vice versa. Of the genes described to be negatively regulated by Hfq, only *NMB1552* was down-regulated at 24 h, 96 h and 21 days when *hfq* was up-regulated. The up-regulation of *NMB1522* at 4 h p.i. might have been a transient response to the down-regulation of *hfq*. It is possible that other undetermined environmental and gene regulatory factors may also contribute to the expression changes of those genes. It remains to be determined if the down-regulation of *hfq* at 4 h p.i. is accompanied by a low level abundance of the protein and the up-regulation at later time points with a high abundance of Hfq. A list of genes described as negatively regulated by Hfq (Pannekoek *et al.*, 2009; Fantappiè *et al.*, 2009) and which were found to be differentially expressed in the present study are shown in Table 5.8.

Table 5.8. Expression profiles of *hfq* and genes regulated by *hfq*.

Gene ID	NMB0748	NMB0881	NMB1017	NMB1315	NMB1552	NMB1472
COG	Not in COGs genes	Post-translational modification genes	Inorganic ion transport and metabolism genes	Nucleotide transport and metabolism genes	Replication recombination and repair	General function prediction only genes
Description (KEGG)	Host factor-I Hfq	Phosphor ABC transporter, permease protein CysT	Phosphor ABC transporter, Sbp	Uracil permease UraA	Pilin gene inverting protein PivNM-1A	ClpB
4h vs 3 h-DMEM	-1.79	-1.09	-1.75	1.20	1.12	-1.91
24 h vs 3 h-DMEM	1.58	1.57	-1.10	-1.25	-1.05	-1.46
96 h vs 3 h-DMEM	1.78	1.76	1.04	-1.09	-1.11	-1.49
21 d vs 3 h-DMEM	3.41	1.7	1.42	1.18	-3.01	1.29
24 h vs 4 h	2.75	1.71	1.59	-1.49	-1.18	1.31
96 h vs 4 h	3.12	1.92	1.82	-1.31	-1.25	1.28
21 dvs 4 h	5.95	1.90	2.48	-1.02	-3.37	2.47
p-value	$1.20*10^{-3}$	$6.30*10^{-3}$	0.0437	0.0253	0.0292	$8.54*10^{-4}$
References	Mellin <i>et al.</i> , 2010	Mellin <i>et al.</i> , 2010	Mellin <i>et al.</i> , 2010	Mellin <i>et al.</i> , 2010	Mellin <i>et al.</i> , 2010	Mellin <i>et al.</i> , 2010

Expression (fold change) of later time points of host cell interaction (4 h, 24 h, 96 h and 21 days) was compared to earlier time points (3 h-DMEM, 4 h) and filtered on a p-value ≤ 0.01 (in all pairs of comparison) and a fold change ≥ 2.0 in at least at one

5.2.5.7. The expression profile over 21 days of meningococcal genes regulated by iron

Involved in meningococcal iron-uptake are the transferrin and lactoferrin binding proteins TbpA, TbpB, LbpA and LbpB encoded by *NMB0461*, *NMB0460*, *NMB1540* and *NMB1541*, respectively. The expression of iron-regulated genes is mainly dependent on iron-availability (Jordan and Saunders, 2009). The culture medium DMEM contains 0.1 mg / l ferric nitrate (www.invitrogen.com) while the quantities of Fe-containing proteins such as lactoferrin and transferrin in the hi-fbs are not known. In the human nasopharynx, lactoferrin is the predominant source of iron (Huettinger *et al.*, 1998).

The ferric uptake regulon (Fur) protein encoded by *NMB0205*, is a negative transcriptional regulator of genes involved in iron-acquisition such as, among others, the transferrin binding proteins (Legrain *et al.*, 1993). In a meningococcal transcriptome analysis of a model of septicaemia, *fur* was found to be up-regulated (Echenique-Rivera *et al.*, 2011) suggesting a role during blood stream infection. The expression profiles of *fur*, genes regulated by *fur* or those involved in iron-uptake, which were differentially expressed in the present study, are shown in Table 5.9. However, the p-value of *fur* was 0.081 which indicates that the data are statistically not robust and that the gene did not qualify to be significantly differentially regulated.

In the present study, *NMB0460* and *NMB1541* were down-regulated at all time points (Table 5.9). The genes *NMB0461* and *NMB1540* were not differentially expressed, probably indicating sufficient iron availability. Iron-limited conditions were described to induce expression of Tbp and Lbp (Schryvers and Morris, 1988).

The TonB-ExbBD system is encoded by *NMB1730*, *NMB1728* and *NMB1729*, respectively and forms cytoplasmic membrane proteins, which are involved in iron and vitamin B12 binding and uptake (Stojiljkovic and Srinivasan 1997). TonB is involved in host iron-uptake during invasion of epithelial cells and TonB, ExbB and ExbD are essential for causing meningococcal invasive disease in infant rats after intraperitoneal challenge (Sun *et al.*, 2000; Larson *et al.*, 2002). In the present study, *tonB* was not found to be differentially expressed. The transcriptional profiles of *exbB* and *exbD* are shown in

Table 5.9. *ExbB* was down-regulated at all time points and *exbD* was up-regulated at 24 h and 96 h.

In summary, the expression profile of genes involved in iron uptake (most of them were down-regulated or not differentially expressed at later time points) might possibly be explained by sufficient iron availability in the experimental conditions.

Table 5.9. Expression profiles of genes regulated by iron.

Gene ID	NMB0205	NMB0460	NMB0949	NMB0950	NMB1398	NMB1541	NMB1728	NMB1729
COG	Iron/zinc uptake regulation proteins	Not in COGs genes	Energy production and conversion genes	Energy production and conversion genes	Not in COGs genes	Not in COGs genes	Intracellular trafficking and secretion genes	Intracellular trafficking and secretion genes
Description (KEGG)	Ferric uptake regulon Fur	Transferrin-binding protein 2 TbpB	Succinate dehydrogenase SdhD	Succinate dehydrogenase SdhA	Cu-Zn-superoxide dismutase SodC	Lactoferrin binding protein B LbpB	Biopolymer transport protein ExbD	Biopolymer transport protein ExbB
4h vs 3 h- DMEI	-1.55	-1.69	1.26	1.02	1.07	-1.39	-1.13	-2.27
24 h vs 3 h- DMEI	-1.36	-1.89	-1.04	-1.18	-1.43	-1.62	1.59	-1.78
96 h vs 3 h- DMEI	1.00	-1.83	1.17	-1.31	-1.28	-1.54	2.01	-1.10
21 d vs 3 h- DMEI	1.15	-2.94	1.45	1.39	-2.31	-2.11	-2.61	-1.78
24 h vs 4 h	1.14	-1.12	-1.31	-1.21	-1.53	-1.17	1.80	1.28
96 h vs 4 h	1.55	-1.10	-1.08	-1.35	-1.37	-1.10	2.28	2.06
21 d vs 4 h	1.79	-1.74	1.14	1.36	-2.47	-1.52	-2.3	1.28
p-value	0.081	4.75×10^{-4}	0.042	7.62×10^{-3}	2.74×10^{-3}	2.89×10^{-4}	0.0036	5.80×10^{-4}
References	Mellin <i>et al.</i> , 2007	Schryvers and Morris 1988	Mellin <i>et al.</i> , 2010	Mellin <i>et al.</i> , 2010	Mellin <i>et al.</i> , 2010	Schryvers and Morris 1988	Sun <i>et al.</i> , 2000	Sun <i>et al.</i> , 2000

Expression (fold change) of later time points of host cell interaction (4 h, 24 h, 96 h and 21 days) was compared to earlier time points (3 h- DMEI, 4 h) and filtered on a p-value ≤ 0.01 (in all pairs of comparison) and a fold change ≥ 2.0 in at least at one pair of comparison (black) or on $p \leq 0.05$ (grey) or on p-value ≤ 0.1 (pale red)

5.2.5.8. The expression profile over 21 days of meningococcal genes involved in tolerance to antibacterial host cell defence

Cationic antimicrobial peptides (CAMPs) such as LL-37 and the β -defensins 1 and 2, which are present in the human nasopharynx, are part of the innate immune system. Although being mainly produced by phagocytes, expression in epithelial cells has been described (Hancock, 2001). Besides the capsule, LOS and type IV pili, *N. meningitidis* produces other negatively charged macromolecular structures which can bind cationic peptides non-specifically at a distance from the outer membrane to neutralize their antibacterial activities (Tzeng *et al.*, 2005b). The MtrC, D and E efflux pumps encoded by *NMB1716*, *NMB1715* and *NMB1714*, respectively; the *lptA* gene (*NMB0082*), involved in lipid A modification; and the *pilMNOPQ* operon (encoded by *NMB1808*-*NMB1812*) have all been found to be involved in rendering *N. meningitidis* highly resistant to CAMPs (Tzeng *et al.*, 2005b).

In the present study, the expression profile of *mtrC* and *mtrD* showed down-regulation at 4 h and at 24 h (Table 5.10). The gene *mtrD* remained down-regulated at 96 h and at 21 days while *mtrC* was up-regulated at 96 h and down-regulated at 21 days. Data for *mtrE*, *lptA*, *pil*, *pilM*, *pilP* and *pilQ* did not pass the filter settings of $p \leq 0.05$ indicating that these genes might not be differentially expressed in the experimental conditions.

Data for *pilN* and *pilO* are presented in Table 5.10. Expression of *pilN* and *pilO* were similar, both genes were down-regulated at all time points compared to 3 h-DMEM. In summary, the expression profile of *mtrC*, *D*, *E*, *lptA*, and *pilMNOPQ* did not indicate a response to CAMPs. A possible explanation for these findings is, that in a transcriptomic study of 16HBE14 cells upon infection with *N. meningitidis* strain MC58, LL-37 and β -defensins 1 and 2 were not found to be differentially expressed (Wong, 2010). It remains to be determined if CAMPs were present in the experimental conditions of this study.

Table 5.10. Expression profiles of genes involved in tolerance of antibacterial host cell defence.

Gene ID	<i>NMB1810</i>	<i>NMB1809</i>	<i>NMB1716</i>	<i>NMB1715</i>
COG	Intracellular trafficking and secretion genes	n / a	Cell wall / membrane biogenesis gene	Defence mechanism genes
Description (KEGG)	Type IV pilus assembly protein PilO	Type IV pilus assembly protein PilN	Membrane fusion protein MtrC	Multiple transferable resistance system protein MtrD
4h vs 3 h-DMEM	-2.25	-1.88	-1.58	-1.52
24 h vs 3 h-DMEM	-1.24	-1.39	-1.12	-1.82
96 h vs 3 h-DMEM	-1.62	-1.59	1.04	-1.51
21 d vs 3 h-DMEM	-1.74	-1.17	-1.35	-2.08
24 h vs 4 h	1.82	1.35	1.41	-1.20
96 h vs 4 h	1.39	1.18	1.65	1.01
21 d vs 4 h	1.29	1.60	1.17	-1.37
p-value	0.0136	8.53×10^{-4}	2.9×10^{-4}	3.24×10^{-5}
References	Tzeng <i>et al.</i> , 2005b	Tzeng <i>et al.</i> , 2005b	Tzeng <i>et al.</i> , 2005b; Spinoso <i>et al.</i> , 2007	Tzeng <i>et al.</i> , 2005b; Spinoso <i>et al.</i> , 2007

n / a : not in COGs functional groups

Expression (fold change) of later time points of host cell interaction (4 h, 24 h, 96 h and 21 days) was compared to earlier time points (3 h-DMEM, 4 h) and filtered on a p-value ≤ 0.01 (in all pairs of comparison) and a fold change ≥ 2.0 in at least at one pair of comparison (black) or on a p-value ≤ 0.05 (grey).

5.2.5.9. The expression profile over 21 days of genes encoding the four meningococcal two-component systems

Two-component systems (Tcs) in bacteria are ATP dependent signal transduction systems used to sense and react to environmental changes (Hoch and Silhavy, 1995). Tcs are composed of a sensory kinase, which is a transmembrane spanning protein with a surface expressed receptor domain and a cytoplasmic ATP binding kinase; and a cytoplasmic response regulator. *N. meningitidis* harbours four sets of two-component systems (Tettelin *et al.*, 2000). NtrY/NtrX, encoded by *NMB0114* and *NMB0115*, is a homolog of the nitrogen regulating system in *Azorhizobium caulinodans* (Pawlowski *et al.*, 1991). MisR/MisS (or PhoP/PhQ) is encoded by *NMB0595/NMB0594* and was shown to be involved in LOS inner core assembly (Tzeng *et al.*, 2004; Newcombe *et al.*, 2005). The homolog of the gonococcal NarQ/NarP is encoded by *NMB1249/NMB1250* and may be involved in anaerobic growth (Lissenden *et al.*, 2000) and the homolog of the PilS/PilR system, known to be involved in piliation of *P. aeruginosa*, is encoded by *NMB1606/NMB1607* (Hobbs *et al.*, 1993).

In the present study, none of the two component systems completely passed the filter setting of $p \leq 0.05$. The expression profile of three genes, *NMB0115*, *NMB0595* and *NMB1250*, each belonging to different Tcs, were found to be differentially regulated over 21 days (see Table B in the Appendix). No robust conclusions about the expression of any of the four two-component systems could be drawn about changes in the expression of any of the four two-component systems on prolonged epithelial cell association. However, one might speculate that up-regulation of *NMB0595* at 24 h, 96 h and 21 days (shown in Table B in the Appendix) and no differential regulation of *NMB0594* indicates expression of this Tcs. Experiments on the protein level, confirming the presence of each of the Tcs would help to understand their transcriptional profile and to conclude about their role in prolonged host cell association.

5.2.5.10. The expression profile over 21 days of meningococcal genes identified in the present

study as having a potential role in prolonged host cell interaction

More than half of the genes differentially expressed at 24 h, 96 h and 21 days compared to 3 h-DMEM, were up-regulated suggesting a possible direct role in host cell interaction of these genes. As described above, the expression of *NMB0345*, a putative cell binding factor, was studied in greater detail as it has attributes favouring candidacy as a vaccine antigen (Bakshi, 2004; Hedman, 2009) and appears to have a role in virulence (Sun *et al.*, 2000). The adjacent genes *NMB0342* to *NMB0344* and *NMB0346* to *NMB0348* showed a similar expression profile in the present study as *NMB0345* (Figure 5.11 and Table 5.11). This locus has accordingly been investigated in greater detail.

5.2.5.10.1. Description and expression profile of the *NMB0342* - *NMB0348* locus

NMB0342 and *NMB0345* were implicated in meningococcal virulence in a genome wide study using signature tagged mutagenesis (STM; Sun *et al.*, 2000). Bakshi (2004) characterized the relevant locus (*NMB0342-NMB0348*) and demonstrated attenuated virulence of each of the *NMB0342-NMB0348* mutants in an infant rat model. *NMB0345* and *NMB0342* mutants showed decreased adherence to Chang cells at 3 h p.i. compared to wild-type bacteria. *NMB0345* was shown to have PPIase (peptidyl-prolyl *cis-trans* isomerase) activity, suggesting a role in protein folding. Several genes within this locus have homologues in other pathogens which are described below.

NMB0342, termed intracellular septation protein A (IspA; KEGG) is a 531 nt (176 aa) gene and has a homolog in *Shigella flexneri* (Mac Siomoin *et al.*, 1996; Hong *et al.*, 1998). In *S. flexneri*, IspA has been shown to be involved in intracellular spread of bacteria during infection of epithelial cells (monkey kidney epithelial cells, MK2). Hong *et al.* (1998) have shown that IspA is located in the cytoplasmic membrane of *S. flexneri* with small amounts being present in the outer membrane. A defect of *Shigella* septation was found in *ispA*- deletion mutants during invasion of host cells

resulting in long filamentous intracellular bacteria especially at 8 h p.i. However, no difference in morphology compared to wild-type bacteria was observed when the *ispA* mutant was grown in standard media (Mac Siomoin *et al.*, 1996). Bakshi (2004) could not observe any morphological changes in her *NMB0342* mutant on immunofluorescence or transmission electron microscopy. However, it should be noted that bacteria were cultured prior to microscopy in the absence of host cells, and the *ispA* mutant in *S. flexneri* presented with long filamentous bacteria exclusively during invasion of epithelial cells (Mac Siomoin *et al.*, 1996). *NMB0342* has been shown to be essential for causing invasive meningococcal disease in an infant rat model (Sun *et al.*, 2000).

NMB0343 and ***NMB0344*** have respectively been classified as a Ycil-like hypothetical protein and a member of the BolA/YrbA family protein (KEGG). Both are short genes (each 279 nt or 92 aa long) with no intergenic space. The Ycil superfamily is characterized by single or multiple repeats of a protein domain containing a conserved histidine and aspartate residue speculatively directing enzymatic function (Marchler-Bauer *et al.*, 2011).

Overexpression of *bolA* in *E. coli* resulted in round cells, contrasting with the usual rod shaped morphology (Aldea *et al.*, 1988). Santos *et al.* (1999) described the *bolA* as being expressed upon carbon starvation, oxidative and acidic stress all of which potentially occurring during biofilm formation.

Vieira *et al.* (2004) investigated the expression of *bolA* in *E. coli* during biofilm formation.

Overexpression or deletion of the gene induced or inhibited biofilm formation, respectively. Adnan *et al.* (2010) suggested that a *bolA* induced round morphology might be advantageous to bacteria through decreasing the surface area and thereby reducing the exposure to potentially harmful environments.

NMB0345 is a 876 nt (288 aa) gene encoding a putative cell-binding factor (KEGG). *NMB0345* has homology to the *Campylobacter jejuni* cell binding factor-2 (Cbf-2), one of the two major cell binding factors (Cbf-1 and Cbf-2) in that organism. Surface expression of *cbf-1* but not of *cbf-2* could be

confirmed by immunogold labelling. Purified Cbf-1 adhered to a greater extent to HeLa cells than Cbf-2 (Kervella *et al.*, 1993). NMB0345 has been shown to be essential for causing invasive meningococcal disease in an infant rat model (Sun *et al.*, 2000).

NMB0346 is a 759 nt (252 aa) long gene encoding for a hypothetical protein of unknown function (KEGG).

NMB0347 encodes for a D-tyrosyl-tRNA(Tyr) deacylase (KEGG), widely found in bacteria and involved in protein synthesis (Calendar and Berg, 1967; Lim *et al.*, 2003).

NMB0348 (1011 nt; 336 aa) encodes tRNA-dihydrouridine synthase (KEGG), involved in 5.6-dihydrouridine ('D') synthesis. D is a modified uridine present in tRNA of many organisms and implicated in structural flexibility of tRNA (Bishop *et al.*, 2002).

Although there are some suggestions that homologs of *NMB0342* (Mac Siomoin *et al.*, 1996; Hong *et al.*, 1998) and *NMB0345* (Kervella *et al.*, 1993) in other organisms are surface expressed, so far, the localization of NMB0342-NMB0348 on the neisserial surface has not been established.

Table 5.11. Expression profiles of genes identified in the present study potentially involved in host cell interaction

Gene ID	NMB0342	NMB0343	NMB0344	NMB0345	NMB0346	NMB0347	NMB0348
COG	Cell cycle control, mitosis, meiosis genes	Not in COGs genes	Not in COGs genes	Carbohydrate transport and metabolism genes	Not in COGs genes	Signal transduction mechanisms genes	Post-translational modification, protein
Description (KEGG)	Intracellular septation protein IspA	Conserved hypothetical protein	BoIA/YrbA family protein	Putative cell-binding factor	Hypothetical protein	Conserved hypothetical protein Dtyrosyl tRNA deacylase	Conserved hypothetical protein
4h vs 3 h-DMEM	1.17	-1.71	-1.29	-1.22	1.17	1.35	-1.22
24 h vs 3 h-DMEM	2.01	1.99	3.05	3.71	3.84	2.42	1.44
96 h vs 3 h-DMEM	3.25	2.23	3.13	2.70	3.82	2.73	1.78
21 d vs 3 h-DMEM	1.15	1.57	1.14	1.15	1.51	-2.71	-1.06
24 h vs 4 h	1.72	3.4	3.93	4.53	3.29	1.8	1.75
96 h vs 4 h	2.78	3.81	4.03	3.3	3.27	2.02	2.16
21 d vs 4 h	-1.02	2.69	1.46	1.41	1.29	-3.65	1.14
p-value	0.0236	2.76×10^{-3}	2.01×10^{-4}	7.19×10^{-4}	1.89×10^{-4}	2.97×10^{-4}	3.07×10^{-4}
References	Sun <i>et al.</i> , 2000; Bakshi, 2004	Bakshi, 2004	Bakshi, 2004	Sun <i>et al.</i> , 2000; Bakshi, 2004	Bakshi, 2004	Bakshi, 2004	Bakshi, 2004

Expression (fold change) of later time points of host cell interaction (4 h, 24 h, 96 h and 21 days) was compared to earlier time points (3 h-DMEM and 4 h) and filtered on a p-value ≤ 0.01 (in all pairs of comparison) and a fold change ≥ 2.0 in at least at one pair of comparison (black) or on $p \leq 0.05$ (grey). Genes highlighted in orange are selected common up-regulated at 24 h vs 3h, 96 h vs 3 h and 21 days vs 3h (obtained from the Venn diagram in Figure 5.5).

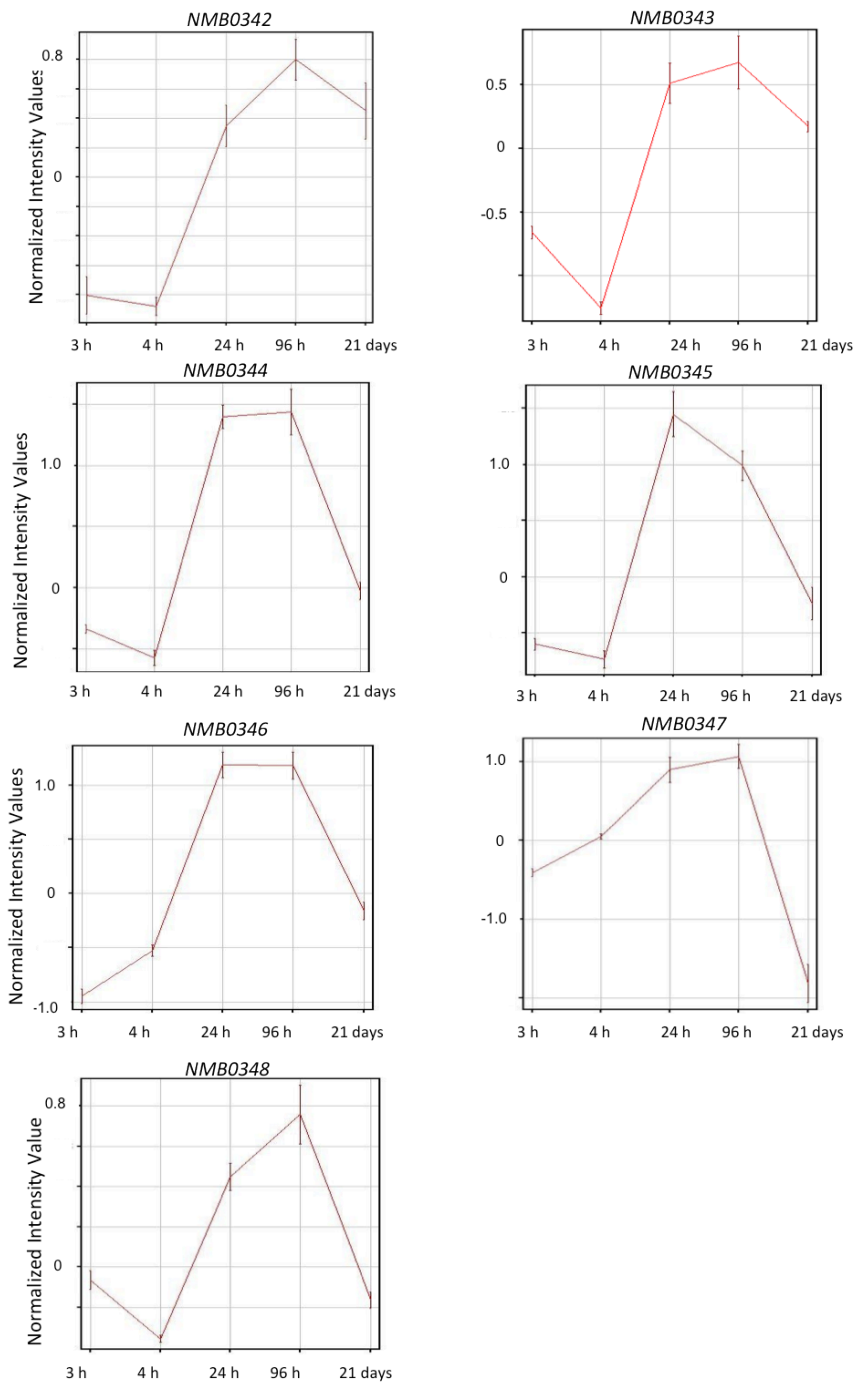


Figure 5.11. Normalized intensity values of *NMB0342-NMB0348* over 21 days. Similar for all genes presented is an initial down-regulation at 4 h (except *NMB0347*) before an up-regulation at 24 h and 96 h and a subsequent down-regulation at 21 days indicating a potential involvement in host cell interaction at 24 h and 96 h. The error bars indicate the SD. The 3 h samples are those obtained from culture in DMEM in absence of host cells (3 h-DMEM). Replicates of each time point were averaged and normalized to the mean intensity values.

5.2.6. Analysis of gene expression using quantitative real time PCR (q-RT-PCR)

Microarrays, convenient for study of the genome wide transcriptome and to identify genes potentially involved in host cell association, are generating hypotheses by providing preliminary evidence, that some genes, for example *siaD*, *porA*, *porB*, and *NMB0345* might be involved in host cell association due to their up-regulation. Q-RT-PCR was performed to gain supportive evidence on the data obtained from the microarray experiments by testing the generated hypotheses and to assure the biological importance of those genes before pursuing further investigations.

The time points selected for q-RT-PCR analysis were the 24 h and 96 h p.i. The microarray data have shown a great difference between early (4 h) and later (24 h and 96 h) time points. The 21 days transcription data again differ from the earlier time points. As the focus of the present study is on the meningococcal gene-expression during prolonged host cell association, and the 24 h and 96 h data were reproducible and of high quality, these two time points were chosen for q-RT-PCR analysis.

For q-RT-PCR experiments, fresh RNA was extracted of three independent biological replicates at each time point in the same manner as for the microarray experiments (Chapter 2, Section 2.9.1). The list of genes chosen for q-RT-PCR analysis is set out in Table 5.12. Q-RT-PCR was performed in quadruplicate on each of the three biological replicates at each time point, which resulted in a total of 12 q-RT-PCR replicates per time point and gene. In addition to the genes shown in Table 5.12, *16S* served as the endogenous control which was used for normalization.

Genes analysed using q-RT-PCR (Table 5.12) encoded i) (potential) surface structures (selected on the basis of their expression profiles obtained from microarray experiments); ii) members of the *NMB0345* cluster; and iii) genes previously described as involved in host cell interaction. *CidA* was included because of its role in meningococcal biofilm formation and *lgtE* and *pglD* because of their

potential role in meningococcal surface modulation upon host cell contact. All genes selected for analysis with q-RT-PCR passed a $p \leq 0.05$ in the microarray experiments.

Table 5.12. Description of genes selected for q-RT-PCR

Gene ID	Description (Kegg)
<i>NMB0067 – siaD</i>	Polysialic acid capsule biosynthesis protein SiaD
<i>NMB0342</i>	IspZ; intracellular septation protein A
<i>NMB0344</i>	BolA/YrbA family protein
<i>NMB0345</i>	Putative cell binding factor
<i>NMB0995</i>	Macrophage infectivity potentiator-related protein
<i>NMB1040</i>	Hypothetical protein
<i>NMB1429 - porA</i>	Outer membrane protein PorA
<i>NMB1822 - pgID</i>	Pilus glycosylation protein PgID
<i>NMB1926 - lgtE</i>	Lacto-N-neotetraose biosynthesis glycosyl transferase LgtE
<i>NMB2003 - cidA</i>	Hypothetical protein; potentially involved in biofilm formation (Lappann <i>et al.</i> , 2010)

A visual quality control of each q-RT-PCR experiment was achieved by use of the amplification plot, generated by the Applied Biosystems software (Figure 5.12). Four replicates of each sample and gene were analysed. Each sample was represented by four lines (one for each replicate) of the same colour (Figure 5.12). All four replicates of each sample should show a similar profile on the plot as shown in Figure 5.12. Potential outliers of the four replicates could be identified on the amplification plot. The concentration of cDNA in each reaction was optimized until the amplification start of the endogenous control (16S) was between cycles three and eight.

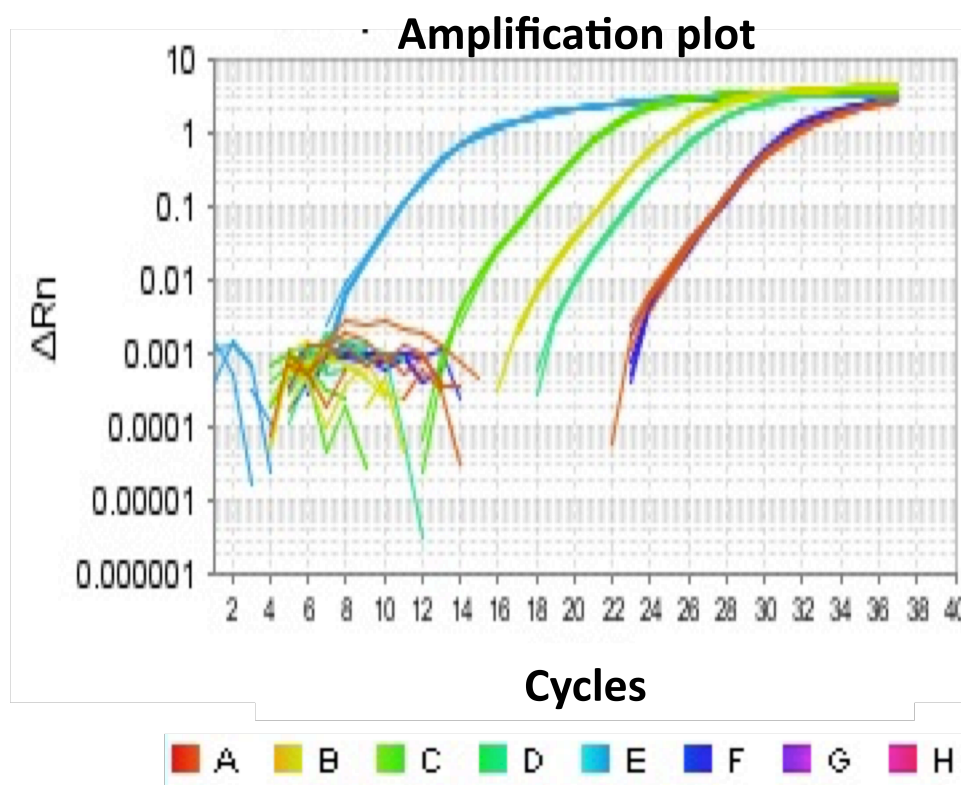
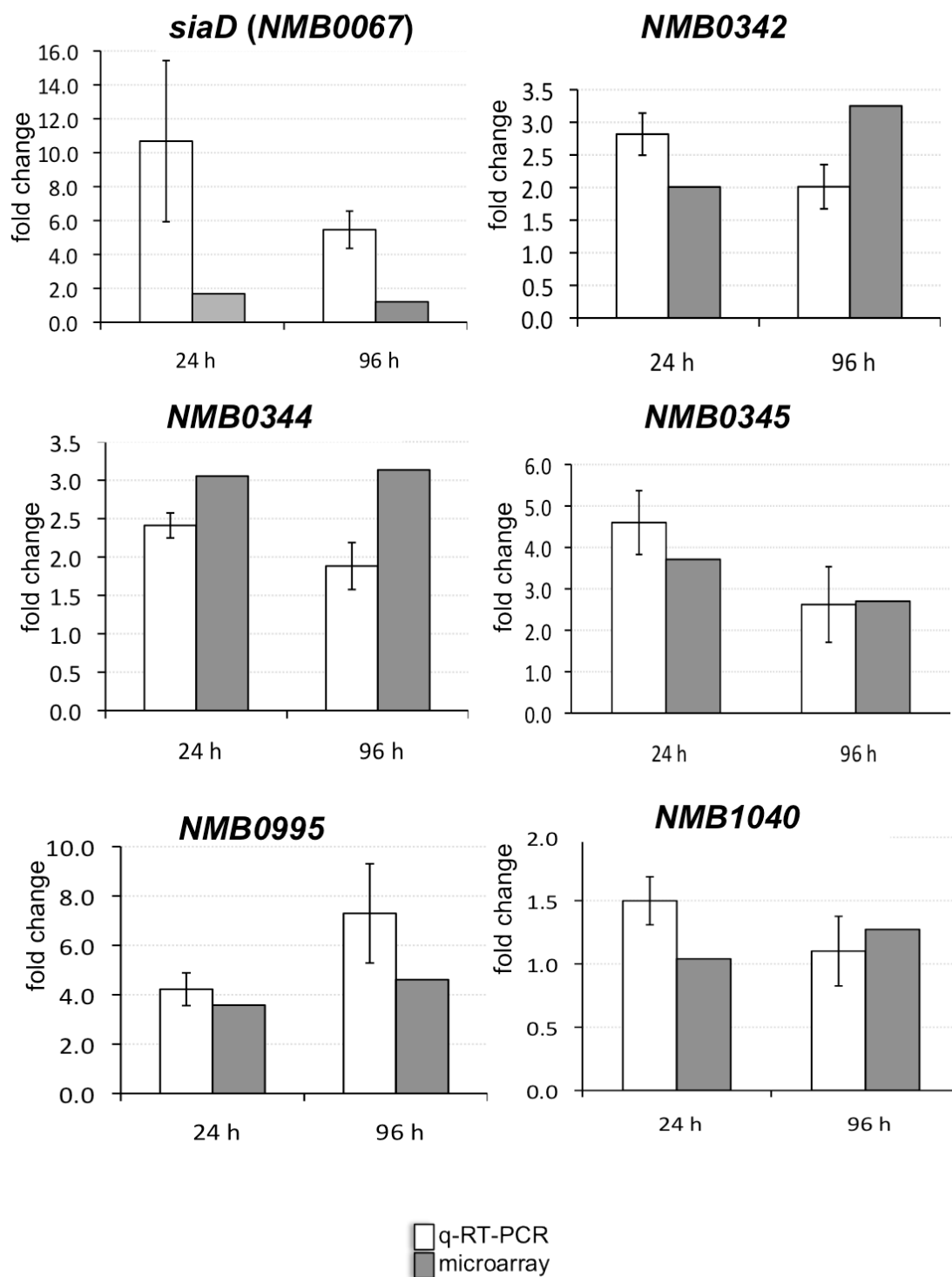


Figure 5.12. Representative amplification plot of a taqman q-RT-PCR experiment. Sample E, starting amplification at cycle 4, is the endogenous control, *16S* RNA. Other samples shown are representative amplifications. Each sample was analysed in four replicates, represented in one colour and each replicate with one line. The relative expression levels of each gene were calculated by comparing the *16S* expression within each sample (time point), then between samples (described in Chapter 2). ΔRn represents the normalized (background subtracted) fluorescence emission intensity of the amplified PCR product.

Q-RT-PCR expression profiles at 24 h and 96 h p.i. were consistent with the data obtained from the microarray experiments. The expression profile of all of the ten genes tested with both techniques showed differential regulation in the same direction (up- or down-regulated). Q-RT-PCR data are shown compared to the microarray data in Figure 5.13.

Microarray and q-RT-PCR analyses of *siaD* (NMB0067), *NMB0342*, *NMB0344*, *NMB0345*, *NMB0995*, *NMB1040*, *porA* (NMB1429), *pglD* (NMB1822) and *lgtE* (NMB1822) showed up-regulation, and of *cidA* (NMB2003) down-regulation at both time points (24 h and 96 h). This congruence reassured the confidence on the data obtained of microarray analysis at 24 h and 96 h.



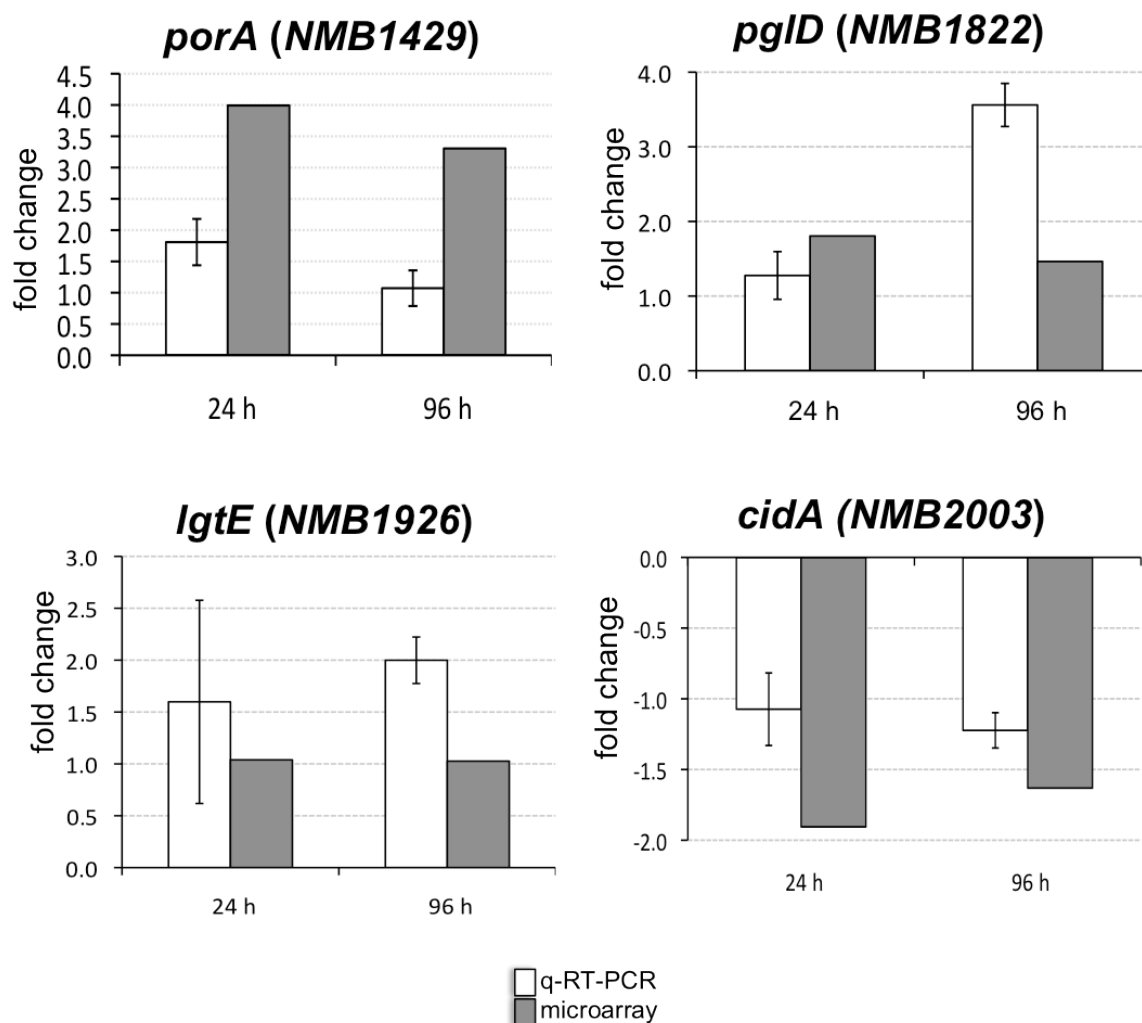


Figure 5.13. Comparison of expression levels of genes over time normalized to 3 h-DMEM using q-RT-PCR (white bars) and microarray technology (grey bars) at 24 h and 96 h. Three independent RNA samples per time point, which were not previously analysed with microarrays, were subjected to q-RT-PCR. Each sample was analysed in quadruplicate. Relative expression levels of each gene were, first, normalized to that of 16S, then compared to the 3 h-DMEM samples. Error bars indicate SEM.

5.3. Discussion

The microarray quality control measurements described in the beginning of this chapter indicate that experiments were satisfactorily reproducible: replicates of each time point correlated $\geq 91\%$. Biological variations, which potentially negatively influence the correlations between replicates of the same time point could result from many factors, including those derived from host cells (for example the age and passage of the host cell monolayers) and bacteria, such as the morphological changes over time of host cell associated meningococci (described in Chapter 2). The scanning EM photographs suggest meningococci adhere as single bacteria and microcolonies to 16HBE14 cells. It may be that the gene expression profile of bacteria within a microcolony, where they lie in close proximity to each other, differs from that of bacteria adhering directly to host cells. Differences in gene expression of bacteria within sub-populations (layers) of a biofilm have been described due to a gradient in the availability of, for example, nutrients and oxygen in different layers of a biofilm (Sternberg *et al.*, 1999; Werner *et al.*, 2004; Yu *et al.*, 2004,).

A plausible explanation for the variation between replicates of the same and of different time points may also be that the extracted bacterial RNA was contaminated at a low level with human RNA as was demonstrated by capillary gel electrophoresis (see Chapter 4). Although efforts to minimize human RNA contamination were performed, such as the separation of host cells and bacteria prior to RNA extraction, interference in terms of non-specific hybridization could not be excluded. Such interference may contribute to the variance between replicates at the same time point as well as to false-positive signals.

A further explanation for the variation might be, for example, differences in availabilities of nutrients. This could influence the state of bacteria as well as epithelial cells, whose change will all dictate the meningococcal transcriptome seen by microarray or q-RT-PCR.

Despite the complex experimental set up of the present study, a good correlation ($\geq 91\%$) between replicates of samples was found. King *et al.* (2005) classified correlation values into high ($>94\%$) and moderate ($>80\%$).

Transcriptomes obtained from meningococcal host cell co-cultivation experiments correlated $\geq 80\%$ between different time points indicating that the time of co-cultivation clearly influences the meningococcal transcriptome. Correlations between samples obtained from host cell co-cultivation and the 3 h samples (where no host cells were present) were $>60\%$ indicating the influence of the presence of host cells on the meningococcal transcriptome. Corroborated by Flight and Wentzell (2010), as anticipated, time points being far away from one another (21 days and 96 h), had a lower correlation than time points being closer together (96 h, 24 h and 4 h).

The meningococcal transcriptome during host cell interaction was analysed at four different time points (4 h, 24 h, 96 h and 21 days) and compared to 3 h-DMEM samples obtained from planktonic bacteria (grown in the absence of host cells). The gene expression profile at 24 h and 96 h was similar but differed substantially from those at 4 h and at 21 days. Grifantini *et al.* (2002) and Dietrich *et al.* (2003) demonstrated that the transition from plate / planktonic to host cell adherent growth was associated with a substantial change in the bacterial transcriptome. This plausibly reflects adaption to a different environment and the further differences found in the present study between 4 h and 24 h transcriptomes suggest that the adaptive process is not complete at 4 h.

On the other hand, the similar transcription profiles found at 24 h and 96 h suggest that bacteria have by this time established a more stable co-existence with the host cells. These findings suggest that transcriptomes assessed at time points of 24 h and 96 h may be better representations of a stable the colonizing state than the early time point of 4 h. Although co-cultivation for 21 days may well represent as even closer model of the *in vivo* colonizing state (which may persist for weeks to months), transcriptional data from the experiments reported here

indicated greater biological variation than at the earlier times. This may plausibly reflect differences in the extent to which different modes of colonisation (scattered cells, microcolonies, biofilm) are established, and the result of accumulated phase variation in critical genes having genome wide consequences.

Future work at these long co-cultivation times will require many more replicates to establish a truly representative 'mean' transcriptome, and should include transmission electron microscopy to establish the pattern of bacterial / host cell interaction.

Up-regulation of bacterial gene expression results in increased protein synthesis and genes found to be up-regulated in the present study were interpreted accordingly. Appropriate caution was given to reflect the obvious point that the level of expression of complex multi-component structures (e.g. pili) or non-protein determinants (capsule or LOS), depend on the coordinate expression of single – even major – subunits or enzymes involved in synthesis. Post translational modifications such as glycosylation of, for example, proteins of type IV pili, involve interaction of more than one gene product and expression profiles of functionally related genes or gene clusters such as, for example the capsule or type IV pilus biosynthesis genes, were interpreted together. For example, the up-regulation of *siaD* at 24 h and 96 h in context with the other capsule biosynthesis genes which were either found to be down-regulated or not differentially expressed, is not evidence enough to speculate about a possible capsule expression.

Interpretations of down-regulated genes remain difficult. One possibility is, that the gene product of the down-regulated gene is not required in the experimental condition. Alternatively, a gene could temporarily be down-regulated as its product is present in sufficient amounts and microarray data represent a snapshot of gene expression. In addition, an up-regulated negative transcriptional regulator can lead to down-regulation of several genes. For clarification, experiments on the protein level, for example antibody staining or ELISA, are required.

Of the 2063 open reading frames of *N. meningitidis* strain MC58, more than 700 have hypothetical / unknown function which complicates interpretation as the functional relationship of these genes is unclear and the expression profiles of these genes could only be interpreted on a single gene basis.

Genes found to be non-differentially expressed were not interpreted in the present study as they did not appear in the lists of differentially regulated genes. Exemptions were genes such as *NMB1508 (pptB)*, which has been shown to be involved in meningococcal host cell interaction (Chamot-Rooke *et al.*, 2011). A possible explanation for a gene known to be involved in host cell interaction but which was not differentially transcribed in the present study might be that it has multiple functions and is also involved in meningococcal survival during planktonic growth. Genes essential for meningococcal survival might also not appear to be differentially expressed in the present study. Future work might include, for example, quantitative immunoprecipitations with specific antibodies to clarify if the products of non-differentially expressed genes are present at a similar level during the time course of the present study.

Microarrays are a highly sensitive method of transcriptome analysis and changes of gene expression can be detected on the ng level of RNA. In the present study, microarray experiments generated hypotheses, i.e. that up-regulated genes (at later time points) are involved in prolonged host cell interaction. To test these hypotheses, the expression of a set of genes of freshly extracted meningococcal RNA (which was not previously used for microarray hybridization) was tested by a different technique, q-RT-PCR. Future work might consider the validation of microarray experiments by q-RT-PCR on the same biological sample and of a number of genes representing the 2063 open reading frames of strain MC58. This would be advantageous to directly confirm the microarray data by a different technique without the possible variation of two separately prepared cDNA samples. In the present study microarray and q-RT-PCR analysis on the same sample was impossible due to the small amount of RNA extracted from each co-cultivation

experiment. Additionally, future work should include experiments on the protein level to assess whether differential gene regulation is accompanied by a parallel change of the encoded protein.

Chapter 6

In silico comparison with other meningococcal
transcriptomes

6.1. Introduction

The results of the present transcriptome analysis were compared to those of other meningococcal gene expression studies relevant in modelling colonization (Grifantini *et al.*, 2002; Dietrich *et al.*, 2003; O'Dwyer *et al.*, 2009), septicaemia (Hedman, 2009) and invasive disease in an infant rat model (Sun *et al.*, 2000) to identify genes potentially involved in colonization and invasive disease. Amongst others, the expression of genes encoding previously identified meningococcal vaccine antigens was of particular interest. Furthermore, this transcriptome comparison aimed to reveal other potential vaccine candidates. It was traditionally believed that an ideal vaccine candidate should be highly expressed when interacting with epithelial cells and thereby possibly inducing herd immunity, such as the ones identified by Grifantini *et al.* (2002). However, as it was stated in my Hypothesis (Chapter 1), a surface protein that is minimally expressed during colonization and highly expressed in blood infection could also be a putative vaccine candidate. It is believed that the extensive diversity of meningococcal outer membrane proteins, which has hampered the development of effective vaccines, is driven by natural selection in the nasopharynx, exemplified by the highly expressed porins PorA and PorB. Proteins with relatively low level of expression in this environment should be subjected to a lesser extent to the mucosal immune pressure, and so would be less diverse in sequence. This principle was used in the present study in an attempt to identify novel vaccine candidates.

The meningococcal transcriptome obtained at 21 days of co-cultivation with host cells had similarities to the transcriptome at 24 h and 96 h, however, a greater variation than the latter two. For greater clarity in the complex transcriptome comparison, data obtained at 21 days was therefore not used in the comparisons presented below.

6.2. Results

6.2.1. Comparison of the meningococcal transcriptome from the present study with that from a similar co-cultivation model (Grifantini *et al.*, 2002)

Grifantini *et al.*, (2002) investigated the meningococcal transcriptome during interaction with 16HBE14 cells for up to 180 min. Data of Grifantini *et al.*, (2002) were normalized to the transcriptome of planktonic bacteria in the absence of host cells as was in the present study.

The 4 h vs 3 h-DMEM data of the present study ($p \leq 0.01$, $fc \geq 2.0$), being the closest one to the 180 min vs 180 min-DMEM of Grifantini *et al.* (2002; $p \leq 0.01$, $fc \geq 2.0$), was used for comparison. Data from both studies were grouped into up- and down-regulated genes and compared in Venn diagrams to identify similarities and differences (Figure 6.1). In both studies, 47 and 19 genes were found to be mutually identified as down- and up-regulated, respectively (of 199 down- and 149 up-regulated genes of Grifantini *et al.*, 2002). Those down-regulated genes included, for example, the pilus genes *pilE* and *pilP*. Complete lists of genes mutually identified in both studies are shown in Table E of the Appendix.

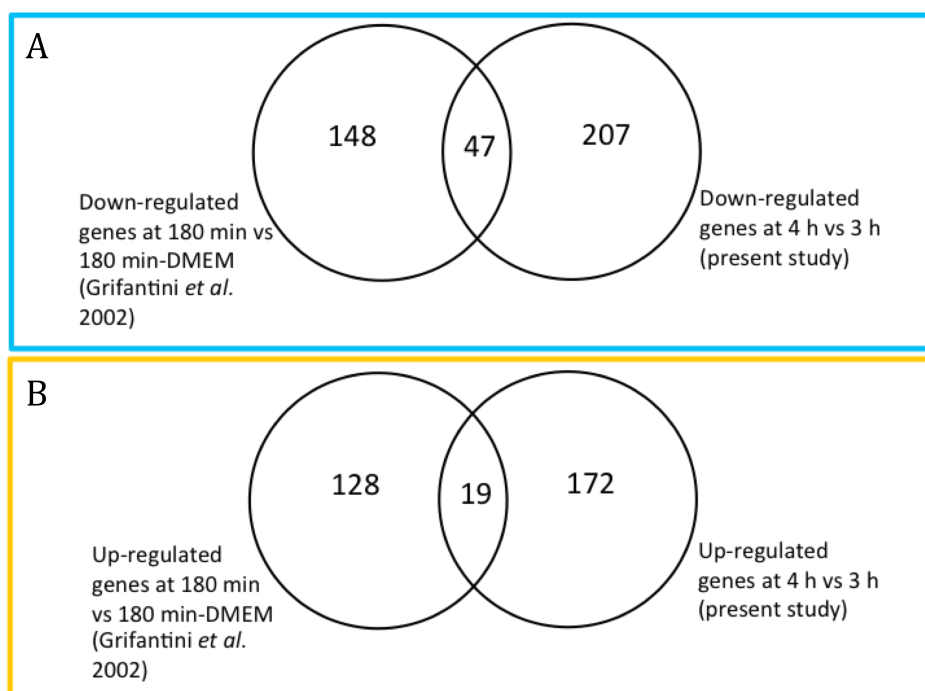


Figure 6.1. Venn diagrams of differentially expressed genes identified in the present study (4 h vs 3 h-DMEM) and in Grifantini *et al.* (2002) at 180 min vs 180 min-DMEM. Both data sets were filtered on $fc \geq 2.0$, $p \leq 0.01$ and grouped into down-regulated genes (panel A) and up-regulated genes (panel B). Lists of the common genes in both studies are shown in Table E of the Appendix.

By comparing the different time points of the present study, significant transcriptional changes were found between early (4 h) and later (24 h and 96 h) stages of host cell interaction as discussed in chapter 5, while the meningococcal gene expression profile at 24 h and 96 h showed much greater similarities, indicating that from 24 h onward the major transcriptome changes for adaptation of colonized habitat were established. The transcriptomes of late time points (present study) were also compared with that of 180 min (Grifantini *et al.*, 2002), to investigate whether a parallel comparison can be drawn with that of 4 h transcriptome (present study). Of the 145 down- and 257 up-regulated genes at 24 h -96 h, 9 and 29 were also found to be down- and up-regulated by Grifantini *et al.* (2002) (The Venn diagram and lists of commonly identified genes are shown in Figure A and Table F of the Appendix). These findings further

support the previous observations that the meningococcal transcriptional profile at early time points of host cell interaction is representative of an acute phase of the transcriptional change, and was different from the transcriptome of later time points, representative of the adaptation being stabilized. Apart from the transcriptional differences reflecting different biological responses under the experimental conditions, other technical factors such as different RNA extraction protocols, microarray platform and hybridization techniques and data analysis software programs may also contribute to the differences between the two studies.

Grifantini *et al.* (2002) identified genes as potential vaccine candidates which were up-regulated during epithelial host cell interaction, conserved among different meningococcal strains and surface expressed (confirmed by FACS analysis). Recombinant proteins of those gene products were able to stimulate bactericidal antibodies in infant mice. By this approach, five vaccine candidates were identified: NMB0315 (conserved hypothetical protein), NMB0652 (MafA protein), NMB0995 (macrophage infectivity potentiator-related protein), NMB1119 (hypothetical protein) and NMB1876 (N-acetylglutamate synthase). In the present study, three of these five genes (*NMB0315*, *NMB0995* and *NMB1876*) were found to be significant differentially expressed (Table 6.1). *NMB0315* was up-regulated at 4 h as was shown at 180 min by Grifantini *et al.*, (2002). However, *NMB0995* and *NMB1876* were relatively down-regulated at 4 h. *NMB1876* was also down-regulated at the other time points, and therefore fulfilling the present definitions of a potential vaccine candidate (described in the Hypothesis, Chapter 1). *NMB0995* was up-regulated at late time points (24 h, 96 h and 21 d p.i.) and in a transcription study modelling blood infection (Hedman, 2009) which might indicate a role in colonization as well as in invasive disease.

Table 6.1. Expression profile of meningococcal genes encoding vaccine candidates identified by Grifantini *et al.* (2002).

Gene ID	COG	Description (Kegg)	p-value	4h vs 3h	24h vs 3h	96h vs 3h	21 days vs 3h
NMB0315	Cell wall/membrane biogenesis genes	Conserved hypothetical protein	0.0408	1.13	-1.02	1.18	-1.45
NMB0995	Function unknown genes	Macrophage infectivity potentiator-related protein	1.08×10^{-4}	-1.30	3.58	4.61	2.20
NMB1876	Amino acid transport and metabolism genes	N-acetylglutamate synthase	0.0059	-1.78	-1.11	-1.10	-2.20

6.2.2. Comparison of the meningococcal transcriptome from the present study with that from interaction with Hep-2 and HBMEC cells (Dietrich *et al.*, 2003)

Dietrich *et al.* (2003) studied the meningococcal transcriptome of the capsule deficient MC58 (*siaD* deletion) strain during 6 h p.i. with epithelial HEp-2 and endothelial HBMEC cells. Data were normalized to the transcriptome during planktonic growth (6 h) in cell culture medium (DMEM), similar to the control used in the present study. In the epithelial cell model (Hep-2 cells), 72 genes, using a fold change cut-off of ≥ 1.6 , including *porA* (NMB1429) were differentially expressed. Five of the 72 genes were found to be down-regulated. Of the 72 genes, 20 were differentially expressed in the present study (4 h vs 3 h-DMEM, $p \leq 0.02$ $fc \geq 1.6$). However, only four (NMB0200, NMB0328, NMB0442 and NMB2000) of the 20 genes showed the same direction of differential regulation. A complete list of the 20 differentially expressed genes is shown in Table G in the Appendix.

The meningococcal transcriptome during infection of HBMEC cells (Dietrich *et al.*, 2003) revealed 48 differentially expressed genes ($fc \geq 1.6$) of which 41 were up-regulated. Of these 48 genes, 15 were found to be differentially expressed in the present study (4 h vs 3 h-DMEM data, $p \leq 0.02$ $fc \geq 1.6$), four (NMB0200, NMB0546, NMB0942, NMB1330) of these 15 in the same direction as in Dietrich *et al.* (2003) study. A complete list of the 15 differentially expressed genes is shown in Table G in the Appendix.

Differences in the data sets obtained by Dietrich *et al.* (2003) and in the present study could be explained by the use of different cell lines. Although 16HBE14 and Hep-2 cells are derived from the human respiratory epithelium, Hep-2 cells are human epidermoid laryngeal carcinoma cells, which might have different characteristics than 16HBE14 cells, which are human bronchial epithelial cells. HBMEC cells are human brain endothelial cells, which differ from the epithelial phenotype and which meningococci encounter at a later stage of invasive disease. Other factors such as the different *N. meningitidis* strain, different RNA extraction protocol and microarray platform could contribute to the discrepancies between the gene lists generated.

6.2.3. Comparison of the meningococcal transcriptome from the present study with that of a biofilm model (O'Dwyer *et al.*, 2009)

A Sorbarod flow system, a cylindric shaped cellulose filter, attached to a continuous flow of broth, was used to study the meningococcal transcriptome after 48 h of inoculation (O'Dwyer *et al.*, 2009). Data were normalized to the transcriptome of plate grown organisms from which the initial bacterial load was seeded. Because the reference sample used in present study were obtained from liquid phase grown meningococci, for comparison purpose, both data sets were normalized to the median value of each transcriptome and filtered on $p \leq 0.05$ (without filtering on f_c). The assumption was that the median value of two systems was equal. The resulting gene lists were then sorted according to the f_c , from high to low. The top 200 (up-regulated) and bottom 200 (down-regulated) genes from each list were taken for comparison. The comparison results are shown in Venn diagrams (Figure B in the Appendix). Co-identified in both studies were seventeen genes being down- and 31 being up-regulated. Lists of co-identified genes are shown in Table H of the Appendix. The results showed that Sorbarod filter colonisation model to a very limited extent resembles the biology of the cell colonization model.

6.2.4. Analyzing the expression profile of meningococcal genes previously identified by Sun *et al.*

(2000) as essential in invasive disease

Sun *et al.* (2000) used a signature-tagged mutagenesis (STM) approach to identify 75 genes that were essential for invasive disease in an infant rat model. In the present study, the transcriptional profile of the 75 identified genes were followed over 21 days of host cell interaction, to identify genes which are essential for invasive disease and potentially involved in colonization (up-regulated in the present study). Of the 75 genes identified by Sun *et al.* (2000), sixteen were differentially expressed in the present study (Table 6.4). Twelve (*NMB0114*, *NMB0342*, *NMB0345*, *NMB0748*, *NMB0758*, *NMB0825*, *NMB0943*, *NMB0994*, *NMB1710*, *NMB1797*, *NMB1814* and *NMB2044*) of the sixteen genes were up-regulated at 24 h, 96 h and 21 days (compared to 3 h-DMEM) suggesting that they are involved in both colonization and invasive disease. *NMB0543*, *NMB1515*, *NMB1729* and *NMB1790* were down-regulated at all time points (compared to 3 h-DMEM) and which therefore were identified as potential vaccine candidate, since these genes may incur less sequence variation and may be up-regulated in blood (see Hypothesis, Chapter 1). *NMB0543* is predicted to have 14 trans-membrane spanning domains and is likely to be expressed on the meningococcal surface (Tettelin *et al.*, 2000). *NMB0543* encodes a conserved lactate permease and was identified by Exley *et al.* (2005 and 2005a) to be essential for meningococcal association with nasopharyngeal cells in human tissue explants. Future work, for example FACS analysis with specific antibodies might demonstrate surface expression of *NMB0543* and the other three vaccine candidates.

Table 6.2. Expression profile of genes identified to be essential for invasive disease in an infant rat model (Sun *et al.*, 2000).

Gene ID	NMB0030	NMB0067	NMB0114	NMB0307	NMB0342	NMB0345	NMB0543	NMB0686	NMB0748	NMB0758	NMB0792	NMB0825
COG	General function prediction only genes; translation	Cell wall/membrane biogenesis genes	n/a	Amino acid transport and metabolism genes	Cell cycle control, mitosis and meiosis genes	Carbohydrate transport and metabolism genes	Intracellular trafficking and secretion genes	Transcription	Not in COGs genes	Cell wall/membrane biogenesis genes	Amino acid transport and metabolism genes	Cell wall/membrane biogenesis genes
Description (KEGG)	Methionyl-tRNA synthetase	Polysialyl transferase SiaD	Nitrogen regulation protein NtrY	Phosphor-2-dehydro-3-deoxyhept	Intracellular septation protein A	Cell-binding factor, putative	L-lactate permease, putative	Ribonuclease III	Host factor-I	Polyribonucleotide nucleotidyl transferase	Transporter, NadC family	Putative ADP-heptose synthase
4 h *	-1.21	-1.08	1.40	-1.02	1.17	-1.22	-1.61	-1.09	-1.75	1.13	-1.13	1.04
24 h *	-1.30	1.68	1.38	-1.39	2.01	3.71	-2.26	-1.13	1.58	3.03	1.72	1.57
96 h v*	-1.53	1.21	1.70	-1.41	3.25	2.70	-2.22	1.02	1.78	2.60	1.95	1.12
21 d *	-1.43	-2.36	1.16	-1.44	1.15	1.15	-1.54	1.71	3.41	1.51	-1.02	1.60
24 h **	-1.08	1.82	-1.01	-1.36	1.72	4.53	-1.41	-1.04	2.75	2.69	1.95	1.52
96 h **	-1.26	1.31	1.21	-1.38	2.78	3.30	-1.38	1.11	3.12	2.31	2.21	1.08
21 d **	-1.18	-2.18	-1.21	-1.41	-1.02	1.41	1.05	1.86	5.95	1.35	1.11	1.54
96 h ***	-1.17	-1.39	1.23	-1.02	1.62	-1.37	1.02	1.15	1.13	-1.16	1.13	-1.40
21 d ***	-1.10	-3.97	-1.19	-1.04	-1.75	-3.22	1.47	1.94	2.16	-2.00	-1.75	1.02
21 d ****	1.07	-2.85	-1.47	-1.02	-2.82	-2.34	1.44	1.68	1.91	-1.72	-1.99	1.43
p-value	0.0325	0.0031	0.0053	0.0268	0.0431	7.19*10 ⁻⁴	0.003179	0.0295	0.0012	0.0027	0.0015	0.0047

* normalized to 3 h-DMEM; ** normalized to 4h p.i.; *** normalized to 24 h p.i.; **** normalized to 96 h p.i.; n/a not assigned into COG

Highlighted in green are genes up-regulated at 4 h, 24 h, 96 h and 21 days; highlighted in pink are up-regulated genes at 24 h, 96 h and 21 days; both indicating genes involved in host cell interaction and invasive disease. Highlighted in yellow are genes down-regulated (potential vaccine candidates) at 4h, 24 h, 96 h and 21 days.

Table 6.2 – continued. Expression profile of genes identified to be essential for invasive disease in an infant rat model (Sun *et al.*, 2000).

Gene ID	NMB0943	NMB0944	NMB1145	NMB1515	NMB1527	NMB1558	NMB1710	NMB1728	NMB1729	NMB1790
COG	Amino acid transport and metabolism genes	Amino acid transport and metabolism genes	Cell wall/membrane biogenesis genes	Not in COGs genes	Inorganic ion transport and metabolism genes	Not in COGs genes	Not in COGs genes	Intracellular trafficking and secretion genes	Intracellular trafficking and secretion genes	Not in COGs genes
Description (KEGG)	5,10-methylenetetrahydrofolate reductase metF	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	UDP-N-acetylglucosamine 6-phosphate deacetylase	Putative transporter; putative integral membrane protein	ADP-heptose-LPS heptosyltransferase II	Diacylglycerol kinase	Glutamate dehydrogenase, NADP-specific	Biopolymer transport protein ExbD	Biopolymer transport protein ExbB	Glutaredoxin 3
4 h *	1.25	-1.41	-1.30	-1.01	1.08	2.18	-1.33	-1.30	-1.30	-2.84
24 h *	1.27	2.26	-1.05	-1.64	-1.29	-1.05	3.32	1.59	-1.78	-1.17
96 h *	1.08	1.55	1.39	-1.51	-1.43	-1.26	2.78	2.01	-1.10	-1.21
21 d *	1.57	1.47	-1.42	-1.64	1.53	-1.30	1.45	-2.61	-1.78	-1.03
24 h **	1.01	3.19	1.24	-1.62	-1.39	-2.28	4.40	1.80	1.28	2.43
96 h **	-1.16	2.18	1.80	-1.49	-1.55	-1.70	3.69	2.28	2.06	2.34
21 d **	1.25	2.07	-1.09	-1.63	1.41	-2.84	1.92	-2.30	1.28	2.75
96 h ***	-1.17	-1.46	1.45	1.09	-1.12	-1.18	-1.19	1.26	1.61	-1.04
21 d ***	1.24	-1.54	-1.35	-1.00	1.96	-1.24	-2.29	-4.15	-1.00	1.13
21 d ****	1.45	-1.05	-1.96	-1.09	2.19	-1.05	-1.92	-5.24	-1.62	1.17
p-value	0.0181	0.0120	0.0298	0.0014	0.0028	0.0161	0.0011	0.0105	0.0029	1.18*10 ⁻⁴

* normalized to 3 h-DMEM; ** normalized to 4 h p.i.; *** normalized to 24 h p.i.; **** normalized to 96 h p.i. n/a not assigned into COG
 Highlighted in green are genes up-regulated at 4 h, 24 h, 96 h and 21 days; highlighted in pink are up-regulated genes at 24 h, 96 h and 21 days; both indicating genes involved in host cell interaction and invasive disease. Highlighted in yellow are genes down-regulated (potential vaccine candidates) at 4h, 24 h, 96 h and 21 days

Table 6.2 – continued. Expression profile of genes identified to be essential for invasive disease in an infant rat model (Sun *et al.*, 2000).

Gene ID	NMB1797	NMB1814	NMB1929	NMB2044
COG	Energy production and conversion genes	Energy production and conversion genes	Cell wall/membrane biogenesis genes	Carbohydrate transport and metabolism genes
Description (KEGG)	Penicillin-binding protein 3	3-dehydroquinase synthase	Lacto-N-neotetraose biosynthesis glycosyl transferase LgtA	Phosphoenolpyruvate-protein phosphotransferase
4 h *	-1.12	1.41	1.05	1.78
24 h *	1.91	1.61	-1.37	1.39
96 h *	2.78	2.05	-1.42	1.66
21 d *	1.05	1.11	-1.14	1.01
24 h **	2.13	1.14	-1.43	-1.28
96 h **	3.11	1.45	-1.49	-1.07
21 d **	1.17	-1.27	-1.20	-1.77
96 h ***	1.46	1.27	-1.04	1.20
21 d ***	-1.82	-1.45	1.20	-1.38
21 d ****	-2.66	-1.84	1.25	-1.65
p-value	0.0020	9.50*10 ⁻⁴	4.65*10 ⁻⁴	0.0081

* normalized to 3 h-DMEV; ** normalized to 4h p.i.; *** normalized to 24 h p.i.; **** normalized to 96 h p.i. n/a not assigned into COG
 Highlighted in green are genes up-regulated at 4 h, 24 h, 96 h and 21 days; highlighted in pink are up-regulated genes at 24 h, 96 h and 21 days; both indicating genes involved in host cell interaction and invasive disease. Highlighted in yellow are genes down-regulated (potential vaccine candidates) at 4h, 24 h, 96 h and 21 days

6.2.5. Comparison of the meningococcal transcriptome during epithelial cell association (present study) with that of an *ex vivo* model of human septicaemia (Hedman, 2009)

Hedman (2009) analysed the meningococcal transcriptome during an *ex vivo* model of human septicaemia using whole blood of healthy donors. By this approach, genes involved in meningococcal survival during blood infection were identified. Surface expressed, conserved up-regulated genes were identified as potential vaccine candidates.

The transcriptional profile of MC58 during host cell association obtained from the present study was compared to that of the model of human septicaemia (Hedman, 2009) to identify genes which are potentially involved in either colonization or invasive disease. As the two different models represent the conditions meningococci might encounter during colonization and septicaemia, similarities were expected to be low. With the aim to identify potential vaccine candidates, the transcriptional profile of those already identified by Hedman (2009) was investigated during host cell interaction in the present study.

For comparison of the present study with data obtained by Hedman (2009), both data sets were normalized to the median spot intensity values and filtered on $p \leq 0.05$. Time points chosen for comparison were the 90 min data of the septicaemia model (Hedman, 2009), as this time point has been shown to be stable and at 90 min bacteria were rapidly multiplying in blood and therefore the transcriptome plausibly reflects growth in blood. Of the present study, the 24 h-96 h data were used. Similar to the comparison method applied in the section above (6.2.3), the top 200 up and down-regulated genes from each list were compared. By using Venn diagrams, 16 commonly identified down- and 33 up-regulated genes were found (Figure 6.2). The 33 common up-regulated genes, including besides *siaC* (NMB0068), *hfq* (NMB0748), OMP class 4 (NMB0382) and *pilP* (NMB1811) mainly genes within the COGs group of 'Energy production and conversion genes', might be involved in survival during both, host cell interaction and blood infection. As for the co-identified 16 down-regulated genes it is possible that none of them is required in either condition (host cell interaction and blood survival). Future work including experiments on the protein level, for example by Western blotting with specific antibodies might answer the questions if down-regulation of the common 16 genes and up-regulation of the 33 genes is accompanied by changes in the abundance of the encoded proteins. The differences between the two studies might indicate that genes that were important during blood infection (up-regulated) were not likely to be involved in host-cell interaction (down-regulated) and vice versa.

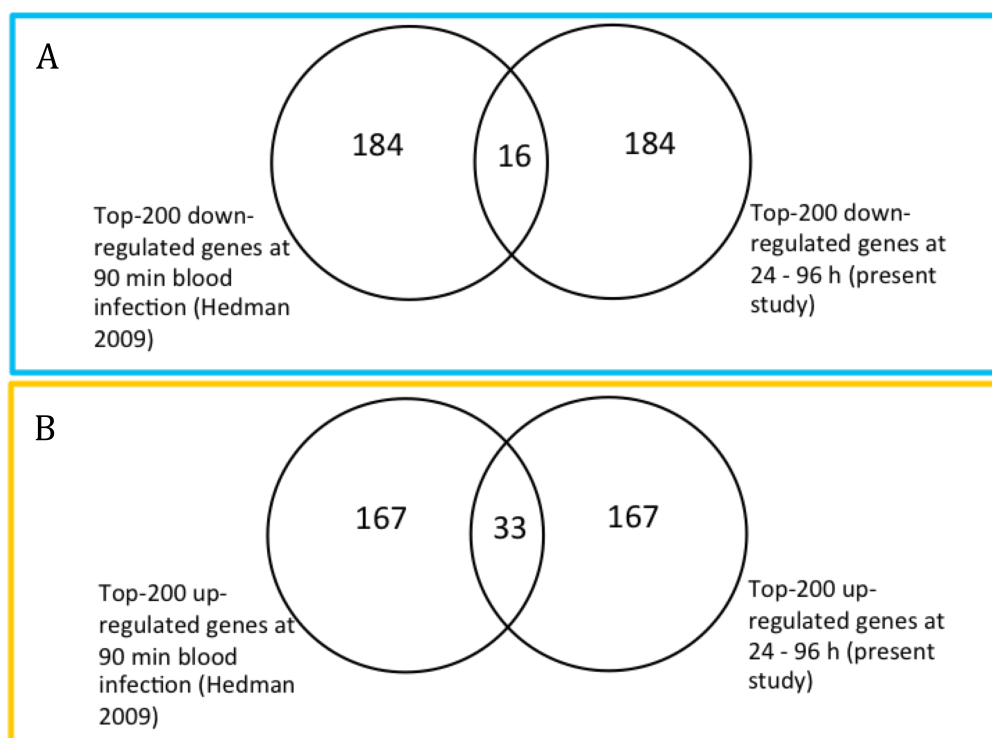


Figure 6.2. Venn diagrams comparing the top-200 down- (panel A) and up-regulated (panel B) genes identified during 90 min blood infection (Hedman, 2009) and of 24 h-96 h p.i. of the present study. Data of both studies were normalized to the median and filtered on $p \leq 0.05$. In both studies, 16 genes were found to be down- and 33 up-regulated. Lists of the genes commonly identified in both studies are shown in Table I in the Appendix.

The transcriptional profiles ($p \leq 0.05$) of potential vaccine candidates identified by Hedman (2009) during host cell interaction over 21 days (of the present study) are shown in Table 6.6. Those genes which were down-regulated during host cell interaction but up-regulated and potentially surface expressed during blood infection were considered to be potential vaccine candidates. One such vaccine candidate was identified, *NMB2135*, which was down-regulated at all time points of the present study. *NMB2135* is a hypothetical protein of 1405 aa (4218 nt) of unknown function. Future work including for example FACS analysis using a specific antibody would reveal the surface location of this protein.

Table 6.3. Expression profile of vaccine candidates identified by Hedman (2009) during a meningococcal transcriptome analysis in a model of septicemia ($p \leq 0.01$, $f_{c \geq 1.5}$). Data obtained from the present study were added in columns to the right. Data were normalized to the median for each time point. Highlighted in yellow is the candidate which was identified as potential vaccine candidate.

Gene ID	NMB0267	NMB0318	NMB0345	NMB0390	NMB0464	NMB0511	NMB0563	NMB0586	NMB0816	NMB1040	NMB1164	NMB1333	NMB1497	NMB1775
COGs	Cell wall/membrane biogenesis genes	Inorganic ion transport and metabolism genes	Defense mechanisms genes	Posttranslational modification, protein turnover, chaperones genes	Carbohydrate transport and metabolism genes	Not in COGs genes	Intracellular trafficking and secretion genes	Intracellular trafficking and secretion genes	Not in COGs genes	Inorganic ion transport and metabolism genes	Not in COGs genes	Not in COGs genes	Inorganic ion transport and metabolism genes	Cell wall/membrane biogenesis genes
Description (KEGG)	Conserved hypothetical protein	TonB-dependent receptor, putative	Fatty acid efflux system protein	Cell-binding factor, putative	Maltose phosphorylase	Phospholipase A1, putative	Hemagglutinin/hemolysin-related protein	Hypothetical protein	Thiamine biosynthesis lipoprotein in ApbE	Adhesin, putative	Hypothetical protein	Hypothetical protein	TonB-dependent receptor	Hypothetical protein
0 to 20' *	X			X	X		X		X		X			
0 to 40' *	X			X	X		X		X		X			
0 to 60' *	X				X		X	X		X		X		
0 to 90' *	X													
0 to 240' *	X	X					X			X				X
20' to 40' *			X					X					X	
20' to 60' *			X					X					X	
20' to 90' *		X				X								X
20' to 4 h *		X												X
4h **	-0.064	-0.003	0.086	0.013	-0.001	-0.028	-0.023	0.008	0.001	-0.020	n/a	0.006	-0.017	n/a
24h **	-0.106	0.135	-0.029	-0.001	0.075	0.106	-0.016	0.002	-0.042	0.005	n/a	-0.051	0.028	n/a
96h **	0.220	-0.053	0.218	0.005	0.087	-0.245	-0.031	0.130	0.049	0.077	n/a	0.086	-0.051	n/a
21d **	0.037	0.019	-0.237	-0.092	0.020	0.046	0.114	-0.066	0.060	-0.024	n/a	0.025	0.130	n/a

* Hedman, 2009; ** present study

Table 6.3 - continued. Expression profile of vaccine candidates identified by Hedman (2009). . Highlighted in yellow is the candidate which was identified as potential vaccine candidate.

Gene ID	NMB0293	NMB0497	NMB0964	NMB1668	NMB1714	NMB1779	NMB1882	NMB1961	NMB2135
COGs	Inorganic ion transport and metabolism genes	Intracellular trafficking and secretion genes; Cell wall/membrane biogenesis genes	Intracellular trafficking and secretion genes	Intracellular trafficking and secretion genes	Inorganic ion transport and metabolism genes	Not in COGs	Cell cycle control, mitosis and meiosis genes	Inorganic ion transport and metabolism genes	Function unknown genes
Description (KEGG)	Hemoglobin receptor	Multidrug efflux pump channel protein	Hypothetical protein	Hemagglutinin/hemolysin-related protein	TonB-dependent receptor	VacJ-related protein	Conserved hypothetical protein	TonB-dependent receptor	Conserved hypothetical protein
0 to 20' *								X	
0 to 40' *								X	
0 to 60' *		X						X	
0 to 90' *							X	X	X
0 to 240' *		X				X			
20' to 40' *			X						
20' to 60' *			X						
20' to 90' *	X	X	X	X	X	X	X		
20' to 4 h *	X	X	X	X	X	X			
4h **	0.004	-0.017	0.015	0.014	-0.031	-0.029	-0.012	0.019	-0.018
24h **	0.065	0.056	0.017	-0.057	-0.027	-0.094	-0.117	0.015	-0.106
96h **	-0.250	-0.083	-0.116	-0.138	0.007	-0.070	0.078	0.181	-0.221
21d **	-0.099	-0.012	-0.094	-0.123	0.013	0.103	-0.072	-0.066	-0.165

* Hedman, 2009; ** present study

6.3. Discussion

In this comparison of meningococcal transcriptomes of different studies, similarities as well as intriguing differences were found. With respect to other host cell interaction studies, similarities were found in

particular to Grifantini *et al.* (2002) study and to a lesser extent to the data of Dietrich *et al.* (2003).

Although the experimental designs of all of these studies were similar, the differences observed in the comparative transcriptome analysis might be resulted from a number of technical factors as described on the example of Grifantini *et al.* (2002) study. In the Grifantini *et al.* (2002) study, the RNA of host cell associated bacteria was obtained after host cell lysis with saponin, whereas in the present study trypsin-EDTA and a differential spin were used. In the present study, the use of saponin was not satisfactory to obtain good quality bacterial RNA. The microarrays used by Grifantini *et al.* (2002) were composed of PCR products of 600 – 800 nt length while in the present study, 40 – 60 nt oligonucleotide arrays were used. The length of the probes of the array might significantly influence non-specific hybridisation. Long sequences are more likely to harbour short regions to which non-specific binding might occur, for example derived from contamination with human RNA in the bacterial RNA extraction or homologous sequences from other meningococcal genes rather than the intended one. The microarrays in the study of Grifantini *et al.* (2002) were hybridised with labelled cDNA from the host cell associated meningococci (sample) mixed with labelled cDNA derived from bacteria grown in liquid cell culture medium in the absence of host cells (control). Sample and control were each labelled directly with Cy-dye (Cy-5/Cy-3) with the dye swap applied and hybridized to two arrays to correct for dye-biases (Cy-3 tends to give a stronger signal than Cy-5 even when the same amount of RNA is used per reaction). The average intensities of each spot from both microarray slides were used for analysis. In contrast, in the present study, microarrays were hybridised with cDNA from the experimental condition (Cy-5 labelled) mixed with Cy-3 labelled MC58 genomic DNA (universal control). Using genomic DNA as control sample has the advantage that it is a stable reference (each gene has in vast majority, only one copy) in contrast to the potentially variable RNA sample. Both data sets (of Grifantini *et al.*, 2002 and of the present study) were initially Cy5/Cy3 normalized before further statistical procedures and normalization to the reference sample (derived from bacteria grown in the absence of host cells).

A biological explanation for the differences observed between the different meningococcal transcriptome studies during host cell interaction might be the time point of analysis. As in the previous

chapter shown, a stable transcriptional profile was obtained at 24 h – 96 h p.i. During the early time points of 3 h (Grifantini *et al.*, 2002), 4 h (the present study) and 6 h (Dietrich *et al.*, 2003) the meningococci might be in the process of a rapid response adapting to the new environment (presence of host cells) which might be reflected in the more variable genome wide transcriptional profile compared to later time points of 24 h and 96 h p.i.

The transcriptional profile at of 48 h of the Sorbarod study (O'Dwyer *et al.*, 2009) might be relatively stable as that of the 24 h-96 h of the present study. The differences observed between the Sorbarod model and the present study might be plausibly explained on the fact that the two experimental models are vastly different. In contrast to the abiotic surface used by O'Dwyer *et al.*, (2009), host cells and a different culture media were used in the present study. Culture conditions were static in the present study, whereas a flow system was applied on the Sorbarods. In the present study, before bacterial RNA was extracted, cell associated bacteria were separated from host cells which was not required in the Sorbarod model. Different microarray slides were used in both studies, which required a slightly different cDNA synthesis protocol, the use of a different slide scanner and software program for analysis.

The data of the present study were compared to the meningococcal transcriptome during a model of septicaemia (Hedman, 2009). The number of genes being coidentified as down-regulated in both models, together modelling most of the meningococcal life styles, were expected to be low. Being down-regulated in both conditions indicates the gene might not be required in either condition. Commonly identified up-regulated genes might be involved in meningococcal survival in both conditions. The main reason for the comparison of these two studies was to identify potential vaccine candidates, i.e. surface expressed genes which are down-regulated in the present study and up-regulated during blood infection. By this approach, *NMB2135* was identified as potential vaccine candidate. Future work including FACS analysis using a specific antibody might confirm location of *NMB2135* on the meningococcal surface. Additional vaccine candidates were identified by comparing data of the present study to those of Grifantini *et al.* (2002) and Sun *et al.* (2000). *NMB1876* (from comparison with Grifantini *et al.*, 2002),

NMB0543, *NMB1515*, *NMB1729* and *NMB1790* (from comparison with Sun *et al.*, 2000) were found to be down-regulated throughout the present study. *NMB1876* was shown to be surface expressed and recombinant protein variations able to induce bactericidal antibodies in infant mice by Grifantini *et al.* (2002). *NMB0543*, *NMB1515*, *NMB1729* and *NMB1790* were shown to be essential for invasive disease in an infant rat model (Sun *et al.*, 2000). Future work might include FACS analysis using specific antibodies to reveal expression of *NMB0543*, *NMB1515*, *NMB1729* and *NMB1790* on the meningococcal surface and stimulation of bactericidal antibodies of recombinant proteins.

Chapter 7

The role of the *NMB0342-NMB0348* locus in prolonged
host cell association

7.1. Introduction

Due to its expression profile, the *NMB0342* - *NMB0348* locus was identified as being potentially involved in host cell interaction (Chapter 5). As described in Chapter 5, *NMB0342* is a homolog to the *S. flexneri* intracellular septation protein A and has been identified to be essential for meningococcal invasive disease in an infant rat model (Sun *et al.*, 2000). A *NMB0342* deletion mutant (in serogroup B strain C311) has been shown decreased adherence to Chang cells at 3 h p.i. (Bakshi, 2004). *NMB0343* and *NMB0344*, both are small open reading frames and without intergenic space, have been assigned Ycil-like hypothetical protein and BolA/YrbA family protein (KEGG), respectively. The *E. coli* BolA protein has been shown to be involved in biofilm formation (Santos *et al.*, 1999; Vieira *et al.*, 2004). *NMB0345* was assigned putative cell binding factor (KEGG) and is a homolog to the *C. jejuni* cell binding factor (Kervella *et al.*, 1993; Bakshi, 2004). In an infant rat model of meningococcal invasive disease, *NMB0345* has been shown to be essential to cause disease (Sun *et al.*, 2000). A *NMB0345* deletion mutant (strain C311) has shown decreased adherence to Chang cells at 3 h p.i. (Bakshi, 2004). *NMB0346* encodes a hypothetical protein of unknown function. *NMB0347* and *NMB0348* have been assigned as D-tyrosyl-tRNA(Tyr) deacylase and tRNA-dihydrouridine synthase, respectively (KEGG).

In order to further characterize this locus, DNA sequence analysis was performed to identify a potential promoter and predicted cellular locations associated with each gene product. RT-PCRs were performed to ascertain operon organization and to confirm experimentally the extent of co-transcription of the seven genes. Mutants of *NMB0342*, *NMB0344*, *NMB0345*, *NMB0347* and *NMB0348* were constructed by insertion of kanamycin resistance cassettes into each of the genes. Transcriptional analysis of each mutant was performed by RT-PCR to ascertain the effect of each mutation on expression of other genes. The mutants were studied in cell association experiments with 16HBE14 monolayers over 21 days.

7.2. Results

7.2.1. DNA sequence analysis of the *NMB0342* - *NMB0348* locus to find potential promoter sequences

The *NMB0342* – *NMB0348* locus comprises a set of seven genes located in close proximity to each other. The longest intergenic space is between *NMB0346* and *NMB0347* (72 nt). *NMB0343* and *NMB0344* show no intergenic sequence (Figure 7.1).

The close proximity to each other, as well as the same direction of transcription and the opposite orientation of genes *NMB0341* and *NMB0349*, the two genes flanking the locus, indicate a possible organisation of this locus in an operon. Gene sequences and adjacent regions of *NMB0342* – *NMB0348* were searched for a promoter sequence. However, the presence of a Pribnow box, a 6 nt T – A rich region (-10 box, typically TATAAT) upstream of each open reading frame (Madigan and Martinko, 2006) was not identified in any genes. The -35 box, a 6 nt sequence (typically TTGACA) was also not found either (Madigan and Martinko, 2006). *N. meningitidis* harbors numerous, up to 250 Correia elements (CE) in its genome. CE are relatively short (ca. 150 nt) transposable elements, flanked by inverted repeats (Tettelin *et al.*, 2000; Siddique *et al.*, 2011) which may promote or prevent transcription of genes (Siddique *et al.*, 2011). By using BLAST, CE were not found within the *NMB0342*-*NMB0348* locus nor in the adjacent regions. However, promoters might have different sequences than those searched for (Travers, 1980). To establish experimentally whether this locus is organised as an operon, co-transcription of the seven genes was tested by RT-PCR.

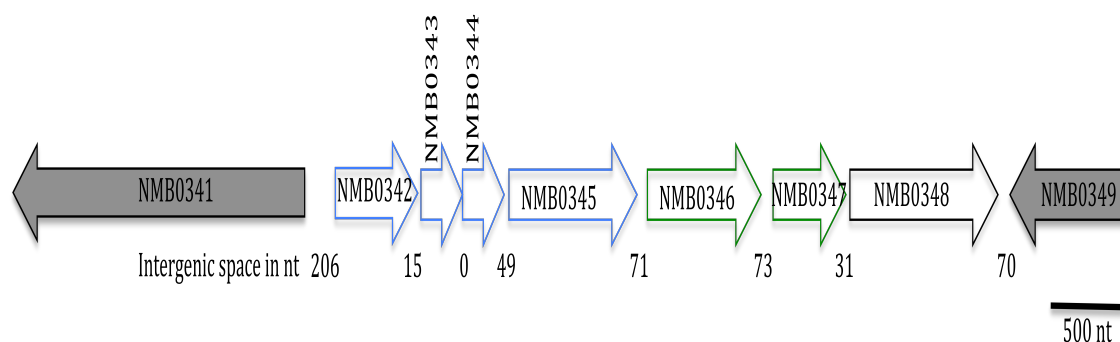


Figure 7.1. Genomic organisation of the *NMB0342-NMB0348* locus. Genes are presented as arrows, numbers in between indicate the length of the intergenic sequence in nt. The bar represents 500 nt. *NMB0341* and *NMB0349* are directed in the opposite orientation to *NMB0342-NMB0348*. Co-transcription of *NMB0342-NMB0345* (blue outline) and of *NMB0346* and *NMB0347* (green outline) was shown by RT-PCR (see Figure 7.3 and next Section).

7.2.2. Defining the co-transcription relation within the *NMB0342 - NMB0348* locus by RT-PCR

RT-PCR was performed using RNA extracted from MC58 wild type harvested from an o/n sGC plate. A set of RT-PCRs was performed as shown in Figure 7.2. After using gene specific reverse-primers (r-primer) in the RT-reaction to generate first strand cDNA, several PCRs were performed using different combinations of gene specific forward (f) and reverse (r) primers (Figure 7.3). Primer pairs and predicted PCR product length are shown in Table 7.1. For each primer pair, a control PCR was performed on MC58 DNA to confirm that the heterologous primer pairs generate a product of the correct size (Figure C in the Appendix).

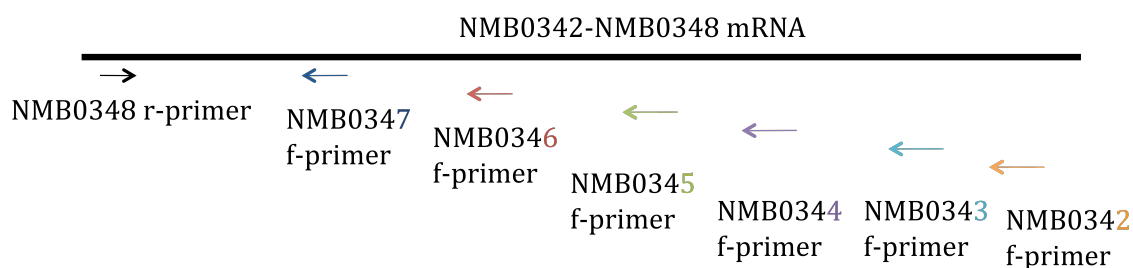


Figure 7.2. Schematic graph of the set of RT-PCRs performed on RNA reverse transcribed using the *NMB0348* gene specific rt-r-primer. On the cDNA (reverse transcribed with *NMB0348* rt-r-primer), six different PCRs were performed using the *NMB0348* rt-r-primer and one of the rt-f-primers of the upstream genes. Similar experiments were performed for all the other genes (*NMB0347-NMB0342*) of this locus.

Table 7.1. Combinations of reverse and forward primers used in RT-PCRs and the length of the predicted product in bp.

rt-r- primer→ rt-f-primer ↓	<i>NMB0342</i>	<i>NMB0343</i>	<i>NMB0344</i>	<i>NMB0345</i>	<i>NMB0346</i>	<i>NMB0347</i>	<i>NMB0348</i>
<i>NMB0342</i>	148	612	928	1471	2438	3011	3748
<i>NMB0343</i>	612	170	486	1029	1996	2569	3306
<i>NMB0344</i>	928	486	155	698	1665	2238	2975
<i>NMB0345</i>	1471	1029	698	292	1259	1832	2569
<i>NMB0346</i>	2438	1996	1665	1259	267	840	1577
<i>NMB0347</i>	3011	2569	2238	1832	840	139	873
<i>NMB0348</i>	3748	3306	2975	2569	1577	873	290

Primer sequences are shown in Table 2.9 (Chapter 2). Primers designed for RT-PCR were used.

Co-transcription of *NMB0343*, *NMB0344* and *NMB0345* was confirmed by RT-PCR (Figure 7.3). In a separate RT-PCR, co-transcription of *NMB0342-NMB0343* was detected, suggesting a polycistronic mRNA of *NMB0342-NMB0345*. These results are schematically incorporated in Figure 7.1. The predicted

product size of *NMB0342 – NMB0345* is of 1471 bp. As RT works best on products of <1000 bp (according to the Invitrogen protocol), the length of the *NMB0342 – NMB0345* product could be an explanation for failure of amplification.

NMB0346 and *NMB0347* were shown to be transcribed in one bicistronic mRNA. A clear band of the expected size was detected (Figure 7.3 lane 3). Co-expression of *NMB0345 – NMB0346* and *NMB0347 – NMB0348* was not detected (although the predicted product size of *NMB0345-NMB0346* is of 1259 bp which might be too long to be amplified in RT-PCR).

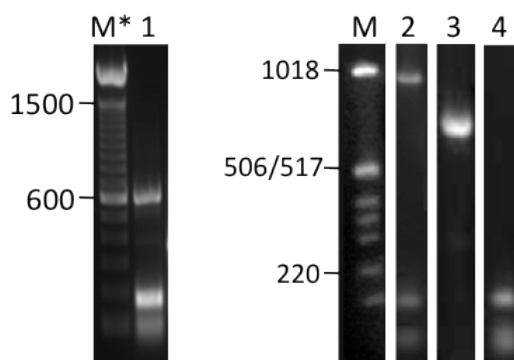


Figure 7.3. Gel images of RT-PCR products performed on MC58 wild type RNA to investigate co-transcription of *NMB0342-NMB0348*. **Lane 1:** Co-transcription of *NMB0342* and *NMB0343*. A clear band of the expected size (612 bp) is shown. **Lane 2:** A band of the expected size (1029 bp) confirms co-transcription of *NMB0343-NMB0345*. **Lane 3:** Co-transcription of *NMB0346-NMB0347* was shown by a band of the expected size (840 bp). **Lane 4:** The amplified product should be of 876 bp which was not detected in three independent experiments indicating that *NMB0348* and *NMB0347* were not co-transcribed. Marker used were the 100 bp DNA ladder (Invitrogen) indicated by M* and the 1 kb DNA ladder (Invitrogen) indicated by M. Numbers indicate the length of the marker bands in bp.

7.2.3. *In silico* analysis of the deduced NMB0342 - NMB0348 amino acid (aa) sequences to identify the potential cellular locations of the proteins

The pSORT algorithm (Nakai and Kanehisa, 1991) was applied to search for transmembrane domains in the amino acid (aa) sequence of each of the proteins encoded by *NMB0342* - *NMB0348* to predict potential surface localization. Based on empiric aa sequence data, the algorithm predicts cytoplasmic, inner or outer membrane or periplasmic location of the protein. It has been successfully used in several meningococcal studies for prediction of the location of proteins (Massignani *et al.*, 2001 and 2003; Fletcher *et al.*, 2002; Ferrari *et al.*, 2006).

For NMB0342, five cytoplasmic membrane helices and a signal peptide were predicted. A final location in the cytoplasmic membrane was suggested. The location of NMB0343 and NMB0344 remains unclear: no structure or signal sequence indicating location in a bacterial membrane was indicated. NMB0345 was predicted to be an integral outer membrane protein. For NMB0346, a signal peptide was detected but its location (inner or outer membrane – cytoplasmic, periplasmic or outer face) was uncertain as multiple localizations were suggested. No evidence for extracellular location of NMB0347 and NMB0348 was predicted, and they are likely to be cytoplasmic proteins. The results of the pSORT analysis are shown in Table J in the Appendix.

7.2.4. Constructing and characterizing mutants of genes within the *NMB0342* - *NMB0348* locus

7.2.4.1. Generating *kanR*-cassette insertions in the *NMB0342* - *NMB0348* locus

Mutants in which a kanamycin resistance cassette was inserted into each of *NMB0342*, *NMB0344*, *NMB0345*, *NMB0347* and *NMB0348* were constructed as described in Chapter 2 (Section 2.11) by transformation of MC58 wild-type with the appropriate DNA prepared from systematically mutated meningococcal strain 8013, carrying a *kanR*-cassette in the homologous target gene. Table 7.2 shows a list of homologous genes of strain MC58 and 8013.

Table 7.2. Homologous target genes of strain MC58 and 8013 used for transformations

Gene ID of MC58	Homologous gene of strain 8013
<i>NMB0342</i>	<i>NMV_0379</i>
<i>NMB0344</i>	<i>NMV_0381</i>
<i>NMB0345</i>	<i>NMV_0382</i>
<i>NMB0347</i>	<i>NMV_0384</i>
<i>NMB0348</i>	<i>NMV_0385</i>

The Pelicic library (Pelicic *et al.*, 2000) did not contain mutants with inserts in the *NMB0343* and *NMB0346* homologs and accordingly, these two genes were excluded from further consideration. Correct insertion of the *kanR* cassette into the target gene in MC58 was confirmed by PCR (Figure 7.4) and sequencing of genomic DNA obtained from kanamycin resistant colonies (mutants of e.g. *NMB0342* are named *NMB0342kanR* hereafter). The *kanR* cassette is 1500 nt long (Pelicic *et al.*, 2000) resulting in a PCR product of the target gene larger by this amount than that obtained from MC58 wild type DNA.

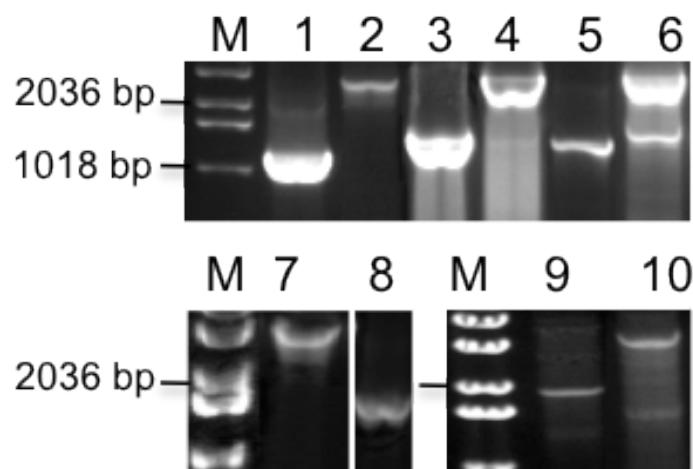


Figure 7.4. Gel images of PCR products to confirm insertion of the kanR cassette into the target genes. As control, PCR was performed on MC58 wild type DNA. **Lane 1:** wild type *NMB0342* (1242 bp), **lane 2:** *NMB0342kanR* (2742 bp), **lane 3:** wild type *NMB0344* (1467 bp), **lane 4:** *NMB0344kanR* (2967 bp), **lane 5:** wild type *NMB0347* (1478 bp), **lane 6:** *NMB0347kanR* (2978 bp), **lane 7:** *NMB0345kanR* (3152 bp), **lane 8:** wild type *NMB0345* (1652 bp), **lane 11:** wild type *NMB0348* (1645 bp), **lanes 12:** *NMB0348kanR* (3145 bp). The line in the bottom right panel indicates the 2036 bp band of the marker.

7.2.4.2. Establishing the fitness of the constructed mutants

Growth curves of *NMB0342kanR*, *NMB0344kanR*, *NMB0345kanR*, *NMB0347kanR* and *NMB0348kanR* mutants in DMEM-10%-hi-fbs in comparison to wild type MC58 were performed over 7.5 h, in triplicate (Figure 7.5). The mutant bacteria grew comparably to MC58 wild type with no significant difference. Using scanning EM, no changes in the morphology of each of the mutants was found compared to wild type MC58 (at 24 h p.i. with 16HBE14 cells, data not shown).

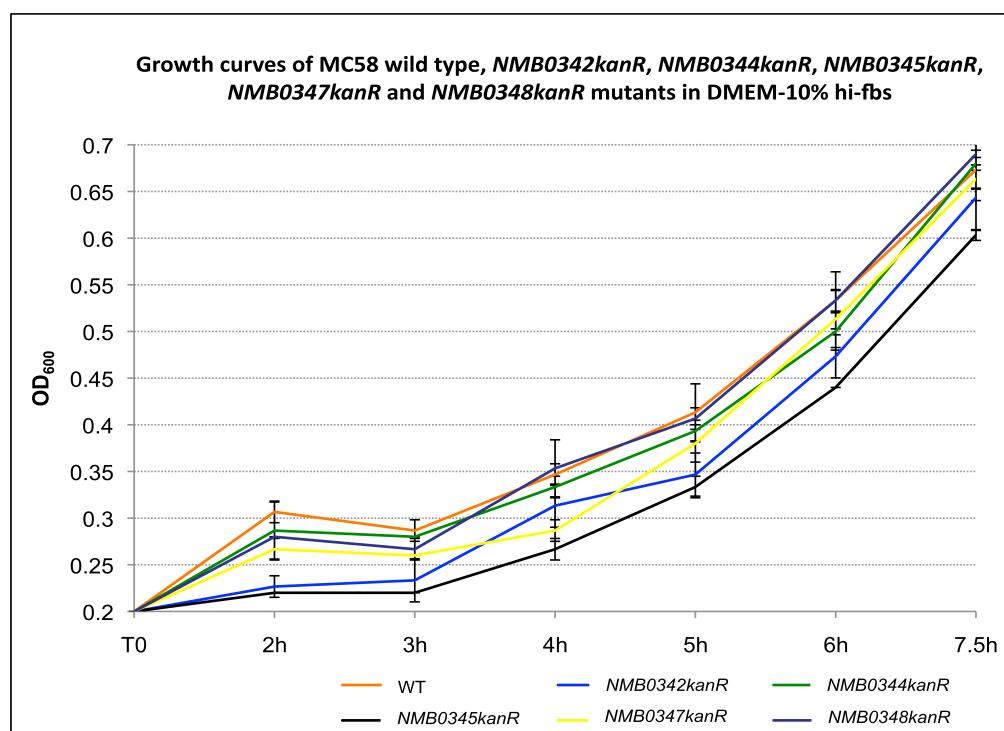


Figure 7.5. Growth curves of MC58 wild type, *NMB0342kanR*, *NMB0344kanR*, *NMB0345kanR*, *NMB0347kanR* and *NMB0348kanR* in DMEM 10%- hi-fbs. Experiments were performed in triplicate for each strain. Error bars indicate the SEM.

7.2.4.3. Investigation of possible polar effects of *kanR* insertion in the *NMB0342-NMB0348* locus

RT-PCR performed on RNA extracted from mutant meningococci was carried out to investigate whether insertion of the *kanR*-cassette into one of the genes *NMB0342*, *NMB0344*, *NMB0345*, *NMB0347* or *NMB0348* affected the expression of any of the rest of the genes at the locus (Figure 7.6). RNA was extracted from mutants grown on sGC plates overnight. As control, all PCR reactions using the RT-primers were performed on MC58 wild type genomic DNA in parallel (shown in the Appendix Figure C).

Expression of undisrupted genes of the *NMB0342 - NMB0348* locus was confirmed for all of the mutants, indicating a lack of polar effect. Decreased adherence to host cells (Figure 7.7 and 7.8) is accordingly the result of interruption of the target gene rather than a down-stream effect of the *kanR* insertion.

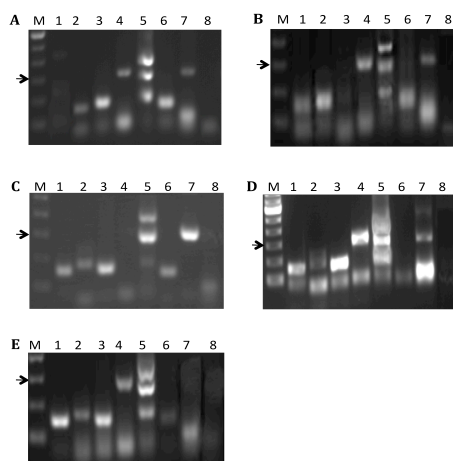


Figure 7.6. Gel images of RT-PCR products of *NMB0342kanR*, *NMB0344kanR*, *NMB0345kanR*, *NMB0347kanR*, *NMB0348kanR* RNA on each of the other genes of the locus. **Panel A:** RT-PCRs on *NMB0342kanR* RNA, **panel B:** RT-PCRs on *NMB0344kanR* RNA, **panel C:** RT-PCRs on *NMB0345kanR* RNA, **panel D:** RT-PCRs on *NMB0347kanR* RNA, **panel E:** RT-PCRs on *NMB0348kanR* RNA. **Lane 1:** *NMB0342* RT-PCR (148 nt), **lane 2:** *NMB0343* RT-PCR (170 nt), **lane 3:** *NMB0344* RT-PCR (155 nt), **lane 4:** *NMB0345* RT-PCR (292 nt), **lane 5:** *NMB0346* RT-PCR (267 nt), **lane 6:** *NMB0347* RT-PCR (139 nt), **lane 7:** *NMB0348* RT-PCR (290 nt) and **lane 8 (negative control):** RNAse treated sample RT-PCR on *NMB0796* (300 nt). The arrow indicates 300 nt. The marker (M) used had bands in 100 nt intervals.

7.2.4.4. Quantitative host cell association experiments over 21 days of mutants compared to wild type MC58 using 16HBE14 monolayers

The effect on host cell association of the mutants with disruption of *NMB0342*, *NMB0344*, *NMB0345*, *NMB0347* and *NMB0348*, compared to wild type, was investigated in host cell association experiments carried out over 21 days (Figure 7.7). The significance of differences in host cell association was calculated using Student's t-test (paired, two-tailed).

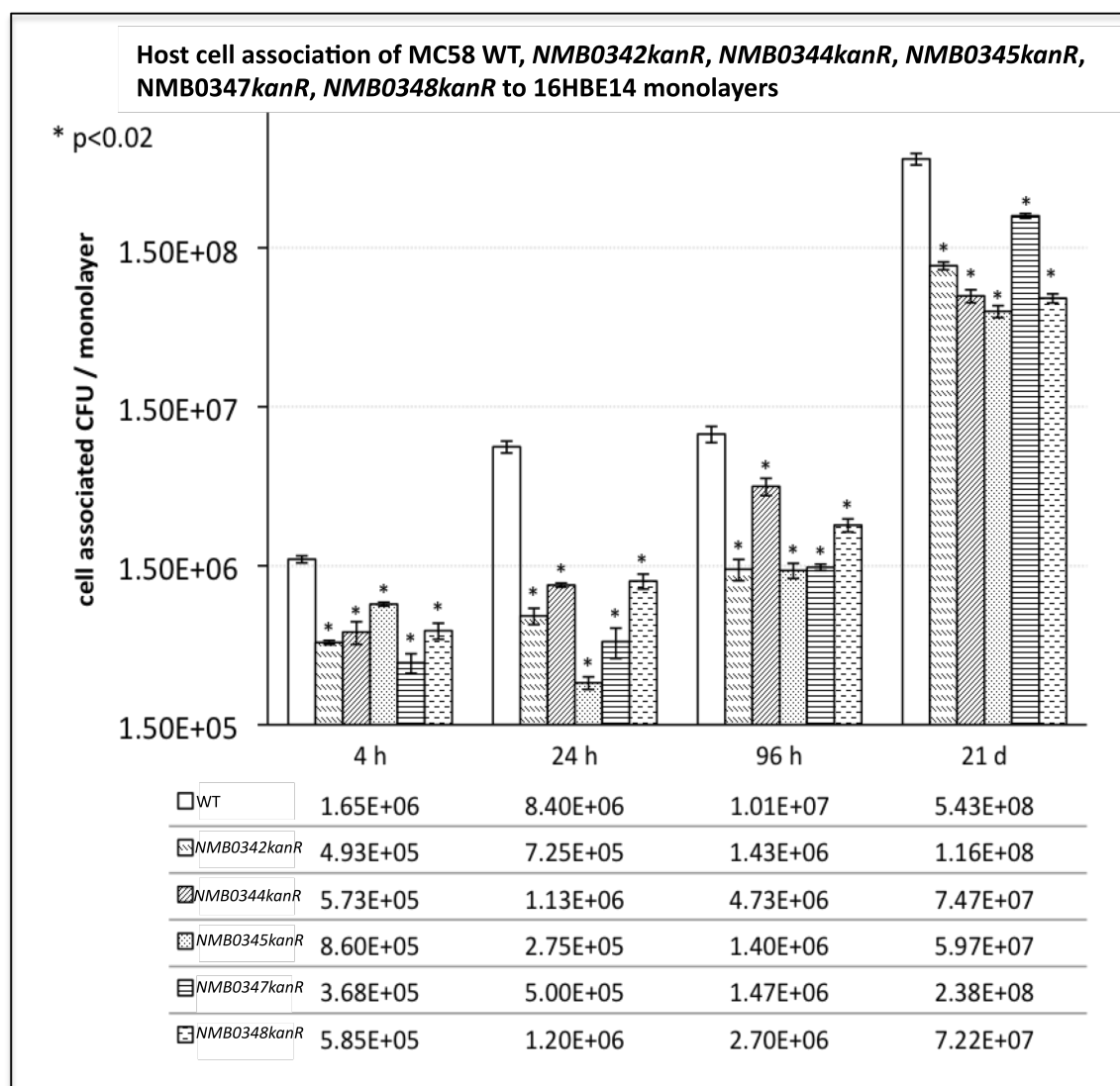


Figure 7.7. Cell association of MC58 wild type, *NMB0342kanR*, *NMB0344kanR*, *NMB0345kanR*, *NMB0347kanR* and *NMB0348kanR* mutants with 16HBE14 monolayers at 4 h, 24 h, 96 h and 21 days. Experiments were performed in three independent replicates each with three technical replicates. Y-error bars represent the SEM. Using Student's t-test (paired, 2-tailed), the p-value was calculated for each mutant in comparison to the corresponding wild type data. Bars marked with an * have a $p < 0.02$.

7.2.4.5. Quantitative adherence experiments over 96 h of *NMB0345kanR* and wild-type meningococci using MucilAir monolayers

Host cell adherence experiments on MucilAir monolayers were performed to explore the implication of the results further. Only six MucilAir monolayers were available, so experiments were limited to a triplicate for each of wild type MC58 and *NMB0345kanR* mutant and a single time point (96 h p.i.). *NMB0345kanR* was chosen as this mutant showed the lowest number of cell associated CFU compared to wild type bacteria in experiments using 16HBE14 cells (Figure 7.7). The results are shown, together with the corresponding results using 16HBE14 monolayers for comparison, in Figure 7.8.

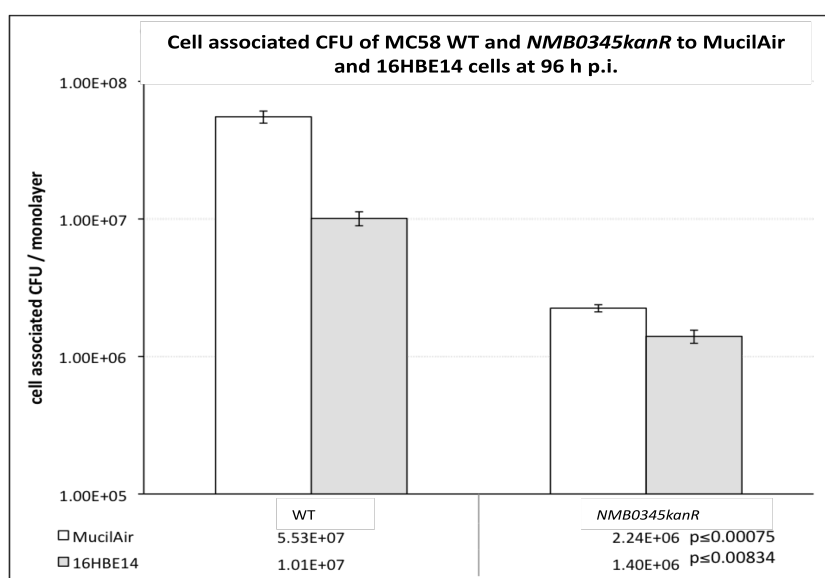


Figure 7.8. Association of MC58 wild type and the *NMB0345kanR* mutant with cells of MucilAir (0.28 cm² growth area) and 16HBE14 monolayers (2 cm² growth area) at 96 h p.i. Each experiment was performed in triplicate. Y-error bars indicate the SEM. Using Student's t-test (paired, 2-tailed), the p-value was calculated for *NMB0345kanR* in comparison to the wild type data for each cell line. A significant difference between cell associated wild type and *NMB0345kanR* CFU was shown ($p=0.0083$ for 16HBE14 cells and $p=0.00075$ for MucilAir cells).

MucilAir monolayers yielded comparable results to 16HBE14 cells (Figure 7.8). A significant decrease was observed for the *NMB0345kanR* mutant compared to wild type bacteria at 96 h p.i. a p-value of 0.0083 for association with 16HBE14 and 0.00075 for association with MucilAir cells was obtained for the *NMB0345kanR* mutant compared to MC58 wild type.

By comparing MC58 wild type association with MucilAir monolayers to that with 16HBE14, it was found that meningococci associated significantly more to MucilAir than to 16HBE14 monolayers ($p=0.01489$). No significance was observed in a similar comparison for the *NMB0345kanR* mutant strain ($p=0.3356$).

7.3. Discussion

The transcriptional profile of all the genes of the *NMB0342* - *NMB0348* locus was similar over 21 days (Chapter 5, Section 5.2.5.10) but they clearly do not form a single continuous operon. A common promoter sequence could not be identified. Co-expression of *NMB0342* - *NMB0343*, of *NMB0343* - *NMB0345* and of *NMB0346* - *NMB0347* was established by RT-PCRs indicating a common regulation of these two sets of genes. Alternatively, a plausible explanation of co-transcription of *NMB0342* to *NMB0343* and of *NMB0343* to *NMB0345* might be the presence of two major promoters. One of them facilitating co-transcription of *NMB0342* to *NMB0343* with little read through to *NMB0343* to *NMB0345* and the other promoter for *NMB0343* to *NMB0345* only.

Whether *NMB0345* and *NMB0346* are co-transcribed remains unclear as the amplified RT-PCR product is of 1259 nt. Reverse transcriptase works less efficient when the transcript is longer than 1000 nt.

Non-polar insertion of a *kanR* cassette into *NMB0342*, *NMB0344*, *NMB0345*, *NMB0347* and *NMB0348* and subsequent adherence experiments demonstrated the apparent involvement of the products of each of these genes in host cell association (invasion experiments are shown in Figure D in the Appendix). A significant decrease in adherence to 16HBE14 cells over 21 days compared to the wild type was observed for each of the mutants. Mutants showed decreased but not abrogated host cell

association, indicates that *NMB0342*, *NMB0344*, *NMB0345*, *NMB0347* and *NMB0348* are involved, rather than essential for meningococcal survival during host cell interaction. The result was confirmed in the case of *NMB0345kanR* in experiments with MucilAir cell monolayers. Decreased adherence of meningococcal *NMB0342* and *NMB0345* deletion mutants at 3 h p.i. of Chang cells has been demonstrated previously (Bakshi 2004).

The *ispA* (homolog to *NMB0342*) and the *bolA* gene (homolog to *NMB0344*) have been described to be morphogenes in *S. flexneri* and *E. coli*, respectively (Mac Siomoin *et al.*, 1996; Aldea *et al.*, 1988).

Deletion of *ispA* in *S. flexneri* abrogated bacterial cell division during invasion of host cells resulting in long filamentous *S. flexneri* (Mac Siomoin *et al.*, 1996). Deletion of *bolA* in *E. coli* resulted in a round shaped morphology (Aldea *et al.*, 1988). By taking scanning EM photographs of 16HBE14 cells infected with either of the mutants constructed in the present study, changes in bacterial morphology were not observed compared to the wild type, indicating no involvement of any of these genes in meningococcal morphology (data not shown).

Chapter 8

General discussion and future work

8.1. Discussion

In this thesis I have presented a study of the meningococcal transcriptome in a model of epithelial colonization maintained for up to 21 days, a length of time which greatly exceeds all previously published studies (the extent of which has been no more than hours). Studying meningococcal host cell interaction over such a prolonged period of time was chosen to closely mimic, within the limitations of a cell culture system, meningococcal colonization *in vivo* which can last for weeks to months. The reason for undertaking the present study was to explore the meningococcal transcriptional profile over a length of time during that bacteria eventually come to a stable relationship with the host cells, and if possible, to identify i) genes that are important for establishing this association and ii) genes encoding surface exposed proteins with the desired expression profile to be potential vaccine candidates. The criteria to select the vaccine candidates were genes relative down-regulated in the colonization model (so reducing the exposure to immune selection, and consequent susceptibility to antigenic variation) while being relatively up-regulated in bacteria persisting in human blood (the focus of previous work in our group (Hedman, 2009)).

A cell line able to survive 21 days of meningococcal infection was required for this study.

16HBE14, an immortalized bronchial epithelial cell line, was chosen from a list of cell lines, through experimental evaluation described in Chapter 3. The same cell line has been used previously in a similar study but for a much shorter, meningococcal - host cell co-cultivation, time span (Grifantini *et al.*, 2002). These cells had the advantage that meningococci adhered in high numbers, which increased over the course of the experiment, and on the epithelial surface bacteria appeared conducive to formation of a biofilm – large bacterial aggregates embedded in a matrix.

Since the first publication of the complete genome sequence of *H. influenzae* (Fleischmann *et al.*, 1995) numbers of available bacteria genome sequences have increased exponentially

(NCBI). Their exploitation in approaches such as microarray technology has allowed analysis of the genome wide transcriptional activity of an organism in one single experiment. Microarrays can be used to study the transcriptome of bacteria harvested from many different models of infection (Bubert *et al.*, 1999; Marshall *et al.*, 2000; Grifantini *et al.*, 2002; Hautefort and Hinton, 2002; Dietrich *et al.*, 2003; Eriksson *et al.*, 2003; Schnappinger *et al.*, 2003; Hamilton, 2004; O'Dwyer *et al.*, 2009; Hedman, 2009; Echenique-Rivera *et al.*, 2011). By careful experimental design, genes involved in specific aspects of host-pathogen interaction can be identified – in the present study, in prolonged interaction with host cells. By this approach, genes that are important for meningococcal – host cell interaction can be discovered leading to a greater understanding, at the molecular level, of host – pathogen interactive biology and pathogenesis. By analyzing the genome wide transcriptional profile of meningococci during prolonged host cell interaction, profound changes in the expression of genes encoding, directly or indirectly, surface structures such as capsule, type IV pili, LOS, PorA and PorB and others, were observed. Some genes encoding products of unknown / hypothetical function were identified which are potentially involved in host cell interaction including those at the *NMB0342-NMB0348* locus. By testing mutants of *NMB0342*, *NMB0344*, *NMB0345*, *NMB0347* and *NMB0348*, carrying a *kanR* resistance cassette in the target genes, the involvement in prolonged host cell interaction was demonstrated. Association experiments of those mutants showed impaired host cell adherence and invasion compared to wild type meningococci.

The transcriptional organization of *NMB0342-NMB0348* was investigated by using RT-PCR. A polycistronic mRNA of *NMB0342-NMB0345* and a dicistronic mRNA of *NMB0346-NMB0347* was discovered. However, a common promoter sequence was not found.

A limitation of this study, which must be conceded is the use of monolayers of the cell line 16HBE14 as a surrogate for respiratory epithelium. 16HBE14 cells do not differentiate into the ciliated and mucus producing cells present *in vivo* in the nasopharynx. In a first attempt to

translate findings made with 16HBE14 cells into a more close to *in vivo* setting, experiments were carried out with MucilAir cells, which are cultured, fully differentiated, human primary nasopharyngeal epithelial cells. By comparing scanning EM photographs of infected and non-infected 16HBE14 and MucilAir cells, similarities in the pattern of adhering bacteria, in form of singular and diplococci at early time points and microcolonies of increasing size with time were found. Both types of host cell monolayers presented prolonged filamentous host cell surface structures, apparently produced in response to associated meningococci. Bacteria appeared to associate to certain cell types in MucilAir monolayers. This may be due to heterogenous receptor expression, or a manifestation of ciliated action or mucus production, detaching bacteria (although these phenotypes could not be discerned in my experiments). A prevalence of meningococcal adherence to non-ciliated cells in human nasopharyngeal tissue explants has previously been shown (Read *et al.*, 1995).

In adherence experiments with MucilAir monolayers using wild type meningococci and *NMB0345kanR* mutants, similar results were obtained with both types of monolayers. In each case, *NMB0345kanR* meningococci showed impaired adherence.

These encouraging results are supportive of the hypothesis that 16HBE14 monolayers may indeed be an ideal surrogate for the human nasopharyngeal epithelium *in vivo*. Clearly a much more extensive analysis will be necessary, examining the whole meningococcal transcriptome from co-culture experiments in the MucilAir cell model. Preliminary experiments have however been unsuccessful, frustrated by the inability to recover sufficient meningococcal RNA. Future work might consider the use of a bacterial RNA amplification kit which likely generate a sufficient amounts of meningococcal mRNA for q-RT-PCR analysis.

The meningococci recovered from a biofilm on the epithelial surface during prolonged co-cultivation are composed of a complex heterogenous population of bacteria from different layers of microcolonies. Those different sub-populations of meningococci would likely produce

different gene expression profiles according to the microenvironments present, such as the availability of nutrients and oxygen (Sternberg *et al.*, 1999; Werner *et al.*, 2004; Yu *et al.*, 2004). Studying the transcriptome of a biofilm provides an average, integrated overview of the gene expression profile of all sub-populations within the biofilm. The transcriptome of minor sub-populations might be overly influenced by that of major sub-populations, which may explain the variation between biological replicates.

As discussed in Chapter 5, microarray results may be interpreted in different ways.

Simplistically, in bacteria, up-regulation of gene expression results in a higher level of translation and protein expression. However, differential stability of mRNAs and protein products complicates the interpretation of transcriptional data. A gene encoding an unstable product might be continuously up-regulated to maintain a functionally adequate level of the protein, while one encoding a stable protein might be down-regulated once the protein is synthesized. By the same argument, the interpretation of gene down-regulation is difficult. A gene can be down-regulated when its product is temporarily not required, or when its product is simply present in a sufficient amount. In addition, up-regulation of negative transcriptional regulators leads to down-regulation of several genes.

To resolve the crucial matter of whether differential gene regulation is accompanied by a change in the amount of gene product, future work will be directed at detecting products themselves, by, e.g., western blotting, direct detection by gold-labeled antibody binding or ELISA.

The extension of the co-cultivation of time points up to 21 days have provided great insights for the present study and for the wider research community, as it is believed that the habitation of meningococci on human nasopharyngeal epithelium can be a very long process, weeks to months (see Chapter 1). The present study attempted to address the questions: How different are meningococcal transcriptomes between the very long (up to 21 days) co-cultivation and short

time points (used in most studies in the past, including 4 hours in the present study)? How far should co-cultivation time points go to be able to represent a prolonged colonization model? Through analyzing the transcriptomes obtained from different time points of co-cultivation (described in Chapter 5 and 6) it has been shown not only that the transcriptome of short co-cultivation time points (few hours) are very different from prolonged time points (>24 h) but also that the transcriptional profiles of prolonged co-cultivation time points (>24 h) are very similar. The implication is that the 24 h time point represents a threshold where the meningococcal transcriptome became established and stabilized. As co-cultivation time increased, more variations were found between the biological replicates such as the ones obtained from 21 days. It was therefore decided to use mainly the 24- and 96 h time points as representing prolonged transcriptional profiles in comparisons with other studies (described in Chapter 6). However, all understandings achieved so far are based on successfully obtaining the transcriptomes of up to 21 days prolonged co-cultivation.

In the present study, potential new meningococcal vaccine candidates were identified by *in silico* comparison of genome-wide transcriptional data with those previously obtained from meningococci propagated in anticoagulated non-bactericidal whole human blood (Hedman, 2009) or in animal model systems (Sun *et al.*, 2000; Grifantini *et al.*, 2002).

As described above, the criterion for the identification of potential vaccine candidates among gene products expressed on the bacterial surface was the down-regulation during epithelial cell interaction but up-regulation (or being essential) during blood infection. A low level of expression during colonization plausibly means that the gene product is less likely to be subject to immune selection in the nasopharynx, which leads to sequence variation particularly in the most exposed (and therefore immunogenic) domains of the protein. One could also argue that the down-regulation of a meningococcal vaccine antigen during nasopharyngeal colonization,

while minimizing immune-mediated selection, also may minimize any protective effect in preventing carriage, which is important in building up a herd immunity.

8.2. Future work

The present study provides a huge set of data potentially leading to further investigations. As discussed above, results of microarray experiments will need to be confirmed with experiments at the phenotypic level.

The initial phenotypic investigations into the *NMB0342-NMB0348* locus provide a basis for further experiments. RT-PCRs performed (Chapter 7) to investigate the organization of this locus revealed a potential presence of several promoters. Using primer extension or 5'RACE technologies, the promoter(s) could be identified and possibly a regulatory pathway, inferred from the specific DNA sequence appeared in the promoter region. Subsequently, the involvement of the potential regulator in meningococcal responses to host cells could be investigated by constructing mutants (for example as described in Chapter 7).

Recombinant NMB0342-NMB0348 proteins could be expressed in *E. coli* and antibodies generated for example in rabbits or mice. The expression of the proteins on the meningococcal surface during host cell interaction as well as during blood infection could be confirmed by FACS analysis. In addition, human serum bactericidal and passive protection assays for example as described by Plested *et al.* (2009) could be performed.

After confirmation of surface expression of one or each of the genes within the locus, the identification of potential host cell receptors would be supportive for understanding the meningococcal colonization process and the development of therapeutic agents. The host cell receptor could be investigated, for example, by a ligand overlay of recombinant protein hybridized to electrophoretically separated human proteins from epithelial cells. Using MALDI-TOF (matrix-assisted-laser desorption/ionization time of flight) the human protein could be

identified as was, for example performed to show meningococcal NspA binding to human factor H (Lewis *et al.*, 2010).

As described in Chapter 6, six gene products have been identified as potential vaccine candidates through *in silico* comparison of the present study with that of others: NMB0543, NMB1515, NMB1729, NMB1790, NMB1876 and NMB2135. NMB1876 was previously identified by Grifantini *et al.* (2002) as vaccine candidate as it was shown to be expressed on the meningococcal surface and induced bactericidal antibodies in a mouse model. In the study of Grifantini *et al.* (2002) *NMB1876* was found to be up-regulated at 3 h p.i. of 16HBE14 cells. In the present study, *NMB1876* was down-regulated at all time points of host cell interaction, thereby fulfilling my initial hypothesis that a vaccine candidate should be down-regulated during host colonization and up-regulated during invasive disease. As shown by Grifantini *et al.* (2002) *NMB1876* was found to be expressed on the meningococcal surface by FACS analysis, able to induce bactericidal antibodies in a mouse model and the conserved among different meningococcal strains, giving further confidence to use *NMB1876* as vaccine candidate in humans. However, conflicting expression profiles were found in the present and Grifantini *et al.* (2002) study. To confirm the expression level of *NMB1876* during host cell interaction and the level of the encoded protein, q-RT-PCR and FACS analysis of samples derived from the colonization model should be performed.

NMB0543, *NMB1515*, *NMB1729*, *NMB1790* were all found to be down-regulated during prolonged host cell interaction with 16HBE14 monolayers (the present study) and mutations in each of these genes attenuated virulence in an infant rat model (Sun *et al.*, 2000). *NMB1515* and *NMB1790* encode, according to KEGG, a putative transporter and a glutaredoxin, respectively. *NMB0543* and *NMB1729*, encode lactate permease and ExbB, respectively. ExbB is involved in iron acquisition and both, *NMB0543* and *NMB1729* were shown to be located on the meningococcal surface (examined by FACS) and recombinant proteins induced bactericidal

antibodies in a rat model. In addition, both genes were found to be conserved and present in meningococcal serogroup A, B and C strains (Sun *et al.*, 2005). These results support the identification of NMB0543 and NMB1729 as vaccine candidates although it remains to be determined if both candidates induce protective immunity in humans. *NMB2135* was identified as encoding a potential vaccine candidate through its being down-regulated at all time points during host cell interaction but up-regulated during a model of septicaemia (Hedman, 2009). Future work of all of the six vaccine candidates might include i) the validation of down-regulation during meningococcal – epithelial host cell interaction by q-RT-PCR; ii) experiments investigating if the down-regulation of gene expression is accompanied by a low level of protein for example using western blots or FACS; iii) experiments confirming location of the proteins on the meningococcal surface for example by FACS or immuno-gold labeling; iv) investigations if there is a population heterogeneity i.e. conservation of the candidate gene sequences among a broad range of strains, for example by using the database of the MRF funded meningococcal whole genome sequencing project which will sequence >400 meningococcal isolates from invasive disease cases in England in the epidemiological year of 2010-2011 (www.meningitis.org); v) investigations into the immunogenicity of the vaccine candidates (if not previously established) for example by ELISA using recombinant proteins and human sera of convalescent patients.

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Appendix

Table A. Correlations of normalized intensity values of all arrays compared to each other. Highlighted in purple are the replicates of each time point

	3 h-1	3 h-2	3 h-3	3 h-4	4 h-1	4 h-2	4 h-3	4 h-4	24 h-1	24 h-2	24 h-3	24 h-4	96 h-1	96 h-2	96 h-3	96 h-4	21 d-1	21 d-2	21 d-3
3 h-1	1.00	0.94	0.90	0.91	0.74	0.75	0.72	0.69	0.80	0.75	0.83	0.81	0.77	0.78	0.83	0.62	0.76	0.76	0.77
3 h-2	0.94	1.00	0.98	0.93	0.77	0.79	0.78	0.74	0.81	0.80	0.84	0.83	0.81	0.81	0.85	0.70	0.77	0.79	0.78
3 h-3	0.90	0.98	1.00	0.91	0.77	0.79	0.79	0.75	0.80	0.80	0.83	0.82	0.81	0.80	0.84	0.70	0.76	0.79	0.78
3 h-4	0.91	0.93	0.91	1.00	0.76	0.80	0.78	0.74	0.80	0.79	0.81	0.82	0.80	0.79	0.84	0.68	0.76	0.77	0.79
4 h-1	0.74	0.77	0.77	0.76	1.00	0.96	0.95	0.97	0.93	0.95	0.90	0.93	0.92	0.89	0.90	0.90	0.82	0.83	0.73
4 h-2	0.75	0.79	0.79	0.80	0.96	1.00	0.99	0.95	0.92	0.94	0.89	0.93	0.93	0.90	0.92	0.86	0.83	0.85	0.80
4 h-3	0.72	0.78	0.79	0.78	0.95	0.99	1.00	0.95	0.90	0.92	0.87	0.91	0.91	0.89	0.90	0.85	0.81	0.84	0.79
4 h-4	0.69	0.74	0.75	0.74	0.97	0.95	0.95	1.00	0.89	0.93	0.87	0.90	0.89	0.85	0.86	0.92	0.77	0.79	0.72
24 h-1	0.80	0.81	0.80	0.80	0.93	0.92	0.90	0.89	1.00	0.98	0.97	0.99	0.96	0.96	0.95	0.88	0.90	0.89	0.80
24 h-2	0.75	0.80	0.80	0.79	0.95	0.94	0.92	0.93	0.98	1.00	0.94	0.98	0.97	0.95	0.95	0.92	0.87	0.87	0.78
24 h-3	0.83	0.84	0.83	0.81	0.90	0.89	0.87	0.87	0.97	0.94	1.00	0.98	0.94	0.94	0.93	0.84	0.91	0.89	0.81
24 h-4	0.81	0.83	0.82	0.82	0.93	0.93	0.91	0.90	0.99	0.98	0.98	1.00	0.96	0.96	0.95	0.87	0.90	0.89	0.81
96 h-1	0.77	0.81	0.81	0.80	0.92	0.93	0.91	0.89	0.96	0.97	0.94	0.96	1.00	0.98	0.97	0.86	0.90	0.89	0.82
96 h-2	0.78	0.81	0.80	0.79	0.89	0.90	0.89	0.85	0.96	0.95	0.94	0.96	0.98	1.00	0.96	0.83	0.92	0.90	0.82
96 h-3	0.83	0.85	0.84	0.84	0.90	0.92	0.90	0.86	0.95	0.95	0.93	0.95	0.97	0.96	1.00	0.84	0.87	0.88	0.84
96 h-4	0.62	0.70	0.70	0.68	0.90	0.86	0.85	0.92	0.88	0.92	0.84	0.87	0.86	0.83	0.84	1.00	0.73	0.75	0.64
21 d-1	0.76	0.77	0.76	0.76	0.82	0.83	0.81	0.77	0.90	0.87	0.91	0.90	0.90	0.92	0.87	0.73	1.00	0.96	0.88
21 d-2	0.76	0.79	0.79	0.77	0.83	0.85	0.84	0.79	0.89	0.87	0.89	0.89	0.89	0.90	0.88	0.75	0.96	1.00	0.88
21 d-3	0.77	0.78	0.78	0.79	0.73	0.80	0.79	0.72	0.80	0.78	0.81	0.81	0.82	0.82	0.84	0.64	0.88	0.88	1.00

Table B. Complete list of differentially expressed genes. Data were filtered on $p \leq 0.01$ in all pairs of comparison and on a $fc \geq 2.0$ in at least one pair of comparison (including comparison of late time points) (black) or only on $p \leq 0.05$ without filtering on fc (grey). Highlighted in orange are up-regulated and in blue down-regulated genes as shown in figure 5.3. (Chapter 5). N/a not annotated in COG.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0001	Not in cogs genes	Putative acetyltransferase	2.33E-04	+ 1.46	- 1.38	- 1.44	- 1.16	- 2.01	- 2.09	- 1.69
NMB0002	Not in cogs genes	Hypothetical protein	2.38E-03	+ 1.37	- 1.21	- 1.30	- 1.06	- 1.66	- 1.79	- 1.46
NMB0003	Translation	Glutamyl-tRNA synthetase	1.45E-02	- 1.16	- 1.60	- 1.53	+ 1.00	- 1.38	- 1.32	+ 1.17
NMB0004	Function unknown genes	EpH/gdmh-related protein	1.15E-02	- 1.14	- 1.76	- 1.62	- 1.33	- 1.55	- 1.42	- 1.17
NMB0005	Inorganic ion transport and metabolism genes	Arsenate reductase	4.07E-04	+ 1.52	- 1.32	- 1.13	- 2.52	- 2.00	- 1.71	- 3.82
NMB0006	Energy production and conversion genes	Thioredoxin-related protein	1.30E-03	+ 1.10	+ 2.62	+ 1.84	+ 1.30	+ 2.39	+ 1.68	+ 1.18
NMB0012	Inorganic ion transport and metabolism genes	Conserved hypothetical protein	1.62E-03	+ 1.55	- 1.13	- 1.06	+ 1.19	- 1.75	- 1.63	- 1.30
NMB0015	Carbohydrate transport and metabolism genes	6-phosphogluconate dehydrogenase	2.32E-02	+ 1.66	+ 1.21	+ 1.38	+ 1.23	- 1.37	- 1.20	- 1.35
NMB0016	Not in cogs genes	Hypothetical protein	7.91E-04	+ 1.92	+ 1.48	+ 1.74	- 1.76	- 1.30	- 1.11	- 3.39
NMB0018	Intracellular trafficking and secretion genes	Pilin pile	2.88E-03	- 1.02	+ 6.66	+ 3.65	+ 6.64	+ 6.82	+ 3.74	+ 6.80
NMB0019	N/a	Pils cassette	2.50E-03	+ 1.71	+ 7.60	+ 7.13	+ 7.81	+ 4.43	+ 4.16	+ 4.56
NMB0020	N/a	Large pils cassette	2.32E-03	+ 2.38	+ 15.32	+ 11.38	+ 17.51	+ 6.43	+ 4.78	+ 7.34
NMB0022	N/a	Pils cassette	4.65E-04	+ 1.01	+ 2.49	+ 3.59	+ 8.65	+ 2.47	+ 3.55	+ 8.57
NMB0023	N/a	Pils cassette	1.26E-02	- 1.00	+ 2.39	+ 1.55	+ 6.64	+ 2.39	+ 1.55	+ 6.64
NMB0024	N/a	Pils cassette	1.55E-03	+ 1.33	+ 5.44	+ 4.24	+ 5.90	+ 4.08	+ 3.18	+ 4.43
NMB0025	N/a	Large pils cassette	1.29E-04	+ 1.37	+ 4.48	+ 2.83	+ 1.72	+ 3.27	+ 2.06	+ 1.26
NMB0026	N/a	Pils cassette	1.89E-03	+ 2.47	+ 13.91	+ 11.44	+ 11.19	+ 5.62	+ 4.62	+ 4.52
NMB0027	Posttranslational modification, protein turnover, chaperones genes	FKBP-type peptidyl-prolyl cis-trans isomerase	6.52E-03	+ 1.28	+ 1.50	+ 1.52	+ 2.33	+ 1.17	+ 1.18	+ 1.81
NMB0028	Function unknown genes	Hypothetical protein	7.07E-03	+ 1.43	+ 1.31	+ 1.43	- 1.30	- 1.09	- 1.00	- 1.86
NMB0029	General function prediction only genes	Glycerate dehydrogenase	7.69E-04	- 1.14	- 1.98	- 1.76	- 1.04	- 1.73	- 1.54	+ 1.10
NMB0030	General function prediction only genes	Methionyl-tRNA synthetase	3.25E-02	- 1.21	- 1.30	- 1.53	- 1.43	- 1.08	- 1.26	- 1.18
NMB0032	Not in cogs genes	Hypothetical protein	1.01E-02	+ 1.60	+ 1.23	+ 1.34	+ 2.00	- 1.29	- 1.19	+ 1.25
NMB0034	Inorganic ion transport and metabolism genes	Conserved hypothetical protein	2.32E-03	- 1.25	- 2.04	- 1.51	- 2.09	- 1.64	- 1.21	- 1.67
NMB0035	Inorganic ion transport and metabolism genes	Conserved hypothetical protein	2.44E-06	- 1.07	- 1.69	- 1.41	- 1.54	- 1.59	- 1.32	- 1.45
NMB0036	Inorganic ion transport and metabolism genes	Conserved hypothetical protein	4.65E-04	+ 1.31	- 1.73	- 1.21	- 1.91	- 2.27	- 1.59	- 2.51
NMB0039	Not in cogs genes	Hypothetical protein	2.46E-04	- 1.63	- 1.81	- 1.67	+ 1.01	- 1.11	- 1.02	+ 1.65
NMB0041	Coenzyme transport and metabolism genes	ABC transporter, periplasmic solute-binding protein	5.51E-03	- 2.70	- 2.80	- 1.98	- 2.22	- 1.04	+ 1.36	+ 1.22
NMB0043	General function prediction only genes	Conserved hypothetical protein	3.67E-03	+ 1.07	- 2.45	- 1.63	- 2.71	- 2.62	- 1.74	- 2.90
NMB0044	Energy production and conversion genes	Peptide methionine sulfoxide reductase	2.76E-03	- 1.48	- 1.56	- 1.54	+ 1.24	- 1.06	- 1.04	+ 1.84
NMB0045	Intracellular trafficking and secretion genes	Signal recognition particle protein	4.25E-02	+ 1.47	+ 1.52	+ 1.99	+ 1.33	+ 1.03	+ 1.35	- 1.11
NMB0046	Not in cogs genes	Hypothetical protein	3.28E-03	+ 2.46	+ 10.80	+ 13.55	+ 11.64	+ 4.39	+ 5.50	+ 4.72
NMB0047	Replication recombination and repair	Conserved hypothetical protein	1.82E-03	+ 1.06	- 1.50	- 1.43	- 2.09	- 1.59	- 1.51	- 2.21

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0052	Intracellular trafficking and secretion genes	Twitching motility protein pili	3.16E-02	-	+ 1.74	+ 1.46	- 1.14	+ 2.10	+ 1.75	+ 1.06
NMB0053	motility genes	Conserved hypothetical protein	6.19E-04	-	- 2.34	- 1.75	- 3.51	- 2.04	- 1.52	- 3.06
NMB0054	General function prediction only genes	Hypothetical protein	1.10E-02	+ 1.44	- 1.31	- 1.25	+ 1.52	- 1.88	- 1.79	+ 1.06
NMB0055	Not in cogs genes	Pyroline-5-carboxylate reductase	1.16E-02	- 1.19	- 1.41	- 1.33	+ 1.36	- 1.19	- 1.12	+ 1.61
NMB0056	Amino acid transport and metabolism genes	Dnak suppressor protein	1.95E-03	- 1.53	+ 1.11	+ 1.30	+ 1.14	+ 1.69	+ 1.99	+ 1.74
NMB0059	Signal transduction mechanisms genes	Dnaj protein	3.98E-04	- 1.49	- 1.44	- 1.39	+ 1.18	+ 1.04	+ 1.07	+ 1.77
NMB0061	Posttranslational modification, protein turnover, chaperones genes	Dtdp-4-keto-6-deoxy-D-glucose-3,6-epimerase	1.18E-04	- 1.12	- 1.97	- 2.04	- 1.43	- 1.75	- 1.81	- 1.27
NMB0062	N/a	Glucose-1-phosphate thymidyltransferase	1.48E-02	+ 1.10	- 1.63	- 1.56	- 1.00	- 1.79	- 1.71	- 1.10
NMB0063	Cell wall/membrane biogenesis genes	Dtdp-D-glucose 4,6-dehydratase	3.25E-02	+ 1.41	- 1.39	- 1.39	- 1.44	- 1.96	- 1.95	- 2.03
NMB0064	Cell wall/membrane biogenesis genes	UDP-glucose 4-epimerase, truncation	1.61E-03	+ 1.50	- 1.36	- 1.22	+ 1.01	- 2.04	- 1.82	- 1.48
NMB0067	Not in cogs genes	Polysialic acid capsule biosynthesis protein siad	3.07E-03	- 1.08	+ 1.68	+ 1.21	- 2.36	+ 1.82	+ 1.31	- 2.18
NMB0068	Cell wall/membrane biogenesis genes	Polysialic acid capsule biosynthesis protein siac	4.46E-03	- 1.38	- 1.04	- 1.10	- 2.29	+ 1.32	+ 1.25	- 1.66
NMB0069	Cell wall/membrane biogenesis genes	Polysialic acid capsule biosynthesis protein siab	8.70E-04	- 1.93	- 1.11	- 1.24	- 2.62	+ 1.74	+ 1.56	- 1.36
NMB0070	Cell wall/membrane biogenesis genes	Polysialic acid capsule biosynthesis protein synx	3.05E-03	- 1.69	- 1.12	- 1.15	- 3.00	+ 1.51	+ 1.47	- 1.78
NMB0075	Transcription	Putative transcriptional accessory protein Tex	1.12E-03	+ 1.28	+ 2.25	+ 2.01	+ 1.36	+ 1.76	+ 1.57	+ 1.06
NMB0076	N/a	Putative 5-methylcytosine methyltransferase	3.52E-03	- 1.17	+ 1.01	- 1.11	- 2.89	+ 1.19	+ 1.06	- 2.46
NMB0077	Replication recombination and repair	Site-specific DNA methylase, truncation	3.93E-02	- 1.22	+ 1.23	- 1.21	- 2.24	+ 1.51	+ 1.01	- 1.83
NMB0078	Cell wall/membrane biogenesis genes	UDP-glucose 4-epimerase, truncation	1.35E-02	+ 1.19	- 1.32	- 1.54	- 1.28	- 1.58	- 1.84	- 1.52
NMB0081	Cell wall/membrane biogenesis genes	Dtdp-4-keto-6-deoxy-D-glucose-3,6-epimerase	2.94E-02	+ 1.87	+ 1.22	+ 1.12	+ 1.76	- 1.52	- 1.66	- 1.06
NMB0085	Amino acid transport and metabolism genes	Sodium/glutamate symporter	4.78E-03	- 1.43	- 2.02	- 1.81	- 1.20	- 1.42	- 1.27	+ 1.19
NMB0086	Function unknown genes	Hypothetical protein	6.01E-03	- 1.43	- 1.91	- 1.59	- 1.54	- 1.34	- 1.12	- 1.08
NMB0087	Not in cogs genes	Hypothetical protein	1.91E-03	+ 2.01	- 1.04	+ 1.10	+ 2.71	- 2.10	- 1.83	+ 1.35
NMB0090	Not in cogs genes	IS1016 transposase	5.53E-03	- 1.35	- 3.11	- 2.65	- 1.93	- 2.30	- 1.96	- 1.43
NMB0091	Not in cogs genes	Hypothetical protein	2.20E-02	- 1.49	+ 1.17	+ 1.14	- 2.27	+ 1.75	+ 1.69	- 1.53
NMB0093	Not in cogs genes	Hypothetical protein	1.91E-03	+ 1.71	+ 2.14	+ 2.36	- 1.35	+ 1.25	+ 1.38	- 2.31
NMB0096	Not in cogs genes	Hypothetical protein	2.80E-02	+ 1.36	- 1.05	- 1.06	- 2.26	- 1.43	- 1.45	- 3.09
NMB0098	Not in cogs genes	Putative protein export protein	3.75E-03	+ 1.64	+ 1.56	+ 1.63	- 1.72	- 1.05	- 1.00	- 2.82
NMB0099	Not in cogs genes	Hypothetical protein	1.23E-02	+ 1.40	+ 1.23	+ 1.34	- 2.67	- 1.14	- 1.05	- 3.75
NMB0100	Not in cogs genes	Hypothetical protein	2.44E-02	+ 1.53	+ 1.15	- 1.03	- 1.84	- 1.32	- 1.57	- 2.81
NMB0102	Not in cogs genes	Hypothetical protein	1.10E-03	+ 2.12	+ 7.10	+ 9.83	+ 3.04	+ 3.35	+ 4.64	+ 1.43
NMB0103	General function prediction only genes	Putative bacteriocin resistance protein	2.94E-02	+ 1.25	+ 1.18	+ 1.31	- 1.66	- 1.06	+ 1.05	- 2.08
NMB0104	Not in cogs genes	Hypothetical protein	4.42E-03	+ 1.08	+ 1.34	+ 1.53	+ 1.15	+ 1.24	+ 1.42	+ 1.06
NMB0105	Not in cogs genes	Phn-related protein	4.65E-02	- 1.42	- 1.52	- 1.16	- 1.46	- 1.07	+ 1.22	- 1.03
NMB0106	Nucleotide transport and metabolism genes	Aspartate carbamoyltransferase, catalytic subunit	2.75E-02	- 1.28	+ 1.07	+ 1.22	+ 1.22	+ 1.37	+ 1.56	+ 1.57
NMB0107	Not in cogs genes	Aspartate carbamoyltransferase, regulatory subunit	4.66E-03	+ 1.59	- 1.08	+ 1.39	+ 1.09	- 1.71	- 1.14	- 1.45
NMB0108	Not in cogs genes	Hypothetical protein	2.91E-03	+ 1.70	+ 1.38	+ 1.64	+ 1.12	- 1.23	- 1.03	- 1.51

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0110	Not in cogs genes	Peptide deformylase	1.54E-02	+ 1.06	+ 1.17	+ 1.50	+ 1.57	+ 1.10	+ 1.42	+ 1.48
NMB0115	Signal transduction mechanisms genes	Nitrogen assimilation regulatory protein ntrX	1.23E-02	+ 1.49	- 1.19	+ 1.13	+ 1.02	- 1.78	- 1.32	- 1.46
NMB0115	Signal transduction mechanisms genes	Nitrogen assimilation regulatory protein ntrX	1.23E-02	+ 1.49	- 1.19	+ 1.13	+ 1.02	- 1.78	- 1.32	- 1.46
NMB0122	Replication recombination and repair	Conserved hypothetical protein	1.61E-03	- 1.12	- 1.89	- 1.93	- 1.18	- 1.69	- 1.73	- 1.05
NMB0124	Translation	Translation elongation factor Tu	7.79E-04	- 1.60	+ 6.80	+ 3.91	+ 2.38	+ 10.89	+ 6.26	+ 3.81
NMB0125	Intracellular trafficking and secretion genes	Preprotein translocase secE subunit	2.96E-03	- 1.51	+ 1.78	+ 1.94	- 2.02	+ 2.69	+ 2.93	- 1.33
NMB0126	Transcription	Transcription antitermination protein nusG	1.50E-02	- 1.48	+ 1.57	+ 2.09	- 1.57	+ 2.33	+ 3.10	- 1.06
NMB0127	Not in cogs genes	50S ribosomal protein L11	1.00E-03	- 2.20	+ 3.04	+ 4.11	+ 1.49	+ 6.69	+ 9.05	+ 3.29
NMB0128	Not in cogs genes	50S ribosomal protein L1	1.93E-03	- 2.41	+ 3.73	+ 3.33	+ 1.56	+ 8.98	+ 8.02	+ 3.76
NMB0129	Not in cogs genes	Hypothetical protein	1.62E-03	- 1.68	+ 5.17	+ 3.87	+ 1.75	+ 8.66	+ 6.48	+ 2.93
NMB0130	Translation	50S ribosomal protein L10	3.48E-03	- 1.55	+ 3.68	+ 3.29	+ 3.03	+ 5.72	+ 5.10	+ 4.70
NMB0131	Translation	50S ribosomal protein L7/L12	2.85E-03	- 3.85	+ 2.73	+ 2.39	+ 1.42	+ 10.48	+ 9.17	+ 5.45
NMB0132	Transcription	DNA-directed RNA polymerase, beta subunit	4.42E-03	+ 1.12	+ 2.12	+ 2.82	- 1.05	+ 1.89	+ 2.52	- 1.18
NMB0133	Transcription	DNA-directed RNA polymerase, beta' subunit	8.10E-03	+ 1.30	+ 2.25	+ 2.33	- 1.05	+ 1.73	+ 1.80	- 1.37
NMB0136	Not in cogs genes	30S ribosomal protein S12	2.42E-04	- 2.58	+ 5.75	+ 3.20	+ 2.56	+ 14.81	+ 8.24	+ 6.59
NMB0137	Translation	30S ribosomal protein S7	3.41E-04	- 1.47	+ 7.05	+ 3.58	+ 2.48	+ 10.35	+ 5.26	+ 3.64
NMB0138	Translation	Elongation factor G (EF-G)	4.89E-04	- 3.60	+ 4.04	+ 2.27	- 1.55	+ 14.53	+ 8.17	+ 2.32
NMB0140	Translation	30S ribosomal protein S10	4.46E-04	- 1.31	+ 4.57	+ 2.17	+ 1.12	+ 5.99	+ 2.84	+ 1.47
NMB0141	IS1560 transposase	Transposase, truncation	1.01E-02	- 2.00	+ 1.74	+ 1.09	+ 1.09	+ 3.47	+ 2.17	+ 2.18
NMB0142	Not in cogs genes	50S ribosomal protein L3	3.22E-04	- 1.94	+ 8.71	+ 4.32	+ 1.67	+ 16.88	+ 8.38	+ 3.25
NMB0143	Not in cogs genes	50S ribosomal protein L4	1.18E-04	- 1.95	+ 8.20	+ 5.96	+ 1.36	+ 15.98	+ 11.62	+ 2.65
NMB0144	Translation	50S ribosomal protein L23	2.00E-03	- 2.10	+ 4.26	+ 4.13	- 1.27	+ 8.94	+ 8.67	+ 1.65
NMB0145	Translation	50S ribosomal protein L2	9.94E-04	- 1.19	+ 10.72	+ 7.31	+ 1.60	+ 12.75	+ 8.70	+ 1.91
NMB0146	Translation	30S ribosomal protein S19	1.37E-03	- 1.69	+ 6.61	+ 4.63	+ 1.38	+ 11.18	+ 7.84	+ 2.34
NMB0147	Not in cogs genes	50S ribosomal protein L22	1.74E-03	- 2.10	+ 5.39	+ 4.35	+ 1.17	+ 11.35	+ 9.16	+ 2.46
NMB0148	Translation	30S ribosomal protein S3	2.22E-03	- 2.94	+ 3.00	+ 3.09	+ 1.22	+ 8.82	+ 9.09	+ 3.58
NMB0149	Translation	50S ribosomal protein L16	9.03E-04	- 2.91	+ 3.39	+ 4.19	+ 1.35	+ 9.86	+ 12.19	+ 3.93
NMB0150	Not in cogs genes	50S ribosomal protein L29	2.89E-03	- 2.59	+ 2.29	+ 2.99	- 1.21	+ 5.93	+ 7.73	+ 2.13
NMB0151	Translation	30S ribosomal protein S17	9.79E-04	- 1.15	+ 3.28	+ 7.22	- 1.35	+ 3.78	+ 8.32	- 1.17
NMB0152	Not in cogs genes	50S ribosomal protein L14	3.34E-03	- 1.57	+ 3.73	+ 5.50	+ 2.08	+ 5.87	+ 8.65	+ 3.26
NMB0153	Translation	50S ribosomal protein L24	6.15E-04	- 1.29	+ 5.99	+ 8.01	+ 1.85	+ 7.72	+ 10.32	+ 2.39
NMB0154	Translation	50S ribosomal protein L5	4.63E-04	- 2.33	+ 3.04	+ 4.78	- 1.10	+ 7.10	+ 11.16	+ 2.13
NMB0155	Translation	30S ribosomal protein S14	1.91E-03	- 2.13	+ 3.54	+ 4.27	+ 1.43	+ 7.55	+ 9.12	+ 3.05
NMB0156	Not in cogs genes	30S ribosomal protein S8	2.51E-03	- 2.20	+ 3.19	+ 4.21	- 1.20	+ 7.03	+ 9.28	+ 1.84
NMB0157	Translation	50S ribosomal protein L6	4.99E-04	- 2.71	+ 2.77	+ 5.51	- 1.30	+ 7.51	+ 14.93	+ 2.09
NMB0158	Translation	50S ribosomal protein L18	2.29E-03	- 2.33	+ 2.97	+ 3.73	- 1.01	+ 6.91	+ 8.68	+ 2.30
NMB0159	Translation	30S ribosomal protein S5	3.18E-03	- 2.39	+ 2.29	+ 3.10	- 1.32	+ 5.48	+ 7.43	+ 1.81

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0160	Translation	50S ribosomal protein L30	9.26E-04	- 2.64	+ 2.92	+ 4.74	- 2.16	+ 7.71	+ 12.53	+ 1.22
NMB0161	Translation	50S ribosomal protein L15	2.02E-03	- 1.66	+ 2.69	+ 3.18	- 1.50	+ 4.46	+ 5.28	+ 1.11
NMB0162	Intracellular trafficking and secretion genes	Preprotein translocase secY subunit	3.88E-04	- 1.55	+ 3.21	+ 3.52	- 1.45	+ 4.99	+ 5.48	+ 1.07
NMB0163	Translation	Translation initiation factor IF-1	1.75E-05	- 2.74	+ 2.22	+ 2.86	- 3.64	+ 6.09	+ 7.85	+ 1.33
NMB0164	Not in cogs genes	50S ribosomal protein L36	2.07E-04	- 1.33	+ 6.28	+ 7.79	- 1.16	+ 8.37	+ 10.39	+ 1.15
NMB0165	Translation	30S ribosomal protein S13	4.73E-04	- 1.83	+ 5.62	+ 6.52	+ 1.64	+ 10.31	+ 11.95	+ 3.00
NMB0166	Translation	30S ribosomal protein S11	3.30E-04	- 2.01	+ 7.43	+ 6.22	+ 3.15	+ 14.91	+ 12.47	+ 6.33
NMB0167	Translation	30S ribosomal protein S4	8.53E-04	- 1.11	+ 8.58	+ 6.26	+ 3.84	+ 9.54	+ 6.96	+ 4.27
NMB0168	Transcription	DNA-directed RNA polymerase, alpha subunit	4.76E-04	- 1.75	+ 6.21	+ 5.18	+ 1.97	+ 10.85	+ 9.05	+ 3.44
NMB0169	Translation	50S ribosomal protein L17	2.92E-03	- 1.40	+ 4.14	+ 4.31	+ 1.92	+ 5.78	+ 6.01	+ 2.68
NMB0170	Not in cogs genes	Septum site-determining protein mnc	3.57E-02	+ 1.01	+ 1.36	+ 1.46	- 1.63	+ 1.34	+ 1.44	- 1.65
NMB0176	Amino acid transport and metabolism genes	D-amino acid dehydrogenase, small subunit	2.74E-02	- 1.36	- 1.45	- 1.54	+ 1.02	- 1.07	- 1.13	+ 1.39
NMB0178	Cell wall/membrane biogenesis genes	Acyl-LUDP-N- acetylglucosamine O-acyltransferase	1.01E-02	+ 1.05	- 1.13	+ 1.09	- 1.56	- 1.19	+ 1.03	- 1.64
NMB0179	Lipid transport and metabolism genes	(3R)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase	6.36E-03	- 1.48	- 1.01	+ 1.00	- 1.68	+ 1.46	+ 1.48	- 1.14
NMB0180	Cell wall/membrane biogenesis genes	UDP-3-O-(3-hydroxymyristoyl)-glucosamine N-acyltransferase	8.10E-03	+ 1.09	+ 1.43	+ 1.31	- 1.10	+ 1.31	+ 1.21	- 1.19
NMB0181	Cell wall/membrane biogenesis genes	Putative outer membrane protein ompH	5.53E-03	+ 1.38	- 1.05	+ 1.10	- 1.15	- 1.45	- 1.26	- 1.59
NMB0184	Not in cogs genes	1-deoxy-D-xylulose 5-phosphate reductoisomerase	3.57E-02	+ 1.09	+ 1.16	+ 1.36	- 1.32	+ 1.07	+ 1.24	- 1.44
NMB0190	Cell wall/membrane biogenesis genes	Glucose inhibited division protein B	2.15E-02	- 1.14	- 1.45	- 1.21	+ 1.44	- 1.28	- 1.06	+ 1.64
NMB0194	Amino acid transport and metabolism genes	Putative amino acid symporter	4.00E-02	+ 1.10	- 1.36	- 1.02	+ 1.04	- 1.50	- 1.13	- 1.06
NMB0196	Translation	Ribonuclease E	2.52E-03	+ 1.08	+ 2.67	+ 1.87	- 1.21	+ 2.46	+ 1.73	- 1.31
NMB0200	Function unknown genes	Hypothetical protein	7.26E-04	+ 2.03	+ 1.88	+ 1.56	- 1.36	- 1.08	- 1.30	- 2.76
NMB0201	Not in cogs genes	Hypothetical protein	2.99E-04	+ 2.29	+ 1.64	+ 1.62	- 1.29	- 1.39	- 1.42	- 2.96
NMB0202	Not in cogs genes	Hypothetical protein	9.08E-03	- 2.01	- 2.09	- 1.30	- 3.03	- 1.04	+ 1.55	- 1.51
NMB0209	Inorganic ion transport and metabolism genes	Glutathione-regulated potassium-efflux system protein	2.00E-03	- 1.19	- 1.77	- 1.60	- 1.54	- 1.48	- 1.34	- 1.29
NMB0210	Not in cogs genes	Pseudogene (modification methylase)	3.52E-04	+ 1.45	+ 2.41	+ 2.42	+ 1.46	+ 1.66	+ 1.67	+ 1.00
NMB0211	Amino acid transport and metabolism genes	L-serine dehydratase//L-serine dehydratase	3.79E-03	+ 1.15	+ 1.65	+ 1.60	+ 1.12	+ 1.44	+ 1.39	- 1.02
NMB0212	Replication recombination and repair	DNA gyrase subunit B	6.36E-04	- 1.26	- 1.76	- 1.20	- 1.11	- 1.40	+ 1.05	+ 1.13
NMB0214	Amino acid transport and metabolism genes	Oligopeptidase A	1.54E-02	- 1.87	- 1.55	- 1.33	- 1.03	+ 1.20	+ 1.41	+ 1.82
NMB0215	Function unknown genes	Conserved hypothetical protein	4.27E-02	- 1.07	+ 1.21	+ 1.21	+ 1.50	+ 1.30	+ 1.29	+ 1.61
NMB0217	Transcription	Putative RNA polymerase sigma-54 factor rpon	7.72E-03	- 1.45	- 1.52	- 1.36	+ 1.32	- 1.05	+ 1.07	+ 1.92
NMB0218	Cell wall/membrane biogenesis genes	Glycosyltransferase	1.41E-02	+ 1.25	- 1.11	+ 1.25	- 1.25	- 1.38	- 1.00	- 1.57
NMB0220	Secondary metabolites biosynthesis, transport and catabolism genes	Acyl carrier protein	8.73E-04	- 3.50	+ 1.40	+ 1.17	+ 2.62	+ 4.90	+ 4.09	+ 9.16
NMB0222	Function unknown genes	Hypothetical protein	4.65E-02	+ 1.40	+ 1.58	+ 1.47	- 1.18	+ 1.13	+ 1.05	- 1.64
NMB0223	Not in cogs genes	Hypothetical protein	2.36E-02	+ 1.47	+ 1.98	+ 1.82	+ 1.29	+ 1.34	+ 1.23	- 1.14
NMB0224	Posttranslational modification, protein turnover, chaperones genes	Glutamate-ammonia-ligase adenylyltransferase	3.74E-02	+ 1.23	+ 1.49	+ 1.44	+ 1.26	+ 1.21	+ 1.17	+ 1.03
NMB0225	Replication recombination and repair	Transposase, IS30 family	8.93E-04	+ 1.04	+ 3.26	+ 2.55	+ 1.83	+ 3.13	+ 2.45	+ 1.76
NMB0231	Not in cogs genes	Hypothetical protein	4.75E-02	- 1.00	+ 1.69	+ 1.68	+ 1.03	+ 1.69	+ 1.68	+ 1.03

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h			
NMB0234	Not in cogs genes	Hypothetical protein	1.91E-02	+	1.15	+	1.11	-	1.19	-	1.24	-	1.21
NMB0236	Not in cogs genes	Hypothetical protein//hypothetical protein	1.45E-02	+	1.06	-	1.12	-	1.48	-	1.57	-	3.65
NMB0237	Not in cogs genes	Hypothetical protein	1.13E-02	+	1.11	+	1.06	-	2.33	-	1.34	-	3.31
NMB0247	Not in cogs genes	Hypothetical protein	1.76E-02	+	1.35	+	1.46	-	1.10	+	1.12	-	1.44
NMB0248	Replication recombination and repair	Conserved hypothetical protein	1.63E-02	+	1.47	+	1.38	-	1.57	+	1.02	-	2.22
NMB0252	Not in cogs genes	Hypothetical protein	2.19E-03	+	1.40	+	1.50	+	1.50	-	1.58	-	1.58
NMB0253	Energy production and conversion genes	NADH dehydrogenase I, I subunit	1.31E-02	+	1.29	+	1.07	+	1.13	+	1.07	+	1.66
NMB0256	Not in cogs genes	Hypothetical protein	2.63E-02	+	1.21	+	1.06	-	1.58	-	1.28	-	1.92
NMB0257	Inorganic ion transport and metabolism genes	NADH dehydrogenase I, I subunit	1.01E-02	+	1.95	+	1.41	+	1.43	-	1.38	-	1.36
NMB0258	Energy production and conversion genes	NADH dehydrogenase I, M subunit	1.96E-02	+	1.58	+	1.25	-	1.11	-	1.27	-	1.75
NMB0261	Coenzyme transport and metabolism genes	Geranyltransferase	4.16E-03	+	1.16	-	1.07	+	1.75	-	1.24	+	1.51
NMB0262	Replication recombination and repair	Exodeoxyribonuclease, small subunit	3.07E-03	+	1.53	+	1.20	-	1.09	-	1.27	-	1.67
NMB0263	General function prediction only genes	Conserved hypothetical protein	1.36E-02	+	1.03	-	1.14	-	1.83	-	1.11	-	1.87
NMB0265	Replication recombination and repair	Holliday junction DNA helicase ruva	3.96E-03	+	1.88	-	1.15	-	2.11	-	2.16	-	3.95
NMB0266	Function unknown genes	Conserved hypothetical protein	2.20E-02	+	1.25	+	1.17	-	2.53	+	1.46	-	3.16
NMB0267	Cell wall/membrane biogenesis genes	Conserved hypothetical protein	2.83E-02	-	1.15	+	1.33	-	1.28	+	1.53	-	1.11
NMB0268	Translation	RNA methyltransferase, trmh family	6.05E-03	-	1.77	+	1.28	-	1.12	+	1.69	+	1.58
NMB0271	Not in cogs genes	Hypothetical protein	5.53E-03	+	1.00	-	1.60	+	1.05	-	1.79	-	1.05
NMB0273	Not in cogs genes	Hypothetical protein	4.40E-02	+	1.01	-	1.02	+	1.15	-	1.23	+	1.14
NMB0274	Replication recombination and repair	ATP-dependent DNA helicase reqq	2.19E-02	+	1.48	+	1.56	+	1.09	+	1.05	-	1.62
NMB0277	General function prediction only genes	Virulence factor mvn	4.81E-02	+	1.18	-	1.12	-	1.19	-	1.06	-	1.40
NMB0279	General function prediction only genes	Conserved hypothetical protein	7.91E-03	+	1.08	-	1.01	+	1.47	-	1.07	+	1.36
NMB0282	Transcription	Ribonuclease II-related protein	2.20E-03	+	1.28	-	1.23	-	1.47	-	1.57	-	1.89
NMB0283	General function prediction only genes	Conserved hypothetical protein	9.85E-04	+	1.61	-	1.98	-	2.55	-	3.18	-	4.09
NMB0288	Not in cogs genes	Hypothetical protein	5.82E-04	+	1.47	-	2.07	-	2.05	-	3.04	-	3.01
NMB0292	General function prediction only genes	Conserved hypothetical protein	7.26E-04	+	1.02	-	2.12	-	1.26	-	2.15	-	1.28
NMB0293	Inorganic ion transport and metabolism genes	Putative tonb-dependent receptor	2.49E-02	-	1.37	-	1.49	-	2.00	-	1.09	-	1.46
NMB0294	Not in cogs genes	Thiol:disulfide interchange protein dsba	1.50E-02	+	1.30	+	3.16	+	2.41	+	2.43	+	1.85
NMB0288	Not in cogs genes	Hypothetical protein	5.82E-04	+	1.47	-	2.07	-	2.05	-	3.04	-	3.01
NMB0292	General function prediction only genes	Conserved hypothetical protein	7.26E-04	+	1.02	-	2.12	-	2.19	-	2.15	-	1.28
NMB0293	Inorganic ion transport and metabolism genes	Putative tonb-dependent receptor	2.49E-02	-	1.37	-	1.49	-	1.11	-	1.09	-	1.46
NMB0294	Not in cogs genes	Thiol:disulfide interchange protein dsba	1.50E-02	+	1.30	+	3.16	+	2.07	+	2.43	+	1.85
NMB0295	Intracellular trafficking and secretion genes	Signal recognition particle protein	2.15E-03	-	1.06	-	1.36	+	1.02	-	1.28	+	1.08
NMB0296	General function prediction only genes	Csa-related protein	1.70E-02	+	1.30	+	1.95	+	2.13	+	1.50	+	1.63
NMB0297	Not in cogs genes	Hypothetical protein	5.77E-03	+	1.15	+	2.15	+	1.32	+	1.86	-	1.10
NMB0298	Not in cogs genes	Hypothetical protein	1.25E-04	-	3.06	+	10.28	+	30.60	+	31.41	+	13.83
NMB0303	Not in cogs genes	Transposase	7.20E-03	+	1.15	-	1.29	-	1.77	-	1.49	-	2.79

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0304	Not in cogs genes	Pseudogene (outer membrane protein)	4.46E-04	+ 1.93	+ 1.40	+ 1.41	+ 1.39	- 1.38	- 1.36	- 1.39
NMB0305	Not in cogs genes	Hypothetical protein	2.06E-02	- 1.23	- 1.06	- 1.01	- 2.68	+ 1.16	+ 1.21	- 2.18
NMB0306	Not in cogs genes	Hypothetical protein	2.79E-02	- 1.60	- 2.00	- 2.04	- 3.91	- 1.25	- 1.28	- 2.45
NMB0307	Amino acid transport and metabolism genes	Phospho-2-dehydro-3-deoxyheptonate aldolase	2.68E-02	- 1.02	- 1.39	- 1.41	- 1.44	- 1.36	- 1.38	- 1.41
NMB0308	Coenzyme transport and metabolism genes	Dihydrofolate reductase	1.31E-04	+ 1.02	- 2.36	- 1.06	+ 2.06	- 2.42	- 1.09	+ 2.01
NMB0312	Not in cogs genes	Virulence associated protein	3.82E-02	+ 1.29	+ 1.62	+ 1.51	+ 1.04	+ 1.26	+ 1.18	- 1.23
NMB0314	Function unknown genes	Hypothetical protein	4.31E-03	- 1.30	+ 1.23	+ 1.37	- 1.02	+ 1.59	+ 1.78	+ 1.28
NMB0315	Cell wall/membrane biogenesis genes	Conserved hypothetical protein	4.08E-02	+ 1.13	- 1.02	+ 1.18	- 1.45	- 1.15	+ 1.04	- 1.64
NMB0316	Function unknown genes	Conserved hypothetical protein	1.25E-03	+ 1.53	+ 1.01	+ 1.24	- 1.35	- 1.52	- 1.24	- 2.07
NMB0321	Translation	50S ribosomal protein L28	3.14E-04	- 3.42	+ 2.11	+ 2.54	+ 2.84	+ 7.20	+ 8.66	+ 9.70
NMB0322	Translation	50S ribosomal protein L33	7.44E-03	- 3.84	- 1.83	+ 1.91	+ 2.64	+ 2.09	+ 7.33	+ 10.14
NMB0324	Not in cogs genes	50S ribosomal protein L27	2.33E-04	- 1.98	+ 3.05	+ 2.48	+ 1.67	+ 6.04	+ 4.91	+ 3.31
NMB0325	Not in cogs genes	50S ribosomal protein L21	8.54E-04	- 1.83	+ 4.57	+ 2.70	+ 5.11	+ 8.38	+ 4.95	+ 9.37
NMB0328	Not in cogs genes	Hypothetical protein	2.73E-02	+ 1.71	+ 1.42	+ 1.28	+ 1.08	- 1.21	- 1.33	- 1.58
NMB0330	Not in cogs genes	Conserved hypothetical protein	3.47E-03	- 1.68	- 1.34	+ 1.12	+ 1.29	+ 1.26	+ 1.89	+ 2.18
NMB0331	Putative kinase	Putative kinase	7.20E-03	+ 1.07	+ 1.55	+ 1.60	+ 1.15	+ 1.45	+ 1.49	+ 1.08
NMB0332	Posttranslational modification, protein turnover, chaperones genes	Type IV prelinin peptidase	1.00E-02	+ 1.30	- 1.13	+ 1.08	- 1.05	- 1.47	- 1.20	- 1.36
NMB0334	Carbohydrate transport and metabolism genes	Glucose-6-phosphate isomerase	1.47E-02	- 1.87	- 1.58	- 1.52	- 1.63	+ 1.18	+ 1.23	+ 1.14
NMB0335	Amino acid transport and metabolism genes	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	6.07E-04	- 1.49	- 1.38	- 1.50	+ 1.01	+ 1.08	- 1.00	+ 1.51
NMB0336	Lipid transport and metabolism genes	Enoyl-(acyl-carrier-protein) reductase	1.84E-02	- 1.67	- 1.20	- 1.12	+ 1.04	+ 1.39	+ 1.49	+ 1.74
NMB0337	Coenzyme transport and metabolism genes	Putative branched-chain amino acid aminotransferase	3.46E-02	- 1.42	- 1.24	- 1.10	+ 1.25	+ 1.14	+ 1.29	+ 1.78
NMB0338	Function unknown genes	Hypothetical protein	3.56E-02	- 1.37	- 1.54	- 1.60	- 1.67	- 1.13	- 1.17	- 1.22
NMB0339	Carbohydrate transport and metabolism genes	Conserved hypothetical protein	3.71E-03	- 1.42	- 1.61	- 1.43	+ 1.43	- 1.13	- 1.00	+ 2.03
NMB0341	Intracellular trafficking and secretion genes	Tspa protein	4.04E-03	- 1.36	- 1.46	- 1.57	- 1.32	- 1.07	- 1.15	+ 1.03
NMB0342	Cell motility genes	Intracellular septation protein A	4.31E-02	+ 1.17	+ 2.01	+ 3.25	+ 1.15	+ 1.72	+ 2.78	- 1.02
NMB0343	Cell cycle control, mitosis and meiosis genes	Conserved hypothetical protein	2.76E-03	- 1.71	+ 1.99	+ 2.23	+ 1.57	+ 3.40	+ 3.81	+ 2.69
NMB0344	Signal transduction mechanisms genes	Bola/Yrba family protein	2.01E-04	- 1.29	+ 3.05	+ 3.13	+ 1.14	+ 3.93	+ 4.03	+ 1.46
NMB0345	Posttranslational modification, protein turnover, chaperones genes	Putative cell-binding factor	7.19E-04	- 1.22	+ 3.71	+ 2.70	+ 1.15	+ 4.53	+ 3.30	+ 1.41
NMB0346	Posttranslational modification, protein turnover, chaperones genes	Hypothetical protein	1.89E-04	+ 1.17	+ 3.84	+ 3.82	+ 1.51	+ 3.29	+ 3.27	+ 1.29
NMB0347	Not in cogs genes	Conserved hypothetical protein	2.97E-04	+ 1.35	+ 2.42	+ 2.73	- 2.71	+ 1.80	+ 2.02	- 3.65
NMB0348	Translation	Conserved hypothetical protein	3.07E-03	- 1.22	+ 1.44	+ 1.78	- 1.06	+ 1.75	+ 2.16	+ 1.14
NMB0349	Translation	Glutamyl-trna synthetase-related protein	2.13E-02	+ 1.02	+ 1.41	+ 1.48	+ 1.20	+ 1.38	+ 1.45	+ 1.18
NMB0350	Not in cogs genes	Hypothetical protein	4.28E-03	- 1.35	+ 1.14	- 1.19	- 2.73	+ 1.54	+ 1.14	- 2.02
NMB0352	General function prediction only genes Cell wall/membrane biogenesis genes	Sugar isomerase, kpsf/gutq family	9.59E-03	- 1.82	- 1.24	- 1.44	- 1.40	+ 1.46	+ 1.26	+ 1.30
NMB0353	General function prediction only genes	Conserved hypothetical protein	2.14E-02	+ 1.58	+ 1.69	+ 1.92	+ 1.56	+ 1.07	+ 1.21	- 1.01

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	P-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h			
NMB0356	General function prediction only genes	ABC transporter, ATP-binding protein	2.88E-03	+	1.30	+	1.50	+	1.26	+	1.15	+	1.03
NMB0360	Not in cogs genes	Ampg-related protein	1.44E-03	-	1.51	-	1.51	-	1.25	-	1.00	-	1.21
NMB0361	Replication recombination and repair	Conserved hypothetical protein	1.68E-02	+	1.26	-	1.15	-	1.57	+	1.45	+	1.25
NMB0362	Not in cogs genes	Hypothetical protein	1.34E-03	+	2.40	+	2.02	+	1.16	+	1.19	-	2.06
NMB0363	Not in cogs genes	Hypothetical protein	3.47E-02	-	1.05	-	1.85	-	2.59	-	1.76	-	2.48
NMB0365	Iron-regulated protein frpc, truncation	Iron-regulated protein frpc	1.67E-02	+	1.04	-	1.77	-	2.16	-	1.84	-	2.24
NMB0373	Not In cogs genes	Hypothetical protein	2.73E-02	+	1.32	+	1.81	+	1.08	+	1.37	-	1.23
NMB0376	Not in cogs genes	Hypothetical protein	4.97E-02	-	1.63	-	1.62	-	3.26	+	1.01	-	2.00
NMB0378	Inorganic ion transport and metabolism genes	Putative phosphate permease	2.13E-03	+	1.43	-	1.33	+	1.59	-	1.90	+	1.11
NMB0380	Signal transduction mechanisms genes	Transcriptional regulator, Crp/Fnr family	2.18E-02	-	1.23	-	1.32	+	1.19	-	1.08	+	1.47
NMB0382	Cell wall/membrane biogenesis genes	Outer membrane protein class 4	1.00E-04	-	2.96	+	2.40	+	2.47	+	7.09	+	7.30
NMB0383	Not in cogs genes	Hypothetical protein	1.31E-02	-	2.31	-	1.91	-	1.63	+	1.21	+	1.41
NMB0386	Lipid transport and metabolism genes	Phosphatidylglycerophosphatase A	4.71E-03	+	1.22	-	1.19	-	1.03	-	1.45	-	1.25
NMB0387	General function prediction only genes	ABC transporter, ATP-binding protein	2.19E-02	-	1.06	-	1.24	-	1.14	-	1.17	-	1.07
NMB0388	Not in cogs genes	Putative sugar transporter	7.52E-05	-	1.03	-	1.42	-	1.46	-	1.38	-	1.41
NMB0389	Carbohydrate transport and metabolism genes	Aldose 1-epimerase	2.77E-03	-	1.05	-	1.20	-	1.61	-	1.14	-	1.54
NMB0392	Coenzyme transport and metabolism genes	L-aspartate oxidase	2.03E-02	-	1.63	-	1.09	-	1.60	+	1.49	+	1.02
NMB0395	General function prediction only genes	Conserved hypothetical protein	1.25E-02	-	1.69	-	1.30	-	1.61	+	1.30	+	1.05
NMB0396	Coenzyme transport and metabolism genes	Nicotinate-nucleotide pyrophosphorylase	5.66E-03	+	1.28	-	1.30	-	2.13	-	1.66	-	2.71
NMB0398	Transcription	Transcriptional regulator, arsR family	2.09E-02	+	1.09	+	1.18	+	1.63	+	1.08	+	1.49
NMB0399	Replication recombination and repair	Exodeoxyribonuclease III	4.31E-02	+	1.11	+	1.66	+	1.38	+	1.50	+	1.25
NMB0401	Energy production and conversion genes	Proline dehydrogenase	1.09E-03	-	1.25	+	1.19	-	1.44	+	1.48	-	1.15
NMB0403	Not in cogs genes	Hypothetical protein	6.97E-03	-	1.98	+	1.22	-	1.32	+	2.41	+	1.51
NMB0404	Not in cogs genes	Conserved hypothetical protein	1.02E-02	-	1.07	-	1.16	-	1.51	+	1.08	+	1.62
NMB0406	Cell cycle control, mitosis and meiosis genes	Conserved hypothetical protein	3.35E-03	-	1.20	-	1.53	-	1.08	-	1.28	+	1.11
NMB0413	Cell wall/membrane biogenesis genes	Penicillin-binding protein 2	1.96E-02	+	1.10	+	1.41	+	1.16	+	1.28	+	1.05
NMB0414	Cell wall/membrane biogenesis genes	UDP-N-acetyl(muramoyl)alanyl-D-glutamate-2, 6-diaminopimelate ligase	2.50E-02	+	1.53	+	1.41	-	1.03	-	1.08	-	1.58
NMB0418	Cell wall/membrane biogenesis genes	Phospho-N-acetylmuramoyl-pentapeptide-transferase	2.76E-02	+	1.14	-	1.08	-	1.42	-	1.23	-	1.62
NMB0419	General function prediction only genes	Conserved hypothetical protein/	6.25E-04	+	1.46	-	1.44	-	1.41	-	2.10	-	2.06
NMB0421	Not in cogs genes	Cell division protein ftsw	2.42E-03	+	1.10	-	1.37	-	1.34	-	1.51	-	1.47
NMB0423	Cell wall/membrane biogenesis genes	UDP-N-acetylmuramate-alanine ligase	1.21E-02	-	2.12	+	1.01	+	1.12	+	2.11	+	2.39
NMB0428	General function prediction only genes	Conserved hypothetical protein	4.39E-02	+	1.13	+	1.39	+	1.47	+	1.23	+	1.30
NMB0429	Not in cogs genes	Hypothetical protein	2.26E-02	+	1.88	+	1.45	+	1.72	-	1.29	-	1.09
NMB0430	Carbohydrate transport and metabolism genes	Putative carboxyphosphoenolpyruvate phosphonotomase	4.44E-03	+	1.83	+	1.56	+	2.61	-	1.17	+	1.42
NMB0436	Translation	Conserved hypothetical protein	1.26E-02	-	1.17	-	1.06	-	1.17	-	1.11	-	1.00
NMB0437	Transcription	Conserved hypothetical protein	6.62E-04	-	1.41	+	1.31	+	1.08	+	1.84	+	1.52

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	P-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0440	Amino acid transport and metabolism genes	Putative prephenate dehydrogenase	4.00E-03	- 1.86	- 1.37	- 1.01	- 1.40	+ 1.36	+ 1.85	+ 1.33
NMB0442	N/a	Opacity protein	1.27E-03	+ 1.67	+ 13.82	+ 7.25	+ 6.27	+ 8.28	+ 4.34	+ 3.75
NMB0445	Not in cogs genes	Putative bicyclomycin resistance protein	3.77E-04	+ 1.16	- 1.44	- 1.34	- 1.45	- 1.67	- 1.56	- 1.69
NMB0446	Amino acid transport and metabolism genes	Chorismate mutase/prephenate dehydratase	5.59E-03	- 1.08	+ 1.34	+ 1.53	- 1.09	+ 1.45	+ 1.65	- 1.01
NMB0447	Replication recombination and repair	DNA repair protein reco	2.05E-02	+ 1.45	+ 1.64	+ 2.01	+ 1.43	+ 1.13	+ 1.39	- 1.01
NMB0448	Not in cogs genes	Pyridoxal phosphate biosynthetic protein pdxj	1.15E-02	- 1.31	+ 1.22	+ 1.67	+ 1.51	+ 1.61	+ 2.19	+ 1.98
NMB0449	Not in cogs genes	Hypothetical protein	1.03E-03	+ 1.31	+ 1.94	+ 1.85	- 1.32	+ 1.47	+ 1.41	- 1.73
NMB0450	Not in cogs genes	Hypothetical protein	5.07E-03	+ 1.03	+ 1.32	+ 1.22	- 2.68	+ 1.28	+ 1.18	- 2.78
NMB0451	Not in cogs genes	Hypothetical protein	2.71E-02	- 1.07	- 1.07	- 1.03	- 2.87	- 1.00	+ 1.04	- 2.69
NMB0455	Replication recombination and repair	Conserved hypothetical protein	6.52E-04	+ 1.32	- 1.01	+ 1.19	- 1.29	- 1.33	- 1.11	- 1.70
NMB0456	Cell wall/membrane biogenesis genes	N-acetylmuramoyl-L-alanine amidase	1.51E-03	+ 1.37	+ 1.51	+ 1.49	+ 1.10	+ 1.11	+ 1.09	- 1.24
NMB0457	General function prediction only genes	Conserved hypothetical protein	4.05E-02	- 1.19	- 1.68	- 1.25	- 1.84	- 1.40	- 1.05	- 1.54
NMB0458	Cell wall/membrane biogenesis genes	Glutamate racemase	6.43E-04	+ 1.18	- 1.32	- 1.19	+ 1.30	- 1.55	- 1.40	+ 1.10
NMB0459	Function unknown genes	Conserved hypothetical protein	3.24E-02	- 1.29	- 1.78	- 1.81	- 1.79	- 1.38	- 1.41	- 1.39
NMB0460	Not in cogs genes	Transferrin-binding protein 2	2.53E-03	- 1.69	- 1.89	- 1.83	- 2.94	- 1.12	- 1.08	- 1.74
NMB0462	Amino acid transport and metabolism genes	Spermidine/putrescine ABC transporter	4.50E-02	- 1.15	- 1.28	- 1.15	- 1.06	- 1.11	- 1.00	+ 1.08
NMB0463	Translation	30S ribosomal protein S20	8.11E-04	- 1.14	+ 3.62	+ 4.29	+ 4.10	+ 4.13	+ 4.89	+ 4.68
NMB0466	Translation	Aspartyl-tRNA synthetase	6.65E-03	+ 1.39	+ 2.35	+ 2.07	+ 1.91	+ 1.69	+ 1.49	+ 1.38
NMB0467	Not in cogs genes	Hypothetical protein	4.98E-03	+ 2.19	+ 1.52	+ 1.76	+ 1.96	- 1.44	- 1.24	- 1.12
NMB0468	Amino acid transport and metabolism genes	Biosynthetic arginine decarboxylase	1.28E-02	+ 1.13	- 1.49	- 1.44	- 1.37	- 1.69	- 1.63	- 1.54
NMB0471	Function unknown genes	Conserved hypothetical protein	3.77E-04	- 1.00	+ 1.26	+ 1.23	- 1.77	+ 1.26	+ 1.23	- 1.77
NMB0472	Coenzyme transport and metabolism genes	8-amino-7-oxonanoate synthase	1.00E-02	+ 1.07	- 1.20	- 1.10	- 1.82	- 1.29	- 1.18	- 1.96
NMB0475	Not in cogs genes	Hypothetical protein	3.01E-02	- 1.82	+ 1.21	+ 1.49	+ 1.13	+ 2.20	+ 2.71	+ 2.06
NMB0477	General function prediction only genes	Conserved hypothetical protein	8.10E-03	- 2.65	- 1.23	- 1.25	- 2.15	+ 2.15	+ 2.13	+ 1.23
NMB0481	Not in cogs genes	Hypothetical protein	3.05E-02	+ 1.06	- 1.55	- 1.47	- 2.02	- 1.64	- 1.56	- 2.14
NMB0482	Not in cogs genes	Hypothetical protein	1.24E-02	- 1.21	+ 1.34	+ 1.50	+ 1.10	+ 1.61	+ 1.81	+ 1.32
NMB0483	Not in cogs genes	Hypothetical protein	4.11E-03	+ 1.02	- 1.85	- 1.91	- 1.47	- 1.90	- 1.96	- 1.50
NMB0484	Not in cogs genes	Hypothetical protein	2.19E-02	+ 1.44	- 1.40	- 1.05	- 1.41	- 2.03	- 1.51	- 2.03
NMB0485	Not in cogs genes	Hypothetical protein	1.90E-02	- 1.28	- 1.88	- 1.87	- 1.50	- 1.47	- 1.46	- 1.17
NMB0486	Not in cogs genes	Putative phage protein	8.08E-03	- 2.00	- 3.49	- 3.38	- 3.05	- 1.75	- 1.69	- 1.53
NMB0487	Not in cogs genes	Hypothetical protein	4.07E-02	+ 2.42	+ 2.51	+ 1.89	+ 3.71	+ 1.03	- 1.28	+ 1.53
NMB0488	Not in cogs genes	Hypothetical protein	3.57E-02	+ 1.43	+ 1.13	+ 1.23	- 1.20	- 1.26	- 1.16	- 1.71
NMB0490	Not in cogs genes	Pspa-related protein	1.04E-03	- 1.29	- 1.85	- 1.73	- 1.38	- 1.44	- 1.35	- 1.07
NMB0491	Not in cogs genes	Hypothetical protein	4.71E-03	+ 1.97	+ 1.22	+ 1.18	- 1.41	- 1.61	- 1.67	- 2.78
NMB0492	Not in cogs genes	Hypothetical protein	3.37E-02	+ 1.06	- 1.39	- 1.45	- 2.35	- 1.48	- 1.55	- 2.49
NMB0494	Replication recombination and repair	DNA helicase, truncation	4.00E-03	+ 1.06	- 1.97	- 1.86	- 1.98	- 2.08	- 1.96	- 2.09
NMB0495	Replication protein	Replication protein	2.11E-02	+ 1.54	+ 1.25	+ 1.22	- 1.80	- 1.24	- 1.26	- 2.77

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	P-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0497	Intracellular trafficking and secretion genes	Hemagglutinin/hemolysin-related protein	2.56E-02	- 1.60	- 2.04	- 2.19	- 2.45	- 1.28	- 1.37	- 1.53
NMB0501	Not in cogs genes	Hypothetical protein	2.38E-02	+ 1.14	+ 1.12	+ 1.14	- 3.07	- 1.01	+ 1.00	- 3.49
NMB0502	Not in cogs genes	Hypothetical protein	1.39E-02	- 1.27	+ 1.43	+ 1.59	- 2.09	+ 1.81	+ 2.02	- 1.65
NMB0503	Not in cogs genes	Hypothetical protein	2.64E-02	+ 1.02	+ 1.01	- 1.06	- 2.40	- 1.01	- 1.09	- 2.45
NMB0504	Not in cogs genes	Hypothetical protein	4.54E-02	+ 1.35	+ 1.69	+ 1.71	+ 1.49	+ 1.25	+ 1.27	+ 1.10
NMB0506	Intracellular trafficking and secretion genes	Hypothetical protein	8.74E-03	+ 1.32	+ 1.41	+ 1.60	- 1.30	+ 1.07	+ 1.21	- 1.72
NMB0507	Not in cogs genes	Hypothetical protein	3.56E-02	+ 1.13	+ 1.26	+ 1.05	- 2.20	+ 1.11	- 1.07	- 2.48
NMB0511	Intracellular trafficking and secretion genes	Hypothetical protein	2.14E-02	- 1.26	+ 1.51	+ 1.76	+ 1.09	+ 1.89	+ 2.22	+ 1.37
NMB0513	Function unknown genes	Hypothetical protein	1.39E-02	+ 1.54	+ 1.12	+ 1.11	- 1.41	- 1.37	- 1.38	- 2.16
NMB0514	Not in cogs genes	Hypothetical protein	5.49E-04	+ 1.85	+ 1.55	+ 1.49	+ 1.25	- 1.19	- 1.24	- 1.48
NMB0517	Not in cogs genes	Hypothetical protein	9.29E-03	+ 1.50	+ 1.19	+ 1.31	- 1.18	- 1.26	- 1.14	- 1.77
NMB0520	Not in cogs genes	Hypothetical protein	4.11E-03	+ 1.46	- 1.78	- 1.69	- 1.15	- 2.60	- 2.48	- 1.68
NMB0523	Not in cogs genes	Hypothetical protein	4.79E-02	- 1.31	- 1.43	- 1.53	- 1.63	- 1.10	- 1.17	- 1.25
NMB0526	Not in cogs genes	Hypothetical protein	5.01E-03	+ 1.08	- 1.13	- 1.06	- 2.81	- 1.22	- 1.15	- 3.03
NMB0528	Function unknown genes	Conserved hypothetical protein	1.32E-02	+ 1.56	+ 1.60	+ 1.54	+ 1.19	+ 1.02	- 1.01	- 1.31
NMB0533	Replication recombination and repair	Endonuclease III	3.46E-03	+ 1.28	- 1.09	- 1.17	+ 1.02	- 1.40	- 1.50	- 1.25
NMB0538	General function prediction only genes	Conserved hypothetical protein	6.50E-03	- 1.34	- 1.39	- 1.44	+ 1.22	- 1.04	- 1.07	+ 1.63
NMB0539	Not in cogs genes	Porphobilinogen deaminase	2.07E-02	+ 1.49	- 1.35	- 1.66	- 1.01	- 2.01	- 2.47	- 1.50
NMB0542	Function unknown genes	Hypothetical protein	3.19E-02	+ 1.53	+ 1.36	+ 1.41	+ 2.81	- 1.13	- 1.09	+ 1.84
NMB0501	Not in cogs genes	Hypothetical protein	2.38E-02	+ 1.14	+ 1.12	+ 1.14	- 3.07	- 1.01	+ 1.00	- 3.49
NMB0502	Not in cogs genes	Hypothetical protein	1.39E-02	- 1.27	+ 1.43	+ 1.59	- 2.09	+ 1.81	+ 2.02	- 1.65
NMB0503	Not in cogs genes	Hypothetical protein	2.64E-02	+ 1.02	+ 1.01	- 1.06	- 2.40	- 1.01	- 1.09	- 2.45
NMB0504	Not in cogs genes	Hypothetical protein	4.54E-02	+ 1.35	+ 1.69	+ 1.71	+ 1.49	+ 1.25	+ 1.27	+ 1.10
NMB0506	Intracellular trafficking and secretion genes	Hypothetical protein	8.74E-03	+ 1.32	+ 1.41	+ 1.60	- 1.30	+ 1.07	+ 1.21	- 1.72
NMB0507	Not in cogs genes	Hypothetical protein	3.56E-02	+ 1.13	+ 1.26	+ 1.05	- 2.20	+ 1.11	- 1.07	- 2.48
NMB0511	Intracellular trafficking and secretion genes	Hypothetical protein	2.14E-02	- 1.26	+ 1.51	+ 1.76	+ 1.09	+ 1.89	+ 2.22	+ 1.37
NMB0513	Function unknown genes	Hypothetical protein	1.39E-02	+ 1.54	+ 1.12	+ 1.11	- 1.41	- 1.37	- 1.38	- 2.16
NMB0514	Not in cogs genes	Hypothetical protein	5.49E-04	+ 1.85	+ 1.55	+ 1.49	+ 1.25	- 1.19	- 1.24	- 1.48
NMB0517	Not in cogs genes	Hypothetical protein	9.29E-03	+ 1.50	+ 1.19	+ 1.31	- 1.18	- 1.26	- 1.14	- 1.77
NMB0520	Not in cogs genes	Hypothetical protein	4.11E-03	+ 1.46	- 1.78	- 1.69	- 1.15	- 2.60	- 2.48	- 1.68
NMB0523	Not in cogs genes	Hypothetical protein	4.79E-02	- 1.31	- 1.43	- 1.53	- 1.63	- 1.10	- 1.17	- 1.25
NMB0526	Not in cogs genes	Hypothetical protein	5.01E-03	+ 1.08	- 1.13	- 1.06	- 2.81	- 1.22	- 1.15	- 3.03
NMB0528	Function unknown genes	Conserved hypothetical protein	1.32E-02	+ 1.56	+ 1.60	+ 1.54	+ 1.19	+ 1.02	- 1.01	- 1.31
NMB0533	Replication recombination and repair	Endonuclease III	3.46E-03	+ 1.28	- 1.09	- 1.17	+ 1.02	- 1.40	- 1.50	- 1.25
NMB0538	General function prediction only genes	Conserved hypothetical protein	6.50E-03	- 1.34	- 1.39	- 1.44	+ 1.22	- 1.04	- 1.07	+ 1.63
NMB0539	Not in cogs genes	Porphobilinogen deaminase	2.07E-02	+ 1.49	- 1.35	- 1.66	- 1.01	- 2.01	- 2.47	- 1.50
NMB0542	Function unknown genes	Hypothetical protein	3.19E-02	+ 1.53	+ 1.36	+ 1.41	+ 2.81	- 1.13	- 1.09	+ 1.84

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	P-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0543	Energy production and conversion genes	Putative L-lactate permease	3.18E-03	- 1.61	- 2.26	- 2.22	- 1.54	- 1.41	- 1.38	+ 1.05
NMB0546	General function prediction only genes	Alcohol dehydrogenase, propanol-preferring	2.38E-03	- 3.74	- 1.42	+ 1.25	- 1.55	+ 2.64	+ 4.67	+ 2.42
NMB0547	Intracellular trafficking and secretion genes	Type IV pilin protein	2.02E-03	- 1.85	- 1.15	- 1.11	+ 1.07	+ 1.61	+ 1.66	+ 1.97
NMB0549	Defense mechanisms genes	ABC transporter, ATP-binding protein	7.96E-04	+ 1.70	+ 1.92	+ 1.95	+ 1.42	+ 1.13	+ 1.15	- 1.19
NMB0550	Cell motility genes	Thiol:disulfide interchange protein dbbc	5.61E-04	- 1.79	+ 1.17	+ 1.18	+ 1.09	+ 2.10	+ 2.12	+ 1.96
NMB0551	Posttranslational modification, protein turnover, chaperones genes	Primosomal protein n ⁺	1.00E-02	- 1.56	- 1.15	- 1.15	- 1.64	+ 1.35	+ 1.35	- 1.05
NMB0554	Replication recombination and repair	Dnak protein	1.84E-03	- 1.57	+ 1.27	+ 1.00	+ 2.36	+ 1.99	+ 1.57	+ 3.70
NMB0557	N/a	Conserved hypothetical protein	4.08E-02	- 1.28	- 1.18	+ 1.51	+ 1.62	+ 1.08	+ 1.94	+ 2.08
NMB0558	Not in cogs genes	Hypothetical protein	9.45E-03	+ 1.27	+ 1.12	+ 1.32	+ 1.98	- 1.14	+ 1.04	+ 1.55
NMB0560	Amino acid transport and metabolism genes	Serine acetyltransferase	3.62E-04	- 1.12	+ 1.68	+ 1.45	+ 1.33	+ 1.89	+ 1.63	+ 1.49
NMB0562	Function unknown genes	Conserved hypothetical protein	1.68E-02	- 1.16	- 1.04	+ 1.32	+ 1.34	+ 1.12	+ 1.53	+ 1.56
NMB0563	Not in cogs genes	Thiamine biosynthesis lipoprotein apbe	2.61E-03	- 1.19	- 1.10	+ 1.13	- 1.36	+ 1.08	+ 1.35	- 1.14
NMB0564	Energy production and conversion genes	Na(+)-translocating NADH-quinone reductase, subunit F	6.83E-03	- 1.16	- 1.18	- 1.19	- 1.66	- 1.02	- 1.03	- 1.43
NMB0567	Energy production and conversion genes	Na(+)-translocating NADH-quinone reductase, subunit C	3.15E-04	+ 1.02	- 1.21	- 1.24	+ 1.36	- 1.23	- 1.26	+ 1.34
NMB0568	Energy production and conversion genes	Na(+)-translocating NADH-quinone reductase, subunit B	4.01E-03	+ 1.01	- 1.26	- 1.34	+ 1.19	- 1.26	- 1.35	+ 1.19
NMB0569	Energy production and conversion genes	Na(+)-translocating NADH-quinone reductase, subunit A	1.01E-02	- 1.19	- 1.38	- 1.30	+ 1.25	- 1.16	- 1.09	+ 1.49
NMB0570	Inorganic ion transport and metabolism genes	Hypothetical protein	9.10E-04	+ 1.24	- 1.07	- 1.17	+ 1.07	- 1.33	- 1.45	- 1.16
NMB0573	Transcription	Transcriptional regulator, asnc family	1.20E-03	- 1.22	- 1.55	- 1.66	- 1.09	- 1.27	- 1.37	+ 1.12
NMB0574	Amino acid transport and metabolism genes	Glycine cleavage system T protein	6.87E-03	+ 1.13	- 1.33	- 1.25	+ 1.54	- 1.51	- 1.42	+ 1.36
NMB0575	Amino acid transport and metabolism genes	Glycine cleavage system H protein	2.80E-02	- 1.26	- 1.57	- 1.81	- 1.07	- 1.25	- 1.43	+ 1.18
NMB0576	Coenzyme transport and metabolism genes	Glutaryl-tRNA reductase	9.26E-04	- 1.26	- 1.30	- 1.34	- 1.06	- 1.04	- 1.06	+ 1.19
NMB0579	Defense mechanisms genes	Copper ABC transporter, ATP-binding protein	4.55E-03	+ 1.89	+ 1.54	+ 1.76	- 1.36	- 1.22	- 1.08	- 2.58
NMB0580	Energy production and conversion genes	Putative protein disulfide isomerase nosl	4.37E-03	+ 1.62	+ 1.23	+ 1.28	+ 2.07	- 1.32	- 1.27	+ 1.28
NMB0581	Energy production and conversion genes	Electron transfer flavoprotein-ubiquinone oxidoreductase	2.85E-03	+ 1.05	- 1.18	- 1.32	+ 1.28	- 1.24	- 1.39	+ 1.21
NMB0582	General function prediction only genes	Putative bacteriocin resistance protein	3.26E-04	- 1.16	- 2.13	- 2.44	- 1.73	- 1.83	- 2.10	- 1.49
NMB0583	Replication recombination and repair	IS1016C2 transposase	1.07E-02	- 1.44	- 2.33	- 2.03	- 2.48	- 1.62	- 1.40	- 1.72
NMB0584	Not in cogs genes	Frcp operon protein	2.76E-02	- 1.65	- 2.42	- 1.78	- 1.94	- 1.47	- 1.07	- 1.17
NMB0585	Secondary metabolites biosynthesis, transport and catabolism genes	Iron-regulated protein frpc	2.12E-02	- 1.23	- 1.87	- 1.49	- 2.41	- 1.52	- 1.21	- 1.96
NMB0589	Not in cogs genes	50S ribosomal protein L19	2.61E-06	- 1.44	+ 4.04	+ 2.24	- 1.12	+ 5.82	+ 3.22	+ 1.29
NMB0590	Not in cogs genes	Trna (guanine-N1)-methyltransferase	3.30E-04	- 1.12	+ 5.91	+ 3.90	+ 2.09	+ 6.65	+ 4.39	+ 2.35
NMB0591	Translation	16S rRNA processing protein rimm	2.69E-03	- 1.59	+ 3.53	+ 2.86	+ 1.76	+ 5.61	+ 4.53	+ 2.79
NMB0592	Not in cogs genes	30S ribosomal protein S16	3.73E-04	- 1.40	+ 2.09	+ 1.96	+ 1.37	+ 2.94	+ 2.75	+ 1.92
NMB0595	Signal transduction mechanisms genes	DNA-binding response regulator	1.29E-04	- 1.23	+ 4.94	+ 3.07	+ 1.77	+ 6.06	+ 3.76	+ 2.16
NMB0601	Hypothetical protein NMB0601	Conserved hypothetical protein	1.42E-03	- 1.45	- 1.64	- 1.67	- 1.44	- 1.13	- 1.15	+ 1.01

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0575	Amino acid transport and metabolism genes	Glycine cleavage system H protein	2.80E-02	- 1.26	- 1.57	- 1.81	- 1.07	- 1.25	- 1.43	+ 1.18
NMB0576	Coenzyme transport and metabolism genes	Glutamyl-trna reductase	9.26E-04	- 1.26	- 1.30	- 1.34	- 1.06	- 1.04	- 1.06	+ 1.19
NMB0579	Defense mechanisms genes	Copper ABC transporter, ATP-binding protein	4.55E-03	+ 1.89	+ 1.54	+ 1.76	- 1.36	- 1.22	- 1.08	- 2.58
NMB0580	Energy production and conversion genes	Putative protein disulfide isomerase nosI	4.37E-03	+ 1.62	+ 1.23	+ 1.28	+ 2.07	- 1.32	- 1.27	+ 1.28
NMB0581	Energy production and conversion genes	Electron transfer flavoprotein-ubiquinone oxidoreductase	2.85E-03	+ 1.05	- 1.18	- 1.32	+ 1.28	- 1.24	- 1.39	+ 1.21
NMB0582	General function prediction only genes	Putative bacteriocin resistance protein	3.26E-04	- 1.16	- 2.13	- 2.44	- 1.73	- 1.83	- 2.10	- 1.49
NMB0583	Replication recombination and repair	IS1016C2 transposase	1.07E-02	- 1.44	- 2.33	- 2.03	- 2.48	- 1.62	- 1.40	- 1.72
NMB0584	Not in cogs genes	Frcp operon protein	2.76E-02	- 1.65	- 2.42	- 1.78	- 1.94	- 1.47	- 1.07	- 1.17
NMB0585	Secondary metabolites biosynthesis, transport and catabolism genes	Iron-regulated protein frpC	2.12E-02	- 1.23	- 1.87	- 1.49	- 2.41	- 1.52	- 1.21	- 1.96
NMB0589	Not in cogs genes	50S ribosomal protein L19	2.61E-06	- 1.44	+ 4.04	+ 2.24	- 1.12	+ 5.82	+ 3.22	+ 1.29
NMB0590	Not in COGs genes	tRNA (guanine-N1)-methyltransferase	3.30E-04	- 1.12	+ 5.91	+ 3.90	+ 2.09	+ 6.65	+ 4.39	+ 2.35
NMB0591	translation	16S rRNA processing protein RimM	2.69E-03	- 1.59	+ 3.53	+ 2.86	+ 1.76	+ 5.61	+ 4.53	+ 2.79
NMB0592	Not in COGs genes	30S ribosomal protein S16	3.73E-04	- 1.40	+ 2.09	+ 1.96	+ 1.37	+ 2.94	+ 2.75	+ 1.92
NMB0595	Signal transduction mechanisms genes	DNA-binding response regulator	1.29E-04	- 1.23	+ 4.94	+ 3.07	+ 1.77	+ 6.06	+ 3.76	+ 2.16
NMB0601	hypothetical protein NMB0601	conserved hypothetical protein	1.42E-03	- 1.45	- 1.64	- 1.67	- 1.44	- 1.13	- 1.15	+ 1.01
NMB0604	General function prediction only genes Amino acid transport and metabolism genes	Alcohol dehydrogenase, zinc-containing	6.36E-03	+ 1.02	+ 1.95	+ 2.03	+ 1.23	+ 1.92	+ 2.00	+ 1.21
NMB0605	Not in cogs genes	Histone deacetylase family protein	6.28E-03	- 1.17	+ 1.15	+ 1.15	+ 1.76	+ 1.35	+ 1.34	+ 2.06
NMB0606	Intracellular trafficking and secretion genes	Conserved hypothetical protein	1.24E-02	- 1.89	- 1.22	- 1.20	- 1.07	+ 1.55	+ 1.58	+ 1.77
NMB0608	Intracellular trafficking and secretion genes	Protein-export membrane protein secf	2.59E-02	+ 1.46	+ 1.26	+ 1.22	+ 1.03	- 1.16	- 1.20	- 1.42
NMB0609	Not in cogs genes	30S ribosomal protein S15	2.72E-04	- 2.00	+ 2.78	+ 1.89	+ 1.51	+ 5.55	+ 3.78	+ 3.01
NMB0611	Amino acid transport and metabolism genes	Spermidine/putrescine ABC transporter, permease protein	4.91E-02	+ 1.41	- 1.05	+ 1.07	+ 1.08	- 1.49	- 1.33	- 1.31
NMB0612	Amino acid transport and metabolism genes	Putative oxidoreductase	6.65E-04	+ 1.82	+ 1.30	+ 1.32	+ 1.37	- 1.40	- 1.38	- 1.33
NMB0614	Amino acid transport and metabolism genes	Putative ammonium transporter ambB	1.50E-02	+ 1.34	- 1.10	- 1.03	- 1.20	- 1.48	- 1.38	- 1.61
NMB0615	Not in cogs genes	IS1016 family transposase, degenerate	2.20E-03	+ 1.57	- 1.88	- 1.37	- 2.07	- 2.95	- 2.15	- 3.25
NMB0616	Not in cogs genes	Transcription termination factor Rho	5.01E-03	+ 1.01	- 1.90	- 1.68	- 2.86	- 1.92	- 1.69	- 2.88
NMB0617	Transcription	Phosphoenolpyruvate synthase	3.91E-02	- 1.00	+ 1.27	- 1.00	+ 1.49	+ 1.27	- 1.00	+ 1.49
NMB0618	Carbohydrate transport and metabolism genes	Conserved hypothetical protein	1.04E-03	+ 1.34	- 1.05	- 1.11	+ 1.59	- 1.40	- 1.48	+ 1.19
NMB0619	Not in cogs genes	Phosphoglycolate phosphatase	2.22E-04	- 1.40	- 1.90	- 1.55	+ 1.28	- 1.36	- 1.10	+ 1.80
NMB0620	General function prediction only genes	Glycosyltransferase pglE	7.01E-03	- 1.04	- 1.44	- 1.25	- 3.15	- 1.39	- 1.21	- 3.04
NMB0624	General function prediction only genes	Conserved hypothetical protein	4.16E-02	- 1.53	+ 1.13	+ 1.21	- 1.76	+ 1.73	+ 1.85	- 1.15
NMB0625	Translation	Peptide chain release factor 3	5.61E-04	+ 1.66	+ 1.84	+ 2.09	- 1.27	+ 1.11	+ 1.26	- 2.10
NMB0626	Not in cogs genes	Phosphoribosyl-AMP cyclohydrolase	1.37E-02	+ 1.00	+ 1.28	+ 1.21	- 1.13	+ 1.27	+ 1.21	- 1.14
NMB0627	Amino acid transport and metabolism genes	Amidotransferase hsh	4.89E-04	+ 2.31	- 1.21	+ 1.05	- 2.69	- 2.80	- 2.20	- 6.22
NMB0630			2.71E-02	+ 1.01	- 1.06	+ 1.05	+ 1.73	- 1.07	+ 1.04	+ 1.72

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0631	General function prediction only genes	Energy production and conversion genes	3.82E-02	- 1.60	+ 1.05	+ 1.27	- 1.25	+ 1.68	+ 2.04	+ 1.29
NMB0632	Iron(III) ABC transporter, ATP-binding protein	Putative phosphate acetyltransferase Pta	1.73E-02	- 1.12	- 1.29	- 1.02	- 1.96	- 1.15	+ 1.10	- 1.75
NMB0634	Inorganic ion transport and metabolism genes	Iron(III) ABC transporter, ATP-binding protein	1.58	- 1.58	+ 3.09	+ 2.18	+ 1.13	+ 4.88	+ 3.45	+ 1.79
NMB0637	Amino acid transport and metabolism genes	Argininosuccinate lyase	2.09E-02	- 1.27	+ 1.27	+ 1.20	+ 1.47	+ 1.61	+ 1.53	+ 1.87
NMB0641	Energy production and conversion genes	Inorganic pyrophosphatase	8.25E-04	- 1.49	+ 1.92	+ 1.40	+ 1.24	+ 2.87	+ 2.09	+ 1.85
NMB0643	Not in cogs genes	MafB-related protein	1.82E-02	+ 1.19	- 1.09	- 1.39	- 1.09	- 1.30	- 1.66	- 1.31
NMB0648	Not in cogs genes	Hypothetical protein	2.89E-03	- 1.19	+ 1.27	+ 1.38	- 2.65	+ 1.52	+ 1.64	- 2.23
NMB0649	Not in cogs genes	Hypothetical protein	2.26E-03	- 2.33	- 1.44	- 1.72	- 2.96	+ 1.62	+ 1.36	- 1.27
NMB0650	Not in cogs genes	Hypothetical protein	4.05E-03	- 2.55	- 3.35	- 3.15	- 2.43	- 1.31	- 1.24	+ 1.05
NMB0657	Not in cogs genes	Hypothetical protein	2.61E-02	- 1.18	+ 1.42	+ 1.53	- 1.29	+ 1.67	+ 1.79	- 1.10
NMB0658	Not in cogs genes	Hypothetical protein	7.96E-03	+ 1.70	+ 2.45	+ 2.27	+ 1.51	+ 1.44	+ 1.34	- 1.13
NMB0659	Not in cogs genes	Hypothetical protein	7.96E-04	+ 2.53	+ 3.22	+ 3.03	+ 2.03	+ 1.27	+ 1.20	- 1.24
NMB0660	Not in cogs genes	Hypothetical protein	1.68E-02	- 1.35	- 1.02	+ 1.25	- 2.41	+ 1.32	+ 1.69	- 1.79
NMB0663	Cell wall/membrane biogenesis genes	Outer membrane protein nspa	1.54E-02	- 1.16	+ 1.45	- 1.02	+ 1.14	+ 1.68	+ 1.14	+ 1.33
NMB0665	Coenzyme transport and metabolism genes	Oxygen-independent coprophorphyrinogen III oxidase family protein	3.39E-03	+ 1.98	+ 1.95	+ 1.90	- 1.05	- 1.02	- 1.04	- 2.07
NMB0667	Not in cogs genes	Hypothetical protein	2.86E-02	- 1.21	- 1.50	- 1.21	- 1.12	- 1.24	+ 1.00	+ 1.08
NMB0669	Not in cogs genes	Conserved hypothetical protein	8.45E-06	+ 1.44	- 1.53	- 1.05	- 3.99	- 2.19	- 1.51	- 5.73
NMB0670	Nucleotide transport and metabolism genes	Thymidylate kinase	8.46E-03	+ 1.51	+ 1.82	+ 1.04	- 1.04	+ 1.20	- 1.46	- 1.57
NMB0672	Cell wall/membrane biogenesis genes	Tetraacyldisaccharide 4'-kinase	7.52E-05	+ 1.19	- 2.31	- 2.32	- 3.87	- 2.75	- 2.77	- 4.62
NMB0673	Function unknown genes	Hypothetical protein	7.24E-03	+ 1.04	+ 1.31	+ 1.74	+ 1.43	+ 1.26	+ 1.68	+ 1.38
NMB0674	Not in cogs genes	Conserved hypothetical protein	4.50E-02	+ 1.32	+ 1.56	+ 1.51	+ 1.54	+ 1.19	+ 1.15	+ 1.17
NMB0676	Not in cogs genes	Hypothetical protein	1.09E-02	- 1.09	+ 1.07	+ 1.55	+ 1.47	+ 1.17	+ 1.70	+ 1.61
NMB0677	Not in cogs genes	Hypothetical protein	1.18E-04	+ 2.47	+ 1.61	+ 1.32	+ 1.02	- 1.53	- 1.88	- 2.41
NMB0681	Posttranslational modification, protein turnover, chaperones genes	Conserved hypothetical protein	9.61E-03	- 1.47	- 1.03	+ 1.08	+ 1.42	+ 1.43	+ 1.59	+ 2.09
NMB0685	Function unknown genes	Hypothetical protein	4.48E-03	- 1.35	+ 1.39	+ 1.37	- 1.12	+ 1.88	+ 1.85	+ 1.21
NMB0686	Transcription	Ribonuclease III	2.94E-02	- 1.09	- 1.13	+ 1.02	+ 1.71	- 1.04	+ 1.11	+ 1.86
NMB0687	General function prediction only genes	GTP-binding protein Era	9.03E-04	+ 1.47	+ 1.18	+ 1.23	+ 1.10	- 1.25	- 1.20	- 1.34
NMB0689	Transcription	Transcription elongation factor greb	8.98E-04	+ 1.63	+ 2.67	+ 1.28	+ 2.17	+ 1.64	- 1.28	+ 1.33
NMB0690	Nucleotide transport and metabolism genes	Amidophosphoribosyltransferase	1.18E-04	+ 1.56	+ 1.13	+ 1.57	- 1.18	- 1.38	+ 1.01	- 1.85
NMB0691	Not in cogs genes	Putative colicin V production protein	6.08E-04	+ 1.32	- 1.42	- 1.10	- 2.53	- 1.87	- 1.45	- 3.35
NMB0692	Not in cogs genes	Tpc protein	1.56E-02	- 1.37	- 1.19	- 1.17	+ 1.14	+ 1.15	+ 1.17	+ 1.56
NMB0693	Coenzyme transport and metabolism genes	Folypolyglutamate synthase/dihydrofolate synthase	3.07E-03	- 1.17	- 1.38	- 1.38	- 1.52	- 1.18	- 1.19	- 1.31
NMB0694	Not in cogs genes	Foli protein	2.02E-03	- 1.34	+ 1.20	- 1.00	+ 1.10	+ 1.61	+ 1.33	+ 1.47
NMB0695	Not in cogs genes	Hypothetical protein	4.98E-03	- 1.46	+ 1.04	- 1.03	+ 1.26	+ 1.51	+ 1.41	+ 1.84
NMB0698	Replication recombination and repair	Hypothetical protein	2.02E-03	- 1.40	- 1.65	- 1.66	+ 1.17	- 1.18	- 1.18	+ 1.64

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0701	Not in cogs genes	Hypothetical protein	3.68E-02	+ 1.02	+ 2.18	+ 1.56	- 2.75	+ 2.13	+ 1.53	+ 2.68
NMB0702	General function prediction only genes	Competence protein comA	5.13E-03	+ 1.09	+ 1.02	- 1.08	- 1.62	- 1.07	- 1.18	- 1.76
NMB0703	General function prediction only genes	Competence lipoprotein comI	1.22E-03	- 2.27	- 1.10	- 1.14	+ 1.01	+ 2.06	+ 1.99	+ 2.29
NMB0704	Translation	Ribosomal large subunit pseudouridine synthase D	4.21E-02	- 1.08	+ 1.12	+ 1.51	+ 1.05	+ 1.21	+ 1.63	+ 1.14
NMB0706	Function unknown genes	Conserved hypothetical protein	1.88E-02	- 1.26	+ 1.07	+ 1.07	- 1.31	+ 1.35	+ 1.35	- 1.05
NMB0707	Not in cogs genes	Putative rare lipoprotein B	4.75E-02	- 1.14	- 1.26	- 1.03	- 1.47	- 1.11	+ 1.10	- 1.29
NMB0709	Not in cogs genes	Hypothetical protein	1.91E-04	- 1.06	- 1.28	- 1.16	+ 1.58	- 1.20	- 1.09	+ 1.68
NMB0711	Function unknown genes	Conserved hypothetical protein	3.57E-02	+ 1.12	+ 1.07	+ 1.02	+ 1.56	- 1.04	- 1.10	+ 1.39
NMB0712	Transcription	RNA polymerase sigma-32 factor	7.01E-04	- 1.18	+ 2.37	+ 1.33	+ 1.89	+ 2.79	+ 1.56	+ 2.22
NMB0714	Function unknown genes	Conserved hypothetical protein	8.98E-04	+ 1.17	- 1.21	- 1.13	- 1.65	- 1.42	- 1.33	- 1.94
NMB0717	Energy production and conversion genes	Putative cytochrome	1.17E-03	+ 1.37	- 1.99	- 1.92	- 3.33	- 2.72	- 2.62	- 4.55
NMB0719	Translation	Queuine tRNA-ribosyltransferase	6.28E-03	- 1.40	- 1.05	- 1.16	+ 1.28	+ 1.34	+ 1.21	+ 1.80
NMB0722	Translation	50S ribosomal protein L35	1.05E-04	- 3.12	+ 8.13	+ 3.07	+ 2.59	+ 25.34	+ 9.57	+ 8.06
NMB0723	Not in cogs genes	50S ribosomal protein L20	1.25E-04	- 2.05	+ 5.96	+ 2.79	+ 2.64	+ 12.23	+ 5.72	+ 5.41
NMB0724	Translation	Phenylalanyl-tRNA synthetase, alpha chain	5.76E-04	- 1.25	+ 1.38	+ 1.26	+ 1.64	+ 1.72	+ 1.58	+ 2.05
NMB0725	Replication recombination and repair	Modification methylase HgaI-1	4.16E-03	+ 1.18	+ 1.63	+ 1.60	- 1.63	+ 1.38	+ 1.35	- 1.92
NMB0729	Not in cogs genes	Integration host factor, alpha subunit	2.83E-02	+ 1.83	+ 1.12	+ 1.83	+ 2.68	- 1.63	+ 1.00	+ 1.46
NMB0730	General function prediction only genes	Hypothetical protein	2.22E-03	+ 1.76	- 2.43	- 1.87	- 1.04	- 4.28	- 3.30	- 1.83
NMB0731	Not in cogs genes	Hypothetical protein	3.98E-04	+ 1.86	+ 1.17	+ 1.42	- 1.10	- 1.59	- 1.31	- 2.04
NMB0736	Carbohydrate transport and metabolism genes Signal transduction mechanisms genes	PTS system, nitrogen regulatory IIA protein	2.14E-02	- 1.33	+ 1.16	+ 1.40	+ 1.37	+ 1.54	+ 1.86	+ 1.82
NMB0737	General function prediction only genes	Putative hpr kinase/phosphatase	4.98E-02	- 1.22	- 1.16	- 1.22	- 1.00	+ 1.06	- 1.00	+ 1.22
NMB0738	General function prediction only genes	Conserved hypothetical protein	8.51E-04	+ 1.38	- 1.10	- 1.21	+ 1.24	- 1.52	- 1.68	- 1.12
NMB0741	Amino acid transport and metabolism genes	Conserved hypothetical protein	2.13E-02	- 1.39	- 1.15	+ 1.07	- 1.59	+ 1.22	+ 1.50	- 1.14
NMB0742	Function unknown genes	Conserved hypothetical protein	3.94E-02	- 1.15	- 1.00	+ 1.04	+ 1.61	+ 1.14	+ 1.19	+ 1.85
NMB0743	Coenzyme transport and metabolism genes	Ubiquinone/menaquinone biosynthesis methyltransferase uibE	1.32E-02	- 1.28	- 1.45	- 1.15	+ 1.36	- 1.13	+ 1.12	+ 1.74
NMB0744	Not in cogs genes	Hypothetical protein	1.18E-04	- 1.81	+ 2.86	+ 3.82	+ 1.04	+ 5.19	+ 6.94	+ 1.88
NMB0745	Coenzyme transport and metabolism genes	2-amino-4-hydroxy-6-hydroxymethylhydropterdine- pyrophosphokinase	1.21E-05	- 1.54	+ 1.32	+ 2.65	- 1.21	+ 2.03	+ 4.07	+ 1.27
NMB0746	Not in cogs genes	Conserved hypothetical protein	1.18E-04	+ 1.72	+ 1.14	+ 1.92	- 1.72	- 1.51	+ 1.12	- 2.95
NMB0747	Not in cogs genes	Conserved hypothetical protein	1.85E-03	+ 1.50	+ 1.10	+ 1.68	- 1.11	- 1.37	+ 1.12	- 1.67
NMB0748	General function prediction only genes	Host factor-I	1.20E-03	- 1.75	+ 1.58	+ 1.78	+ 3.41	+ 2.75	+ 3.12	+ 5.95
NMB0749	Cell wall/membrane biogenesis genes	Penicillin-binding protein 4	2.88E-03	- 1.35	- 1.29	+ 1.38	- 1.55	+ 1.05	+ 1.86	- 1.15
NMB0750	Posttranslational modification, protein turnover, chaperones genes	Bacterioferritin comigratory protein	4.79E-02	- 1.00	- 1.26	- 1.10	+ 1.15	- 1.25	- 1.10	+ 1.16
NMB0752	Inorganic ion transport and metabolism genes	Putative bacterioferritin-associated ferredoxin	1.58E-02	+ 1.03	+ 2.08	+ 2.44	+ 2.35	+ 2.01	+ 2.36	+ 2.27
NMB0753	Not in cogs genes	Conserved hypothetical protein	6.05E-03	+ 1.62	+ 3.63	+ 1.74	+ 2.65	+ 2.24	+ 1.07	+ 1.63

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h-DMEM	24 h vs 3 h-DMEM	96 h vs 3 h-DMEM	21 d vs 3 h-DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0754	Not in cogs genes	Hypothetical protein	1.13E-03	- 2.06	+ 2.70	+ 2.77	+ 3.30	+ 5.58	+ 5.72	+ 6.82
NMB0756	Cell wall/membrane biogenesis genes	Putative dtdp-L-rhamnose synthase	6.91E-03	- 1.13	- 1.12	- 1.06	+ 1.58	+ 1.01	+ 1.07	+ 1.79
NMB0757	Not in cogs genes	Phosphoribosylaminoimidazole-succinocarboxamide synthase	1.36E-02	- 1.04	- 1.52	- 1.53	+ 1.00	- 1.47	- 1.47	+ 1.04
NMB0761	Not in cogs genes	Hypothetical protein	4.88E-02	- 1.01	- 1.31	- 1.09	+ 1.50	- 1.31	- 1.09	+ 1.51
NMB0762	Not in cogs genes	Hypothetical protein	4.46E-03	- 1.75	+ 1.52	+ 1.56	+ 1.51	+ 2.66	+ 2.74	+ 2.64
NMB0763	Amino acid transport and metabolism genes	Cysteine synthase	3.47E-03	- 1.96	+ 3.09	+ 3.58	+ 1.43	+ 6.05	+ 7.01	+ 2.79
NMB0764	General function prediction only genes	Conserved hypothetical protein	1.36E-03	- 1.50	- 1.41	- 1.61	- 1.02	+ 1.06	- 1.07	+ 1.47
NMB0767	Nucleotide transport and metabolism genes	5-methylthioadenosine nucleosidase	4.00E-02	+ 1.14	+ 1.08	+ 1.25	+ 1.75	- 1.06	+ 1.10	+ 1.53
NMB0768	Intracellular trafficking and secretion genes	Twitching motility protein pili	1.26E-02	- 1.18	+ 1.07	+ 1.19	+ 1.53	+ 1.26	+ 1.40	+ 1.80
NMB0771	Cell motility genes	Conserved hypothetical protein	6.68E-03	- 1.11	+ 1.00	- 1.21	+ 1.53	+ 1.11	- 1.09	+ 1.70
NMB0773	Posttranslational modification, protein turnover, chaperones genes	Conserved hypothetical protein	8.11E-04	- 1.59	- 1.03	- 1.30	+ 1.97	+ 1.54	+ 1.22	+ 3.12
NMB0775	Function unknown genes	Hypothetical protein	1.91E-02	+ 1.29	- 1.02	- 1.05	+ 1.78	- 1.32	- 1.36	+ 1.37
NMB0776	Not in cogs genes	Conserved hypothetical protein	3.39E-02	+ 1.34	+ 1.11	+ 1.08	+ 2.27	- 1.21	- 1.24	+ 1.69
NMB0778	Coenzyme transport and metabolism genes	Putative uroporphyrin-III C-methyltransferase hemx	1.26E-02	- 1.42	- 1.29	- 1.31	+ 1.22	+ 1.10	+ 1.08	+ 1.72
NMB0779	Coenzyme transport and metabolism genes	Hypothetical protein	3.99E-03	+ 1.90	- 1.23	- 1.16	- 1.44	- 2.35	- 2.20	- 2.74
NMB0780	Not in cogs genes	Hypothetical protein	1.50E-02	- 1.01	- 1.05	- 1.28	- 2.01	- 1.04	- 1.26	- 1.98
NMB0782	Posttranslational modification, protein turnover, chaperones genes	DNA repair protein rada	2.52E-03	- 1.05	+ 1.44	+ 1.00	- 1.34	+ 1.52	+ 1.06	- 1.27
NMB0783	Function unknown genes	Conserved hypothetical protein	4.25E-04	- 1.13	- 1.61	- 1.09	- 3.78	- 1.43	+ 1.04	- 3.35
NMB0784	Inorganic ion transport and metabolism genes	Putative phage shock protein E precursor	1.19E-02	- 1.18	+ 1.06	+ 1.40	- 1.83	+ 1.25	+ 1.65	- 1.55
NMB0787	Amino acid transport and metabolism genes	Amino acid ABC transporter, periplasmic amino acid-binding protein	7.96E-04	- 1.07	+ 5.08	+ 6.17	+ 2.49	+ 5.42	+ 6.58	+ 2.65
NMB0788	Amino acid transport and metabolism genes	Amino acid ABC transporter, permease protein	3.47E-03	+ 1.01	+ 2.16	+ 2.91	+ 1.59	+ 2.15	+ 2.89	+ 1.58
NMB0789	Amino acid transport and metabolism genes	Amino acid ABC transporter, ATP-binding protein	3.11E-04	+ 1.23	+ 2.95	+ 3.00	+ 1.40	+ 2.40	+ 2.43	+ 1.13
NMB0790	Carbohydrate transport and metabolism genes	Phosphoglucosyltransferase	1.25E-04	- 1.00	+ 2.71	+ 3.32	+ 2.06	+ 2.72	+ 3.33	+ 2.07
NMB0791	Posttranslational modification, protein turnover, chaperones genes	Peptidyl-prolyl di-trans isomerase	5.64E-07	- 1.72	+ 1.29	+ 1.50	+ 5.68	+ 2.22	+ 2.57	+ 9.75
NMB0792	Inorganic ion transport and metabolism genes	Transporter, nadc family	1.46E-03	- 1.13	+ 1.72	+ 1.95	- 1.02	+ 1.95	+ 2.21	+ 1.11
NMB0793	Not in cogs genes	Hypothetical protein	1.29E-02	+ 1.48	+ 2.07	+ 2.36	- 1.03	+ 1.40	+ 1.59	- 1.53
NMB0794	Not in cogs genes	Hypothetical protein	8.86E-03	+ 1.03	+ 1.09	+ 1.48	- 1.22	+ 1.06	+ 1.44	- 1.26
NMB0795	Not in cogs genes	Peptidyl-trna hydrolase	2.90E-02	- 1.11	+ 1.14	+ 1.35	+ 1.65	+ 1.27	+ 1.50	+ 1.83
NMB0796	Function unknown genes	Conserved hypothetical protein	1.12E-02	+ 1.53	+ 1.20	+ 1.15	- 1.94	- 1.27	- 1.33	- 2.96
NMB0797	Lipid transport and metabolism genes	Conserved hypothetical protein	2.00E-02	+ 1.11	+ 1.06	+ 1.07	+ 1.30	- 1.04	- 1.03	+ 1.17
NMB0798	Posttranslational modification, protein turnover, chaperones genes	Cell division protein ftsj	3.50E-02	- 1.18	- 1.20	- 1.06	+ 1.10	- 1.01	+ 1.12	+ 1.30
NMB0799	Translation	Cell division protein ftsj	1.91E-03	- 1.27	- 1.28	- 1.24	+ 1.40	- 1.00	+ 1.03	+ 1.78
NMB0800	Not in cogs genes	Conserved hypothetical protein	1.48E-02	+ 1.15	+ 1.08	+ 1.20	+ 1.90	- 1.07	+ 1.04	+ 1.65

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0801	Not in cogs genes	Delta-aminolevulinic acid dehydratase	3.50E-03	-	2.10	-	1.63	-	1.28	-
NMB0802	Amino acid transport and metabolism genes	Cystathionine gamma-synthase	1.96E-02	-	1.10	+	1.13	+	1.01	+
NMB0804	Energy production and conversion genes	Putative nad(pH) nitroreductase	1.45E-02	+	1.19	-	1.23	-	1.95	-
NMB0810	Transcription	Transcriptional regulator, tetR family	6.19E-04	-	1.33	-	1.53	+	1.09	+
NMB0814	Amino acid transport and metabolism genes	Histidyl-tRNA synthetase	2.09E-03	-	1.37	+	1.07	+	1.79	+
NMB0816	Not in cogs genes	Hypothetical protein	1.15E-02	+	2.69	+	1.84	+	1.22	-
NMB0817	Not in cogs genes	Hypothetical protein	4.61E-03	+	1.21	+	1.53	+	1.11	-
NMB0818	Not in cogs genes	Hypothetical protein	1.14E-02	+	1.08	-	1.00	-	1.01	-
NMB0819	Not in cogs genes	Hypothetical protein	1.23E-02	-	1.19	-	1.19	-	1.03	-
NMB0820	Not in cogs genes	Hypothetical protein	2.38E-02	-	1.34	-	1.44	-	1.15	-
NMB0821	Not in cogs genes	Hypothetical protein	4.20E-02	+	1.07	+	1.08	-	1.25	-
NMB0822	Posttranslational modification, protein turnover, chaperones genes	Heat shock protein htpx	6.46E-07	-	2.61	+	2.71	+	4.34	+
NMB0823	Nucleotide transport and metabolism genes	Adenylate kinase	2.22E-04	-	1.06	-	1.10	+	1.15	+
NMB0825	Cell wall/membrane biogenesis genes	Putative ADP-heptose synthase	4.74E-03	+	1.57	+	1.12	+	1.52	+
NMB0831	Not in cogs genes		9.02E-03	+	1.70	+	1.58	+	1.16	-
NMB0836	Posttranslational modification, protein turnover, chaperones genes	ATP-dependent Clp protease, ATP-binding subunit clpA	9.08E-03	-	1.14	+	1.04	+	1.20	+
NMB0837	Not in cogs genes	Conserved hypothetical protein	8.68E-03	-	1.08	-	1.23	+	1.09	-
NMB0840	Not in cogs genes	Conserved hypothetical protein	3.99E-03	-	1.42	+	1.78	+	1.72	+
NMB0841	Not in cogs genes	Hypothetical protein	2.07E-03	-	1.04	-	1.12	+	1.31	+
NMB0844	Not in cogs genes	Hypothetical protein	1.49E-02	-	1.09	+	1.41	+	1.15	+
NMB0845	Signal transduction mechanisms genes	Phob-related protein	1.29E-02	-	1.01	+	1.02	+	1.09	+
NMB0847	Not in cogs genes	Hypothetical protein	4.46E-04	-	2.11	-	1.95	-	1.84	-
NMB0848	Function unknown genes	Hypothetical protein	3.20E-04	-	1.38	-	1.51	+	1.15	+
NMB0850	Function unknown genes	Hypothetical protein	2.93E-03	-	1.73	-	1.86	-	1.16	-
NMB0854	Translation	Histidyl-tRNA synthetase	5.71E-03	+	1.29	-	1.28	-	1.30	+
NMB0856	Not in cogs genes	Hypothetical protein	3.11E-02	+	1.22	-	1.34	-	1.36	-
NMB0857	Not in cogs genes	Hypothetical protein	4.11E-03	-	1.12	+	1.25	+	1.26	-
NMB0858	Not in cogs genes	Hypothetical protein	6.98E-03	-	1.25	-	1.77	-	1.14	-
NMB0859	Not in cogs genes	Hypothetical protein	1.06E-02	+	1.26	+	1.37	+	1.08	-
NMB0861	Not in cogs genes	Hypothetical protein	1.18E-04	-	2.35	-	1.93	-	1.54	-
NMB0862	Not in cogs genes	Hypothetical protein	6.97E-03	+	1.14	+	1.11	-	1.14	-
NMB0863	Not in cogs genes	Hypothetical protein	4.87E-02	-	1.23	-	1.04	-	1.17	-
NMB0864	Not in cogs genes	Hypothetical protein	5.17E-03	-	1.92	-	1.61	-	1.12	-
NMB0865	Not in cogs genes	Hypothetical protein	1.91E-03	-	1.75	-	1.87	+	1.06	-
NMB0866	Not in cogs genes	Hypothetical protein	1.46E-04	-	2.22	-	1.70	+	1.41	+

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0867	Translation	Yabo/yeec/sfih family protein	2.51E-03	+ 1.56	+ 1.20	+ 1.04	- 1.17	- 1.29	- 1.50	- 1.82
NMB0868	General function prediction only genes	Conserved hypothetical protein	4.00E-03	- 1.45	- 1.46	- 1.34	- 1.26	- 1.00	+ 1.08	+ 1.15
NMB0869	Amino acid transport and metabolism genes	Hypothetical protein	1.30E-02	+ 1.05	- 1.16	- 1.15	+ 1.21	- 1.21	- 1.20	+ 1.16
NMB0870	Coenzyme transport and metabolism genes	3-methyl-2-oxobutanoate hydroxymethyltransferase	2.77E-03	+ 1.50	+ 1.05	+ 1.11	- 3.13	- 1.43	- 1.36	- 4.70
NMB0871	Not in cogs genes	Pantoate-β-alanine ligase	8.50E-04	- 1.09	- 1.68	- 1.68	- 1.13	- 1.55	- 1.55	- 1.04
NMB0873	Not in cogs genes	Putative outer membrane lipoprotein l0lb	4.27E-02	+ 1.46	+ 1.16	+ 1.28	+ 1.91	- 1.26	- 1.14	+ 1.31
NMB0874	Lipid transport and metabolism genes	Conserved hypothetical protein	2.57E-02	- 1.98	- 1.60	- 1.54	- 1.38	+ 1.24	+ 1.29	+ 1.43
NMB0875	Nucleotide transport and metabolism genes	Ribose-phosphate pyrophosphokinase	2.60E-03	- 1.08	+ 2.63	+ 2.36	+ 1.47	+ 2.84	+ 2.56	+ 1.59
NMB0876	Amino acid transport and metabolism genes	50S ribosomal protein l25	3.40E-04	- 1.62	+ 3.30	+ 2.09	+ 2.01	+ 5.36	+ 3.39	+ 3.26
NMB0878	Translation	Threonine dehydratase	1.05E-02	+ 1.49	+ 1.05	+ 1.15	+ 1.56	- 1.42	- 1.30	+ 1.04
NMB0879	Inorganic ion transport and metabolism genes	Sulfate ABC transporter, ATP-binding protein	2.14E-02	+ 1.18	+ 1.39	+ 1.40	+ 1.56	+ 1.18	+ 1.19	+ 1.32
NMB0880	Inorganic ion transport and metabolism genes	Sulfate ABC transporter, permease protein	7.14E-03	- 1.63	- 1.01	+ 1.05	+ 1.19	+ 1.61	+ 1.71	+ 1.95
NMB0881	Posttranslational modification, protein turnover, chaperones genes	Sulfate ABC transporter, permease protein	6.30E-03	- 1.09	+ 1.57	+ 1.76	+ 1.74	+ 1.71	+ 1.92	+ 1.90
NMB0883	Not in cogs genes	Conserved hypothetical protein	6.28E-03	+ 1.27	+ 1.11	+ 1.29	- 1.71	- 1.14	+ 1.02	- 2.16
NMB0885	Replication recombination and repair	Replicative dna helicase	4.58E-03	- 1.13	- 1.07	+ 1.07	+ 1.27	+ 1.06	+ 1.21	+ 1.44
NMB0886	Intracellular trafficking and secretion genes	Finbrial protein limit	1.54E-03	- 1.92	- 2.13	- 2.07	- 2.94	- 1.11	- 1.08	- 1.53
NMB0887	Cell motility genes	Putative type IV pilus assembly protein pilv	3.37E-02	- 1.72	- 1.52	- 1.82	- 1.46	+ 1.13	- 1.06	+ 1.17
NMB0891	Not in cogs genes	Hypothetical protein	1.16E-02	+ 2.13	+ 5.49	+ 4.33	+ 4.77	+ 2.58	+ 2.03	+ 2.24
NMB0893	Not in cogs genes	Deoxyuridine 5'-triphosphate nucleotidohydrolase	3.78E-02	+ 1.71	- 1.24	- 1.15	+ 1.39	- 2.12	- 1.96	- 1.23
NMB0894	Amino acid transport and metabolism genes	Aminotransferase, class I	2.13E-02	+ 1.33	- 1.33	- 1.40	- 1.02	- 1.78	- 1.86	- 1.36
NMB0896	Not in cogs genes	Putative Phage integrase	1.37E-05	+ 1.04	- 1.39	- 1.48	+ 1.42	- 1.44	- 1.54	+ 1.37
NMB0897	Function unknown genes	Hypothetical protein	4.02E-03	+ 1.06	- 1.07	+ 1.09	+ 1.93	- 1.13	+ 1.04	+ 1.82
NMB0898	Not in cogs genes	Hypothetical protein	3.47E-03	+ 1.23	- 1.43	- 1.23	+ 1.60	- 1.76	- 1.51	+ 1.30
NMB0899	Not in cogs genes	Hypothetical protein	1.52E-02	- 1.20	+ 1.53	+ 1.20	+ 1.19	+ 1.84	+ 1.44	+ 1.43
NMB0900	Not in cogs genes	Hypothetical protein	3.31E-03	- 1.40	+ 1.36	+ 1.49	- 1.37	+ 1.91	+ 2.10	+ 1.02
NMB0904	Not in cogs genes	Hypothetical protein	2.66E-02	+ 1.73	+ 1.47	+ 1.37	- 1.44	- 1.18	- 1.27	- 2.49
NMB0909	Not in cogs genes	Hypothetical protein	4.24E-02	- 1.41	- 1.62	- 1.57	- 1.79	- 1.15	- 1.11	- 1.27
NMB0910	Transcription	Transcriptional regulator	1.83E-03	- 1.13	- 1.65	- 1.86	- 1.08	- 1.47	- 1.65	+ 1.05
NMB0912	Not in cogs genes	Hypothetical protein	1.31E-04	+ 1.91	- 1.23	- 1.50	- 1.54	- 2.35	- 2.85	- 2.94
NMB0913	Signal transduction mechanisms genes	Pemk protein	5.61E-04	- 1.12	- 1.47	- 1.35	+ 1.24	- 1.31	- 1.21	+ 1.39
NMB0914	Signal transduction mechanisms genes	Pemk protein	6.62E-04	+ 1.07	- 1.33	- 1.26	+ 2.17	- 1.42	- 1.34	+ 2.03
NMB0915	Not in cogs genes	Hypothetical protein	2.66E-02	+ 1.01	+ 1.54	+ 1.60	- 1.22	+ 1.52	+ 1.58	- 1.24
NMB0917	General function prediction only genes	Death-on-curing protein	1.94E-03	+ 1.33	- 1.11	- 1.12	+ 2.31	- 1.47	- 1.48	+ 1.74
NMB0918	Not in cogs genes	Hypothetical protein	1.20E-03	+ 1.30	- 1.31	- 1.56	+ 1.88	- 1.70	- 2.03	+ 1.45

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h-DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0919	Replication recombination and repair	Putative IS1106 transposase	1.39E-02	- 1.83	+ 2.02	+ 1.60	+ 1.56	+ 3.70	+ 2.92	+ 2.84
NMB0921	Not in cogs genes	Hypothetical protein	3.28E-02	- 1.14	- 1.27	- 1.45	+ 1.00	- 1.12	- 1.28	+ 1.14
NMB0922	Not in cogs genes	Alpha-2,3-sialyltransferase	1.20E-02	+ 1.21	+ 1.09	- 1.01	+ 1.27	- 1.11	- 1.22	+ 1.05
NMB0923	Not in cogs genes	Cytochrome c	2.32E-02	- 1.19	+ 1.16	+ 1.11	+ 1.37	+ 1.38	+ 1.31	+ 1.63
NMB0924	General function prediction only genes	Oxidoreductase	1.17E-02	- 1.17	- 1.60	- 1.50	+ 1.48	- 1.37	- 1.28	+ 1.73
NMB0925	Lipid transport and metabolism genes	Acyl coa thioester hydrolase family protein	3.61E-02	+ 1.23	- 1.19	- 1.22	+ 1.23	- 1.47	- 1.49	+ 1.00
NMB0930	Nucleotide transport and metabolism genes	Xanthine/uracil permease family protein	7.91E-04	- 1.24	- 1.75	- 1.78	- 1.67	- 1.41	- 1.44	- 1.35
NMB0937	Translation	Elongation factor p (ef-p)	1.96E-03	- 1.13	+ 2.63	+ 1.80	+ 2.00	+ 2.98	+ 2.03	+ 2.27
NMB0939	General function prediction only genes	Conserved hypothetical protein	9.45E-03	+ 1.16	+ 1.13	- 1.04	+ 1.66	- 1.03	- 1.21	+ 1.43
NMB0940	Amino acid transport and metabolism genes	Homoserine O-acetyltransferas	4.71E-03	+ 1.27	- 1.21	- 1.30	+ 1.24	- 1.55	- 1.66	- 1.03
NMB0941	Translation	50s ribosomal protein l36	2.30E-03	+ 1.56	+ 4.20	+ 1.77	+ 1.38	+ 2.69	+ 1.14	- 1.13
NMB0942	Translation	Putative 50s ribosomal protein l31	1.51E-03	+ 2.06	+ 5.13	+ 1.99	+ 1.63	+ 2.49	- 1.04	- 1.26
NMB0943	Amino acid transport and metabolism genes	5,10-methylenetetrahydrofolate reductase	1.81E-02	+ 1.25	+ 1.27	+ 1.08	+ 1.57	+ 1.01	- 1.16	+ 1.25
NMB0944	Amino acid transport and metabolism genes	5-methyltetrahydropteroyltrimethyl- homocysteine methyltransferase	1.20E-02	- 1.41	+ 2.26	+ 1.55	+ 1.47	+ 3.19	+ 2.18	+ 2.07
NMB0945	Not in cogs genes	Hypothetical protein	2.61E-02	- 2.10	+ 1.26	- 1.21	- 1.63	+ 2.66	+ 1.74	+ 1.29
NMB0946	Posttranslational modification, protein turnover, chaperones genes	Peroxisome 2 family protein/glutaredoxin	1.75E-05	- 3.58	+ 2.54	+ 1.63	+ 4.06	+ 9.10	+ 5.83	+ 14.53
NMB0950	Energy production and conversion genes	Succinate dehydrogenase,	8.19E-03	- 1.01	- 1.23	- 1.36	+ 1.34	- 1.21	- 1.35	+ 1.36
NMB0952	Function unknown genes	Conserved hypothetical protein	4.68E-02	+ 1.44	+ 1.43	+ 1.54	+ 1.52	- 1.01	+ 1.07	+ 1.06
NMB0953	Not in cogs genes	Hypothetical protein	1.02E-02	+ 1.48	+ 3.60	+ 1.04	+ 6.01	+ 2.43	- 1.42	+ 4.05
NMB0956	Energy production and conversion genes	2-oxoglutarate dehydrogenase	2.56E-02	+ 1.03	+ 1.40	+ 1.28	+ 1.60	+ 1.36	+ 1.25	+ 1.56
NMB0957	Energy production and conversion genes	2-oxoglutarate dehydrogenase	7.89E-04	- 1.22	- 1.62	- 1.89	+ 1.16	- 1.33	- 1.54	+ 1.42
NMB0963	Lipid transport and metabolism genes	Phosphatidylserine decarboxylase precursor- related protein	6.09E-04	- 1.05	- 1.22	- 1.47	+ 1.67	- 1.16	- 1.39	+ 1.76
NMB0964	Inorganic ion transport and metabolism genes	TonB-dependent receptor	1.95E-03	+ 1.60	+ 1.56	+ 1.67	+ 1.14	- 1.02	+ 1.04	- 1.41
NMB0966	Coenzyme transport and metabolism genes	Para-aminobenzoate synthase	4.40E-02	- 1.12	- 1.04	- 1.03	+ 1.32	+ 1.08	+ 1.09	+ 1.48
NMB0967	Amino acid transport and metabolism genes	Anthranilate phosphoribosyltransferase	6.28E-04	- 1.22	- 1.17	- 1.55	- 1.01	+ 1.04	- 1.28	+ 1.20
NMB0968	Not in cogs genes	Hypothetical protein conserved hypothetical protein	3.13E-03	+ 1.65	+ 1.06	- 1.01	- 1.23	- 1.55	- 1.66	- 2.03
NMB0969	Replication recombination and repair	Conserved hypothetical protein	3.47E-03	+ 2.07	+ 1.24	+ 1.26	- 1.81	- 1.67	- 1.64	- 3.75
NMB0978	Not in cogs genes	NAD(P) transhydrogenase, beta subunit	7.96E-04	+ 1.55	+ 1.71	+ 1.29	+ 1.23	+ 1.10	- 1.20	- 1.26
NMB0981	Amino acid transport and metabolism genes	Phosphoserine phosphatase	4.84E-02	- 1.04	- 1.06	- 1.42	+ 1.05	- 1.02	- 1.37	+ 1.09
NMB0982	Inorganic ion transport and metabolism genes	Chloride channel protein-related protein	6.62E-03	- 1.69	- 1.96	- 2.07	- 2.73	- 1.16	- 1.22	- 1.62
NMB0983	Not in cogs genes	Phosphoribosylaminoimidazolecarboxamide formyltransferase	7.71E-03	- 1.69	+ 1.04	+ 1.26	+ 1.26	+ 1.75	+ 2.14	+ 2.13
NMB0985	Not in cogs genes	E16-related protein	1.31E-03	+ 1.12	- 1.40	- 1.48	- 1.04	- 1.56	- 1.66	- 1.17

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB0986	Not in cogs genes	Hypothetical protein conserved hypothetical protein	4.16E-03	+ 1.57	- 1.19	- 1.11	+ 1.16	- 1.87	- 1.74	- 1.35
NMB0994	Lipid transport and metabolism genes	Acyl-coa dehydrogenase family protein	1.38E-03	- 1.42	+ 1.57	+ 1.80	+ 1.47	+ 2.24	+ 2.56	+ 2.10
NMB0995	Function unknown genes	Macrophage infectivity potentiator-related protein	1.08E-04	- 1.30	+ 3.58	+ 4.61	+ 2.20	+ 4.65	+ 5.99	+ 2.86
NMB0998	Energy production and conversion genes	Putative oxidoreductase oxidoreductase	3.91E-03	+ 1.29	- 1.04	+ 1.01	- 1.07	- 1.34	- 1.27	- 1.37
NMB1001	Not in cogs genes	Phage integrase putative phage integrase	5.29E-05	- 1.16	- 2.16	- 2.10	- 4.97	- 1.86	- 1.81	- 4.28
NMB1007	Transcription	Transcriptional regulator	3.57E-02	- 1.42	+ 1.05	- 1.11	+ 1.47	+ 1.49	+ 1.28	+ 2.08
NMB1012	General function prediction only genes	Hypothetical protein	2.83E-02	+ 1.55	+ 2.26	+ 2.14	+ 1.83	+ 1.46	+ 1.38	+ 1.18
NMB1017	Inorganic ion transport and metabolism genes	Sulfate ABC transporter, periplasmic sulfate-binding protein	4.36E-02	- 1.75	- 1.10	+ 1.04	+ 1.42	+ 1.59	+ 1.82	+ 2.48
NMB1020	Not in cogs genes	Hypothetical protein conserved hypothetical protein	1.31E-02	+ 1.04	+ 1.38	- 1.17	- 1.30	+ 1.33	- 1.22	- 1.35
NMB1023	Cell cycle control, mitosis and meiosis genes	Conserved hypothetical protein	6.46E-04	+ 1.31	- 1.27	- 1.23	- 1.28	- 1.67	- 1.61	- 1.68
NMB1024	General function prediction only genes	Conserved hypothetical protein	1.95E-02	- 1.32	+ 1.03	+ 1.14	+ 1.76	+ 1.36	+ 1.51	+ 2.32
NMB1027	Putative transmembrane protein-dnaj protein, truncation	Dnaj protein, truncation	4.89E-02	- 1.16	- 1.59	- 1.12	- 1.18	- 1.36	+ 1.04	- 1.01
NMB1028	Not in cogs genes	Conserved hypothetical protein integral membrane protein	1.24E-02	- 1.15	- 1.49	- 1.45	- 1.24	- 1.30	- 1.26	- 1.08
NMB1029	Amino acid transport and metabolism genes	Aspartate ammonia-lyase putative aspartate ammonia-lyase	4.29E-02	+ 1.16	- 1.14	- 1.12	- 1.02	- 1.33	- 1.31	- 1.19
NMB1030	Not in cogs genes	Conserved hypothetical protein periplasmic protein	2.85E-03	- 1.57	- 1.85	- 1.63	- 1.26	- 1.18	- 1.04	+ 1.24
NMB1032	Not in cogs genes	Type II restriction enzyme nlaiv	2.19E-02	- 1.38	- 1.02	- 1.11	- 2.78	+ 1.36	+ 1.24	- 2.02
NMB1033	Replication recombination and repair	Modification methylase nlaiv	1.68E-02	- 1.16	+ 1.53	+ 1.02	- 2.01	+ 1.77	+ 1.18	- 1.73
NMB1035	Not in cogs genes	Hypothetical proteinputative lipoprotein	1.51E-03	- 1.40	+ 1.06	+ 1.37	- 2.07	+ 1.48	+ 1.91	- 1.49
NMB1036	Not in cogs genes	3-isopropylmalate dehydratase, large subunit	6.36E-03	- 1.37	- 1.04	- 1.06	+ 1.01	+ 1.32	+ 1.29	+ 1.38
NMB1037	Not in cogs genes	Glutamate--cysteine ligase	2.35E-02	+ 1.01	+ 1.09	- 1.27	+ 1.75	+ 1.08	- 1.28	+ 1.73
NMB1038	Replication recombination and repair	Dna repair protein radc	2.39E-02	+ 1.59	+ 1.24	+ 1.18	+ 1.86	- 1.29	- 1.36	+ 1.17
NMB1039	Function unknown genes	Conserved hypothetical protein	1.31E-02	+ 1.15	+ 1.17	+ 1.06	+ 1.66	+ 1.01	- 1.09	+ 1.44
NMB1040	Not in cogs genes	Hypothetical protein periplasmic protein	3.77E-04	+ 1.42	+ 1.04	+ 1.27	- 1.33	- 1.37	- 1.12	- 1.88
NMB1044	Energy production and conversion genes	Ferredoxin--NADP reductase	5.48E-03	- 1.63	- 1.04	- 1.16	- 1.17	+ 1.58	+ 1.40	+ 1.40
NMB1046	Amino acid transport and metabolism genes	Threonine synthase	1.84E-03	- 1.84	- 1.31	- 1.22	- 1.21	+ 1.41	+ 1.51	+ 1.52
NMB1048	Not in cogs genes	Hypothetical protein integral membrane protein	3.77E-04	- 1.01	- 1.17	- 1.19	+ 1.58	- 1.17	- 1.18	+ 1.59
NMB1049	Transcription	Putative transcriptional regulator	1.49E-02	- 1.26	- 1.57	- 1.36	- 1.06	- 1.25	- 1.08	+ 1.19
NMB1051	General function prediction only genes	ABC transporter, ATP-binding protein	2.44E-02	+ 1.40	+ 1.01	- 1.03	+ 1.38	- 1.40	- 1.45	- 1.01
NMB1054	Not in cogs genes	IS1106 transposase	3.88E-02	- 1.43	+ 1.76	+ 1.45	- 1.16	+ 2.52	+ 2.08	+ 1.24
NMB1055	Amino acid transport and metabolism genes	Serine hydroxymethyltransferase	1.08E-03	- 1.29	+ 2.43	+ 1.93	+ 1.67	+ 3.12	+ 2.48	+ 2.15
NMB1056	Not in cogs genes	Hypothetical protein conserved hypothetical protein	7.52E-05	- 2.57	+ 1.15	+ 1.04	- 1.30	+ 2.96	+ 2.69	+ 1.98

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h-DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1057	Gamma-glutamyltranspeptidase	Gamma-glutamyltranspeptidase	2.49E-02	- 2.06	- 1.37	- 1.19	- 2.12	+ 1.51	+ 1.73	- 1.03
NMB1058	Not in cogs genes		2.14E-02	- 1.40	- 1.44	- 1.14	- 2.63	- 1.03	+ 1.23	- 1.88
NMB1060	Carbohydrate transport and metabolism genes	Fructose-1,6-bisphosphatase	1.29E-02	- 1.22	- 1.23	- 1.32	- 1.02	- 1.02	- 1.08	+ 1.19
NMB1062	Function unknown genes	Conserved hypothetical protein	3.31E-03	+ 1.04	- 2.63	- 1.74	- 3.00	- 2.74	- 1.81	- 3.13
NMB1063	Coenzyme transport and metabolism genes	Dihydropyrimidin aldolase	5.29E-05	- 1.18	- 1.53	- 1.61	+ 1.54	- 1.30	- 1.37	+ 1.81
NMB1064	General function prediction only genes replication recombination and repair	Conserved hypothetical protein	3.16E-03	+ 1.14	- 2.89	- 2.84	- 1.62	- 3.31	- 3.25	- 1.85
NMB1067	Cell cycle control, mitosis and meiosis genes	Cell division protein ftsk	2.22E-04	- 1.20	- 1.34	- 1.49	+ 1.19	- 1.12	- 1.24	+ 1.43
NMB1068	Amino acid transport and metabolism genes	Gamma-glutamyl phosphate reductase	1.03E-02	+ 1.56	+ 1.18	+ 1.27	+ 1.26	- 1.32	- 1.22	- 1.24
NMB1070	Amino acid transport and metabolism genes	2-isopropylmalate synthase	2.53E-03	- 1.61	- 2.15	- 2.18	- 1.84	- 1.34	- 1.36	- 1.14
NMB1072	Not in cogs genes	Prolipoprotein diacylglycerol	6.10E-06	+ 1.65	- 1.16	- 1.20	+ 1.09	- 1.91	- 1.97	- 1.51
NMB1073	Amino acid transport and metabolism genes	Conserved hypothetical protein	2.71E-02	- 1.25	- 1.42	- 1.43	+ 1.20	- 1.14	- 1.15	+ 1.50
NMB1074	Amino acid transport and metabolism genes	Acetylglutamate kinase	4.57E-03	+ 1.63	+ 1.08	+ 1.19	+ 2.22	- 1.50	- 1.37	+ 1.36
NMB1075	Amino acid transport and metabolism genes	Conserved hypothetical protein	1.62E-03	- 1.20	- 1.13	- 1.28	- 1.01	+ 1.06	- 1.07	+ 1.19
NMB1076	Replication recombination and repair	Dnaa-related protein	6.75E-04	- 2.14	- 1.50	- 1.66	- 1.07	+ 1.43	+ 1.29	+ 2.01
NMB1077	ABC transporter domain protein-ATP-binding protein, truncation	ABC transporter, ATP-binding protein, truncation	3.26E-02	- 1.05	+ 1.34	+ 1.39	- 1.27	+ 1.40	+ 1.46	- 1.21
NMB1078	Not in cogs genes	Transcriptional regulator, umud/lexa family	2.20E-03	+ 1.26	+ 1.44	+ 1.73	+ 1.31	+ 1.14	+ 1.37	+ 1.04
NMB1088	Not in cogs genes	Conserved hypothetical protein	3.27E-03	- 1.47	- 1.79	- 1.58	- 1.22	- 1.22	- 1.08	+ 1.20
NMB1091	Not in cogs genes	Hypothetical protein conserved hypothetical protein	5.86E-04	+ 2.29	+ 3.41	+ 3.14	+ 2.61	+ 1.49	+ 1.37	+ 1.14
NMB1092	Not in cogs genes	Hypothetical protein DNA-binding protein	4.04E-03	+ 1.57	+ 2.42	+ 2.21	+ 2.19	+ 1.54	+ 1.41	+ 1.40
NMB1094	Not in cogs genes	Hypothetical protein conserved hypothetical protein	5.25E-03	+ 2.00	+ 3.04	+ 2.83	+ 2.26	+ 1.52	+ 1.41	+ 1.13
NMB1096	Function unknown genes	Conserved hypothetical protein	6.56E-03	+ 2.13	+ 2.26	+ 2.11	+ 1.63	+ 1.06	- 1.01	- 1.30
NMB1098	General function prediction only genes	Putative I protein	4.93E-03	+ 1.81	+ 2.43	+ 2.31	+ 1.79	+ 1.34	+ 1.27	- 1.01
NMB1100	Not in cogs genes	Hypothetical protein	2.76E-02	- 1.96	- 2.24	- 1.67	- 2.11	- 1.14	+ 1.17	- 1.08
NMB1106	Not in cogs genes	Hypothetical protein	3.88E-03	+ 1.31	+ 1.49	+ 1.09	- 1.05	+ 1.13	- 1.20	- 1.37
NMB1107	Not in cogs genes	Hypothetical protein	2.96E-03	- 1.78	- 1.43	- 1.32	- 2.22	+ 1.24	+ 1.35	- 1.25
NMB1108	Not in cogs genes	Hypothetical protein	2.51E-03	+ 2.35	+ 3.08	+ 2.87	+ 2.56	+ 1.31	+ 1.22	+ 1.09
NMB1109	General function prediction only genes	Putative phage virion protein	3.16E-02	+ 1.45	+ 1.78	+ 1.70	+ 1.35	+ 1.22	+ 1.17	- 1.08
NMB1111	Function unknown genes	Putative baseplate assembly protein V	5.25E-03	+ 1.82	+ 2.11	+ 1.93	+ 2.25	+ 1.16	+ 1.06	+ 1.24
NMB1114	Function unknown genes	Conserved hypothetical protein	5.82E-04	- 1.02	+ 1.56	+ 2.08	- 2.68	+ 1.59	+ 2.11	- 2.63
NMB1116	Not in cogs genes	Hypothetical protein	2.12E-03	+ 1.97	+ 7.62	+ 6.97	+ 9.60	+ 3.86	+ 3.53	+ 4.86
NMB1118	Function unknown genes	Conserved hypothetical protein	3.83E-02	- 1.07	+ 1.29	+ 1.61	+ 1.61	+ 1.38	+ 1.72	+ 1.72
NMB1120	Not in cogs genes	Hypothetical protein conserved hypothetical protein	2.44E-02	+ 1.56	+ 2.20	+ 2.05	+ 1.46	+ 1.41	+ 1.31	- 1.07
NMB1124	Function unknown genes	Hypothetical protein genome-derived Neisseria antigen 1162	2.11E-02	- 1.11	- 1.31	- 1.44	- 1.08	- 1.18	- 1.30	+ 1.02

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1125	Function unknown genes	Hypothetical protein putative periplasmic protein	5.66E-04	- 1.14	- 1.61	- 1.54	+ 1.47	- 1.41	- 1.35	+ 1.68
NMB1126	Cell wall/membrane biogenesis genes	Hypothetical protein periplasmic protein	1.31E-04	- 1.29	- 1.49	- 1.59	+ 1.31	- 1.23	- 1.23	+ 1.70
NMB1128	Function unknown genes General function prediction only genes	Conserved hypothetical protein putative oxidoreductase	7.87E-03	+ 1.10	+ 1.57	+ 1.14	- 1.25	+ 1.42	+ 1.03	- 1.38
NMB1131	Posttranslational modification, protein turnover, chaperones genes	Chaperone protein HscA	3.47E-03	+ 1.19	+ 1.69	+ 1.90	+ 1.55	+ 1.42	+ 1.60	+ 1.31
NMB1132	Not in COGs genes	Conserved hypothetical protein	2.23E-03	+ 1.63	+ 1.91	+ 1.86	+ 1.63	+ 1.17	+ 1.14	+ 1.00
NMB1133	Function unknown genes General function prediction only genes	Conserved hypothetical protein / ankyrin-related protein	1.48E-02	+ 1.03	+ 1.04	- 1.03	+ 1.70	+ 1.00	- 1.07	+ 1.65
NMB1134	Energy production and conversion genes	Ferredoxin, 2Fe-2S type	1.48E-02	- 1.19	+ 1.08	+ 1.11	+ 1.31	+ 1.28	+ 1.32	+ 1.56
NMB1135	Not in COGs genes	Conserved hypothetical protein	3.92E-03	+ 2.37	+ 7.51	+ 6.56	+ 4.89	+ 3.17	+ 2.77	+ 2.06
NMB1136	Not in COGs genes	Hypothetical protein	2.05E-04	- 1.65	+ 1.05	+ 1.25	- 1.28	+ 1.73	+ 2.06	+ 1.29
NMB1137	Not in COGs genes	Conserved hypothetical protein	2.37E-02	- 1.29	- 1.06	- 1.02	+ 1.40	+ 1.21	+ 1.26	+ 1.80
NMB1138	Translation	Conserved hypothetical protein	1.68E-06	- 2.15	- 1.22	- 1.03	- 1.20	+ 1.76	+ 2.08	+ 1.78
NMB1139	Lipid transport and metabolism genes	Acetyl-CoA carboxylase, carboxyl transferase alpha subunit	9.42E-03	- 1.84	- 1.04	- 1.13	- 1.23	+ 1.77	+ 1.62	+ 1.49
NMB1140	tRNA(lle)-lysine synthetase	tRNA(lle)-lysine synthetase hypothetical protein	6.68E-03	+ 1.05	- 1.18	- 1.22	- 1.28	- 1.23	- 1.27	- 1.34
NMB1145	Cell wall/membrane biogenesis genes	UDP-N-acetylmurametyl-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	2.98E-02	- 1.30	- 1.05	+ 1.39	- 1.42	+ 1.24	+ 1.80	- 1.09
NMB1146	Coenzyme transport and metabolism genes	Biotin synthetase	9.39E-03	+ 1.09	- 1.17	+ 1.12	- 1.49	- 1.28	+ 1.02	- 1.63
NMB1148	Not in COGs genes	Hypothetical protein	2.68E-02	+ 1.04	+ 1.82	+ 1.30	+ 1.41	+ 1.76	+ 1.26	+ 1.36
NMB1151	Inorganic ion transport and metabolism genes	Sulfite reductase hemoprotein	2.88E-03	+ 1.17	+ 1.43	+ 1.44	- 1.11	+ 1.22	+ 1.23	- 1.29
NMB1152	Inorganic ion transport and metabolism genes	Sulfite reductase (NADPH) flavoprotein	1.95E-03	+ 1.51	+ 1.79	+ 1.83	- 1.04	+ 1.18	+ 1.21	- 1.58
NMB1153	Inorganic ion transport and metabolism genes	Sulfate adenylyltransferase, subunit 1	2.51E-04	+ 1.44	+ 2.63	+ 2.96	+ 1.47	+ 1.82	+ 2.05	+ 1.02
NMB1154	Coenzyme transport and metabolism genes	Sulfate adenylyltransferase, subunit 2	1.65E-03	+ 1.08	+ 1.69	+ 2.23	+ 1.33	+ 1.58	+ 2.07	+ 1.24
NMB1155	Amino acid transport and metabolism genes	Phosphoadenosine phosphosulfate reductase	3.68E-03	+ 1.00	+ 1.23	+ 1.80	- 1.01	+ 1.23	+ 1.79	- 1.01
NMB1156	Coenzyme transport and metabolism genes	Siroheme synthase sirohaem synthase	7.96E-04	+ 1.27	+ 2.41	+ 3.47	+ 1.64	+ 1.89	+ 2.73	+ 1.29
NMB1157	Not in COGs genes	Hypothetical protein	2.44E-02	+ 2.16	+ 3.54	+ 3.48	+ 3.24	+ 1.64	+ 1.61	+ 1.50
NMB1159	Carbohydrate transport and metabolism genes	Conserved hypothetical protein	1.76E-02	- 1.37	- 1.44	- 1.28	- 1.29	- 1.06	+ 1.07	+ 1.06
NMB1199	Signal transduction mechanisms genes	GTP-binding protein TypA	2.00E-02	- 1.25	+ 1.26	+ 1.49	+ 1.19	+ 1.58	+ 1.86	+ 1.49
NMB1201	General function prediction only genes	IMP dehydrogenase	1.27E-02	- 1.42	+ 1.13	+ 1.07	- 1.23	+ 1.60	+ 1.52	+ 1.15
NMB1204	Nucleotide transport and metabolism genes	Transcriptional regulator	3.87E-02	- 1.19	- 1.63	- 1.58	- 1.06	- 1.37	- 1.32	+ 1.12
NMB1205	Not in COGs genes	Hypothetical protein	2.88E-03	- 2.88	- 3.23	- 3.14	- 4.96	- 1.12	- 1.09	- 1.72
NMB1206	Inorganic ion transport and metabolism genes	Bacterioferritin B	1.00E-02	+ 1.02	- 1.01	- 1.38	+ 1.37	- 1.03	- 1.41	+ 1.34
NMB1207	Inorganic ion transport and metabolism genes	Bacterioferritin A	7.96E-04	- 1.51	- 1.65	- 1.79	+ 1.21	- 1.09	- 1.18	+ 1.82
NMB1210	Posttranslational modification, protein turnover, chaperones genes	Putative toxin-activating protein	7.46E-03	+ 1.27	- 1.35	- 1.29	- 1.20	- 1.72	- 1.64	- 1.52

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h-DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1211	Not in COGs genes	Hypothetical protein	6.20E-03	- 1.82	- 2.01	- 2.02	- 3.64	- 1.10	- 1.11	- 2.00
NMB1212	Not in COGs genes	Hypothetical protein	4.49E-02	- 1.38	- 1.64	- 1.56	- 1.89	- 1.19	- 1.14	- 1.37
NMB1213	Not in COGs genes	Putative lipoprotein	2.08E-02	- 1.06	- 1.26	- 1.40	- 1.92	- 1.19	- 1.33	- 1.82
NMB1215	Not in COGs genes	Hypothetical protein	7.01E-03	+ 1.86	+ 2.47	+ 2.32	+ 1.11	+ 1.33	+ 1.25	- 1.68
NMB1216	Coenzyme transport and metabolism genes	Lipoidic acid synthetase	2.18E-02	+ 1.06	+ 1.62	+ 1.30	- 1.15	+ 1.53	+ 1.23	- 1.22
NMB1217	Coenzyme transport and metabolism genes	Lipoate-protein ligase B	1.16E-02	- 1.25	+ 1.11	- 1.01	+ 1.17	+ 1.39	+ 1.24	+ 1.47
NMB1218	Not in COGs genes	Conserved hypothetical protein	5.64E-07	- 1.92	- 1.08	- 1.17	- 1.05	+ 1.77	+ 1.63	+ 1.82
NMB1219	General function prediction only genes	Putative transporter	3.77E-04	+ 1.12	- 1.62	- 1.44	+ 1.02	- 1.81	- 1.61	- 1.10
NMB1220	Posttranslational modification, protein turnover, chaperones genes	Stomatin/Mec-2 family protein	4.00E-03	- 1.15	- 1.28	- 1.24	+ 1.25	- 1.11	- 1.08	+ 1.44
NMB1221	Posttranslational modification, protein turnover, chaperones genes	Hypothetical protein membrane protein	6.07E-04	- 2.19	- 1.57	- 1.66	- 1.10	+ 1.40	+ 1.32	+ 2.00
NMB1224	Not in COGs genes	Hypothetical protein	2.18E-02	+ 1.27	- 1.02	- 1.11	+ 1.29	- 1.30	- 1.41	+ 1.01
NMB1225	Not in COGs genes	Hypothetical protein	2.54E-03	- 1.52	- 1.57	- 1.43	- 1.13	- 1.03	+ 1.06	+ 1.35
NMB1226	General function prediction only genes	ABC transporter, ATP-binding protein	3.49E-02	+ 1.11	- 1.11	- 1.20	+ 1.22	- 1.23	- 1.33	+ 1.10
NMB1228	Amino acid transport and metabolism genes	Homoserine dehydrogenase	1.65E-02	- 1.12	- 1.27	- 1.18	+ 1.31	- 1.14	- 1.05	+ 1.46
NMB1229	Not in COGs genes	Hypothetical protein membrane protein	6.28E-03	- 1.26	- 1.16	+ 1.01	+ 1.02	+ 1.09	+ 1.27	+ 1.28
NMB1230	Not in COGs genes	DNA-binding protein HU-beta	1.37E-05	- 3.74	+ 2.48	+ 2.31	+ 3.08	+ 9.28	+ 8.66	+ 11.55
NMB1231	Posttranslational modification, protein turnover, chaperones genes	ATP-dependent protease La	4.00E-03	- 1.07	+ 1.37	+ 1.20	+ 1.98	+ 1.47	+ 1.28	+ 2.12
NMB1232	Translation	Conserved hypothetical protein	1.04E-03	+ 1.05	+ 1.36	+ 1.83	+ 1.15	+ 1.29	+ 1.74	+ 1.09
NMB1233	Replication recombination and repair	Exodeoxyribonuclease V, alpha subunit	8.09E-03	- 1.28	- 1.28	+ 1.15	- 1.45	- 1.00	+ 1.47	- 1.14
NMB1235	Cell wall/membrane biogenesis genes	Conserved hypothetical protein	2.64E-02	- 1.08	- 1.44	- 1.27	- 1.03	- 1.33	- 1.17	+ 1.05
NMB1237	Replication recombination and repair	Recombination protein RecR	2.88E-03	+ 1.02	- 1.42	- 1.45	- 1.19	- 1.45	- 1.49	- 1.22
NMB1238	Not in COGs genes	Peptidyl-prolyl cis-trans isomerase-related protein	2.77E-02	+ 1.18	+ 1.16	- 1.05	+ 1.25	- 1.01	- 1.24	+ 1.06
NMB1239	Inorganic ion transport and metabolism genes	Conserved hypothetical protein	2.53E-03	- 1.33	- 1.16	- 1.19	+ 1.18	+ 1.14	+ 1.11	+ 1.56
NMB1241	translation	tRNA nucleotidyltransferase	4.27E-02	- 1.16	+ 1.52	+ 1.70	+ 1.09	+ 1.77	+ 1.97	+ 1.26
NMB1244	Carbohydrate transport and metabolism genes	Ribulose-phosphate 3-epimerase	1.26E-02	+ 1.02	- 1.29	- 1.14	+ 1.26	- 1.33	- 1.16	+ 1.23
NMB1245	Not in COGs genes	Hypothetical protein periplasmic protein	3.49E-06	- 3.94	- 1.24	+ 1.03	- 1.14	+ 3.19	+ 4.07	+ 3.44
NMB1247	Coenzyme transport and metabolism genes	Riboflavin synthase, alpha subunit	5.64E-07	- 1.31	- 1.76	- 1.85	+ 1.16	- 1.34	- 1.41	+ 1.52
NMB1248	Not in COGs genes	Molybdopterin-guanine dinucleotide biosynthesis protein A	1.02E-02	+ 1.48	+ 1.23	+ 1.61	+ 1.83	- 1.20	+ 1.09	+ 1.24
NMB1250	Signal transduction mechanisms genes	Transcriptional regulator, LuxR family	4.21E-03	- 1.19	- 1.34	- 1.32	+ 1.14	- 1.12	- 1.11	+ 1.36
NMB1255	Not in COGs genes	Pseudogene (putative glycosyl transferase)	2.92E-03	+ 1.28	+ 1.60	+ 1.58	+ 1.13	+ 1.25	+ 1.23	- 1.14
NMB1257	Not in COGs genes	Conserved hypothetical protein	3.47E-03	+ 1.48	+ 1.43	+ 1.51	- 1.33	- 1.03	+ 1.02	- 1.97
NMB1258	Replication recombination and repair	Conserved hypothetical protein	3.45E-02	+ 1.47	+ 1.38	+ 1.15	+ 1.33	- 1.06	- 1.27	- 1.10

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 hrs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1260	Defense mechanisms genes	Type III restriction-modification system EcoPI enzyme, subunit res	1.74E-02	+ 1.22	+ 1.49	+ 1.45	+ 1.26	+ 1.22	+ 1.19	+ 1.04
NMB1261	Not in COGs genes	Pseudogene	2.73E-02	+ 1.10	+ 1.65	+ 1.54	+ 1.16	+ 1.51	+ 1.40	+ 1.06
NMB1264	Function unknown genes	Conserved hypothetical protein	4.05E-02	- 1.05	+ 1.27	+ 1.04	- 1.76	+ 1.34	+ 1.09	- 1.67
NMB1266	Inorganic ion transport and metabolism genes	Putative zinc uptake fc protein	1.69E-02	+ 1.28	+ 2.06	- 1.10	+ 1.06	+ 1.62	- 1.40	- 1.20
NMB1269	Not in COGs genes	Conserved hypothetical protein	1.52E-02	- 1.35	- 1.06	+ 1.26	+ 1.03	+ 1.28	+ 1.70	+ 1.38
NMB1270	General function prediction only genes Secondary metabolites biosynthesis, transport and catabolism genes	Conserved hypothetical protein	2.91E-03	- 1.57	- 1.40	- 1.50	- 1.20	+ 1.12	+ 1.05	+ 1.31
NMB1271	Inorganic ion transport and metabolism genes	Putative mercury transport periplasmic protein	3.90E-04	- 1.08	- 1.43	- 1.46	+ 1.25	- 1.32	- 1.35	+ 1.35
NMB1272	Not in COGs genes	Hypothetical protein conserved hypothetical protein	2.37E-02	+ 1.50	+ 1.27	+ 1.52	+ 1.38	- 1.18	+ 1.01	- 1.09
NMB1276	Acyl-CoA synthetase	Long-chain-fatty-acid-CoA ligase	2.75E-02	+ 1.42	+ 1.22	+ 1.05	+ 1.40	- 1.17	- 1.35	- 1.01
NMB1277	Cell wall/membrane biogenesis genes	Transporter, BCCT family high-affinity choline transport protein	2.33E-04	- 1.61	- 1.62	- 1.46	- 1.09	- 1.01	+ 1.10	+ 1.48
NMB1282	Not in COGs genes	Aspartate 1-decarboxylase	2.26E-03	- 1.74	- 1.05	- 1.21	+ 1.10	+ 1.66	+ 1.44	+ 1.91
NMB1283	Cell wall/membrane biogenesis genes	2-dehydro-3-deoxyphosphooctonate aldolase	2.53E-03	- 1.42	+ 1.22	+ 1.04	- 1.28	+ 1.73	+ 1.48	+ 1.11
NMB1285	Carbohydrate transport and metabolism genes	Enolase	1.51E-02	- 1.17	+ 1.51	+ 1.32	+ 1.49	+ 1.76	+ 1.54	+ 1.74
NMB1286	Cell cycle control, mitosis and meiosis genes	Conserved hypothetical protein	6.26E-04	- 1.02	- 1.98	- 1.88	- 1.66	- 1.94	- 1.84	- 1.62
NMB1287	Energy production and conversion genes	Putative ferredoxin	2.42E-02	+ 1.26	- 1.18	- 1.24	+ 1.54	- 1.49	- 1.56	+ 1.22
NMB1289	Not in COGs genes	Putative type II restriction enzyme	3.32E-02	- 1.18	+ 1.13	- 1.12	- 2.95	+ 1.34	+ 1.06	- 2.50
NMB1290	Replication recombination and repair	C-5 cytosine-specific DNA-methylase	3.99E-03	- 1.03	+ 1.79	+ 1.25	- 1.76	+ 1.84	+ 1.28	- 1.71
NMB1291	Nucleotide transport and metabolism genes	Ribonucleoside-diphosphate reductase, alpha subunit	3.85E-02	+ 1.09	+ 1.11	+ 1.14	+ 1.56	+ 1.02	+ 1.04	+ 1.43
NMB1296	Not in COGs genes	Hypothetical protein	8.54E-04	+ 1.29	- 2.06	- 2.04	- 1.66	- 2.65	- 2.62	- 2.13
NMB1299	Not in COGs genes	Sodium- and chloride-dependent transporter	4.83E-02	+ 1.55	+ 1.31	+ 1.26	+ 1.14	- 1.18	- 1.23	- 1.36
NMB1300	Nucleotide transport and metabolism genes	cytidylate kinase	3.78E-02	+ 1.18	- 1.03	+ 1.13	- 1.33	- 1.21	- 1.04	- 1.56
NMB1301	Translation	30S ribosomal protein S1	1.44E-03	- 1.44	+ 3.41	+ 2.15	+ 1.21	+ 4.91	+ 3.10	+ 1.75
NMB1302	Not in COGs genes	Integration host factor, beta subunit	2.31E-02	- 1.99	+ 1.35	+ 1.30	+ 1.57	+ 2.68	+ 2.59	+ 3.12
NMB1303	Transcription	Transcriptional regulator, MerR family	3.92E-03	+ 1.22	+ 1.51	+ 1.64	- 1.19	+ 1.23	+ 1.34	- 1.46
NMB1307	Not in COGs genes	Nucleoside diphosphate kinase	3.94E-02	- 1.15	+ 1.97	+ 1.21	+ 1.36	+ 2.26	+ 1.38	+ 1.56
NMB1309	Intracellular trafficking and secretion genes; Cell motility genes	Putative fimbrial biogenesis and twitching motility protein	1.43E-02	- 1.79	- 1.58	- 1.85	+ 1.01	+ 1.13	- 1.04	+ 1.81
NMB1311	Not in COGs genes	Hypothetical protein	5.25E-03	+ 1.34	+ 1.38	+ 1.20	- 1.87	+ 1.03	- 1.12	- 2.51
NMB1313	Posttranslational modification, protein turnover, chaperones genes	Trigger factor	2.00E-03	- 1.48	+ 1.96	+ 1.46	+ 1.51	+ 2.89	+ 2.16	+ 2.23
NMB1314	Cell cycle control, mitosis and meiosis genes	Cell division protein FtsK	1.16E-03	+ 1.36	- 1.06	- 1.06	+ 1.43	- 1.44	- 1.43	+ 1.05
NMB1315	Nucleotide transport and metabolism genes	Uracil permease	2.53E-02	+ 1.20	- 1.24	- 1.09	+ 1.18	- 1.49	- 1.31	- 1.02
NMB1316	Not in COGs genes	Hypothetical protein Conserved hypothetical protein	5.99E-03	+ 1.96	+ 1.45	+ 1.49	+ 1.44	- 1.35	- 1.32	- 1.36

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h-DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1317	Not in COGs genes	Conserved hypothetical protein	5.59E-03	+ 1.06	- 2.31	- 1.43	- 2.99	- 2.45	- 1.52	- 3.18
NMB1318	Lipid transport and metabolism genes	CDP-diacylglycerol--serine O-phosphatidyltransferase	2.82E-02	- 1.09	- 1.11	+ 1.22	- 1.65	- 1.02	+ 1.32	- 1.51
NMB1319	General function prediction only genes	Conserved hypothetical protein	1.61E-03	- 1.08	+ 1.12	+ 1.57	+ 1.20	+ 1.21	+ 1.70	+ 1.30
NMB1320	translation	50S ribosomal protein L9	8.77E-04	- 1.54	+ 3.24	+ 2.02	+ 2.16	+ 4.98	+ 3.10	+ 3.32
NMB1321	Not in COGs genes	30S ribosomal protein S18	1.90E-04	- 2.84	+ 3.96	+ 2.43	+ 1.44	+ 11.24	+ 6.90	+ 4.07
NMB1322	Replication recombination and repair	Putative primosomal replication protein n	4.25E-04	- 1.81	+ 5.64	+ 3.05	+ 1.82	+ 10.20	+ 5.51	+ 3.29
NMB1323	Translation	30S ribosomal protein S6	1.18E-04	- 1.77	+ 5.21	+ 3.55	+ 2.33	+ 9.24	+ 6.30	+ 4.14
NMB1326	Replication recombination and repair	Excinuclease ABC, subunit C	3.28E-02	+ 1.41	+ 1.38	+ 1.58	+ 1.07	- 1.02	+ 1.13	- 1.31
NMB1329	Not in COGs genes	Hypothetical protein	1.65E-02	+ 1.60	+ 1.14	+ 1.10	- 1.53	- 1.40	- 1.45	- 2.44
NMB1330	Not in COGs genes	Hypothetical protein	4.85E-02	+ 1.65	+ 1.21	+ 1.15	- 1.25	- 1.36	- 1.44	- 2.07
NMB1333	Cell cycle control, mitosis and meiosis genes	Conserved hypothetical protein	4.53E-03	- 1.53	- 1.31	- 1.47	+ 1.15	+ 1.17	+ 1.04	+ 1.76
NMB1334	Not in COGs genes	Hypothetical protein	1.80E-02	- 1.28	- 1.14	- 1.12	+ 1.45	+ 1.13	+ 1.14	+ 1.86
NMB1335	Not in COGs genes	CreA protein	6.21E-04	- 1.26	+ 1.21	+ 1.21	+ 3.73	+ 1.53	+ 1.53	+ 4.71
NMB1309	Intracellular trafficking and secretion genes;	Putative fibrillar biogenesis and twitching motility protein	1.43E-02	- 1.79	- 1.58	- 1.85	+ 1.01	+ 1.13	- 1.04	+ 1.81
NMB1311	Cell motility genes	Hypothetical protein	5.25E-03	+ 1.34	+ 1.38	+ 1.20	- 1.87	+ 1.03	- 1.12	- 2.51
NMB1313	Posttranslational modification, protein turnover, chaperones genes	Trigler factor	2.00E-03	- 1.48	+ 1.96	+ 1.46	+ 1.51	+ 2.89	+ 2.16	+ 2.23
NMB1314	Cell cycle control, mitosis and meiosis genes	Cell division protein FtsK	1.16E-03	+ 1.36	- 1.06	- 1.06	+ 1.43	- 1.44	- 1.43	+ 1.05
NMB1315	Nucleotide transport and metabolism genes	Uracil permease	2.53E-02	+ 1.20	- 1.24	- 1.09	+ 1.18	- 1.49	- 1.31	- 1.02
NMB1316	Not in COGs genes	Hypothetical protein Conserved hypothetical protein	5.99E-03	+ 1.96	+ 1.45	+ 1.49	+ 1.44	- 1.35	- 1.32	- 1.36
NMB1317	Not in COGs genes	Conserved hypothetical protein	5.59E-03	+ 1.06	- 2.31	- 1.43	- 2.99	- 2.45	- 1.52	- 3.18
NMB1318	Lipid transport and metabolism genes	CDP-diacylglycerol--serine O-phosphatidyltransferase	2.82E-02	- 1.09	- 1.11	+ 1.22	- 1.65	- 1.02	+ 1.32	- 1.51
NMB1319	General function prediction only genes	Conserved hypothetical protein	1.61E-03	- 1.08	+ 1.12	+ 1.57	+ 1.20	+ 1.21	+ 1.70	+ 1.30
NMB1320	translation	50S ribosomal protein L9	8.77E-04	- 1.54	+ 3.24	+ 2.02	+ 2.16	+ 4.98	+ 3.10	+ 3.32
NMB1321	Not in COGs genes	30S ribosomal protein S18	1.90E-04	- 2.84	+ 3.96	+ 2.43	+ 1.44	+ 11.24	+ 6.90	+ 4.07
NMB1322	Replication recombination and repair	Putative primosomal replication protein n	4.25E-04	- 1.81	+ 5.64	+ 3.05	+ 1.82	+ 10.20	+ 5.51	+ 3.29
NMB1323	Translation	30S ribosomal protein S6	1.18E-04	- 1.77	+ 5.21	+ 3.55	+ 2.33	+ 9.24	+ 6.30	+ 4.14
NMB1326	Replication recombination and repair	Excinuclease ABC, subunit C	3.28E-02	+ 1.41	+ 1.38	+ 1.58	+ 1.07	- 1.02	+ 1.13	- 1.31
NMB1329	Not in COGs genes	Hypothetical protein	1.65E-02	+ 1.60	+ 1.14	+ 1.10	- 1.53	- 1.40	- 1.45	- 2.44
NMB1330	Not in COGs genes	Hypothetical protein	4.85E-02	+ 1.65	+ 1.21	+ 1.15	- 1.25	- 1.36	- 1.44	- 2.07
NMB1333	Cell cycle control, mitosis and meiosis genes	Conserved hypothetical protein	4.53E-03	- 1.53	- 1.31	- 1.47	+ 1.15	+ 1.17	+ 1.04	+ 1.76
NMB1334	Not in COGs genes	Hypothetical protein	1.80E-02	- 1.28	- 1.14	- 1.12	+ 1.45	+ 1.13	+ 1.14	+ 1.86
NMB1335	Not in COGs genes	CreA protein	6.21E-04	- 1.26	+ 1.21	+ 1.21	+ 3.73	+ 1.53	+ 1.53	+ 4.71
NMB1336	transcription	Conserved hypothetical protein	2.80E-04	- 1.60	- 1.68	- 1.57	+ 1.01	- 1.05	+ 1.02	+ 1.61
NMB1337	Not in COGs genes	Conserved hypothetical protein Putative Holliday junction resolvase	5.43E-03	- 2.32	- 2.20	- 2.07	- 1.54	+ 1.05	+ 1.12	+ 1.51

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1341	Energy production and conversion genes	Pyruvate dehydrogenase, E1 component	2.60E-03	-	1.41	+	1.32	+	1.86	+
NMB1342	Energy production and conversion genes	Pyruvate dehydrogenase, E2 component, dihydrolipoamide acetyltransferase	1.31E-02	-	1.02	+	1.50	+	1.52	+
NMB1343	Not in COGs genes	Hypothetical protein	2.34E-03	+	1.17	+	2.11	+	1.81	-
NMB1344	Energy production and conversion genes	Pyruvate dehydrogenase, E3 component, lipoamide dehydrogenase	4.75E-03	-	1.03	+	1.56	+	1.60	-
NMB1352	Not in COGs genes	Hypothetical protein	5.35E-03	+	1.66	-	1.82	-	3.02	-
NMB1355	Translation	Putative glutamyl-HRNA (Gln) amidotransferase subunit C	2.05E-03	-	1.85	+	1.56	+	2.88	+
NMB1356	Not in COGs genes	Glu-tRNA(Gln) amidotransferase, subunit A	6.30E-03	-	1.56	+	1.13	+	1.77	+
NMB1357	Not in COGs genes	Conserved hypothetical protein	1.25E-02	-	1.01	+	1.34	+	1.36	-
NMB1358	Translation	Glu-tRNA(Gln) amidotransferase, subunit B	3.38E-03	+	1.35	+	1.32	+	1.03	-
NMB1360	Coenzyme transport and metabolism genes	Pyridoxamine 5-phosphate oxidase	1.25E-03	+	1.50	-	1.88	-	2.82	-
NMB1363	Replication recombination and repair	Exodeoxyribonuclease, large subunit	4.81E-02	+	1.28	+	1.02	-	1.26	-
NMB1366	Energy production and conversion genes; Posttranslational modification	Thioredoxin thioredoxin I	1.05E-04	-	1.43	+	1.09	+	1.31	+
NMB1368	Replication recombination and repair transcription	Putative ATP-dependent RNA helicase	7.58E-05	+	1.10	+	3.90	+	3.53	+
NMB1369	Not in COGs genes	Hypothetical protein gputate membrane lipoprotein	5.36E-03	-	1.66	-	1.09	+	1.67	+
NMB1370	Not in COGs genes	Hypothetical protein	5.81E-03	-	1.25	+	1.29	+	1.61	+
NMB1375	Not in COGs genes	Type III restriction/modification system modification methylase	5.86E-04	+	1.22	+	2.08	+	1.80	-
NMB1378	Transcription	Conserved hypothetical protein	7.39E-05	-	1.28	+	2.11	+	3.29	+
NMB1379	Amino acid transport and metabolism genes	NifS protein	1.90E-04	-	1.31	+	2.04	+	2.68	+
NMB1380	Energy production and conversion genes	NifU protein	2.72E-04	-	1.63	+	1.81	+	2.35	+
NMB1381	Function unknown genes	HesB/YadR/YnfF family protein	6.80E-04	+	1.14	+	3.24	+	2.01	+
NMB1382	Not in COGs genes	Conserved hypothetical protein	2.53E-02	-	1.35	+	2.00	+	2.17	+
NMB1384	Replication recombination and repair	DNA gyrase subunit A	1.33E-03	-	1.32	+	1.15	+	1.49	+
NMB1385	Not in COGs genes	IS1106 transposase	1.61E-03	+	1.07	-	2.93	-	2.52	-
NMB1386	Not in COGs genes	Putative transposase	1.62E-02	-	1.06	-	1.60	-	1.40	-
NMB1387	Not in COGs genes	Hypothetical protein	3.05E-03	-	1.02	-	1.23	-	1.22	+
NMB1391	Carbohydrate transport and metabolism genes	Oxidoreductase, So/DevB family	1.77E-02	-	1.11	-	1.14	-	1.06	+
NMB1392	Not in COGs genes	Glucose-6-phosphate 1-dehydrogenase	2.48E-04	-	1.44	-	1.36	+	1.08	+
NMB1393	Not in COGs genes	Phosphoglucuronate dehydratase	2.00E-02	-	1.42	+	1.14	+	2.09	+
NMB1395	General function prediction only genes Amino acid transport and metabolism genes	Alcohol dehydrogenase, zinc-containing	7.03E-04	-	1.15	-	1.18	-	1.10	+
NMB1397	Not in COGs genes	Hypothetical protein	1.18E-02	+	1.74	+	1.43	+	1.22	-
NMB1398	Not in COGs genes	Cu-Zn-superoxide dismutase	3.78E-02	+	1.29	-	1.19	-	1.37	-
NMB1399	Replication recombination and repair	IS1106 transposase	1.24E-02	-	2.24	+	1.37	+	2.29	+
NMB1402	Not in COGs genes	Hypothetical protein	4.60E-02	+	1.42	+	1.16	-	1.39	-
NMB1403	Not in COGs genes	FrpA/C-related protein	4.17E-02	+	1.20	+	1.71	+	1.39	-

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1404	Not in COGs genes	Hypothetical protein	2.92E-03	+ 1.91	- 1.24	- 1.29	- 1.07	- 2.35	- 2.46	- 2.03
NMB1407	Not in COGs genes	Putative iron-regulated protein FrpC outer membrane (pseudogene)	2.01E-02	+ 1.39	- 1.18	- 1.16	- 1.41	- 1.64	- 1.61	- 1.96
NMB1409	Not in COGs genes	FrpA/C-related protein	1.50E-02	+ 1.36	+ 1.38	+ 1.42	- 1.19	+ 1.01	+ 1.04	- 1.62
NMB1412	Not in COGs genes	FrpC operon protein	2.55E-02	- 1.69	- 2.31	- 1.77	- 1.85	- 1.36	- 1.05	- 1.09
NMB1417	Replication recombination and repair	Conserved hypothetical protein	4.68E-02	+ 1.39	- 1.04	+ 1.20	+ 1.54	- 1.45	- 1.16	+ 1.11
NMB1418	Cell wall/membrane biogenesis genes	HtrB/MsbB family protein	1.24E-02	+ 1.71	+ 1.73	+ 1.27	+ 2.13	+ 1.01	- 1.34	+ 1.25
NMB1420	Replication recombination and repair transcription	Putative factor-for-inversion stimulation protein Fis	7.10E-03	+ 1.61	+ 1.63	+ 1.32	+ 1.16	+ 1.01	- 1.22	- 1.38
NMB1424	Not in COGs genes	Conserved hypothetical protein	8.17E-03	- 1.51	- 1.70	- 1.61	- 1.35	- 1.13	- 1.07	+ 1.12
NMB1426	Inorganic ion transport and metabolism genes	Hypothetical protein putative integral membrane protein	1.57E-02	+ 1.50	+ 1.24	+ 1.53	+ 1.09	- 1.21	+ 1.02	- 1.38
NMB1427	Not in COGs genes	Hypothetical protein	3.16E-03	+ 2.19	+ 1.74	+ 1.71	- 1.10	- 1.26	- 1.28	- 2.41
NMB1429	Cell wall/membrane biogenesis genes	Outer membrane protein PorA	7.39E-05	+ 3.54	+ 3.99	+ 3.31	+ 1.81	+ 14.12	+ 11.69	+ 6.40
NMB1434	Lipid transport and metabolism genes	Cardiolipin synthetase family protein	3.74E-02	- 1.13	- 1.06	+ 1.18	- 1.31	+ 1.07	+ 1.33	- 1.16
NMB1435	Not in COGs genes	Drug resistance translocase family protein	3.68E-02	+ 1.14	+ 1.13	+ 1.20	+ 1.32	- 1.01	+ 1.05	+ 1.16
NMB1436	Energy production and conversion genes	Conserved hypothetical protein	5.40E-03	- 1.53	- 1.16	- 1.16	- 1.04	+ 1.33	+ 1.32	+ 1.47
NMB1437	Function unknown genes	Conserved hypothetical protein	7.13E-03	- 1.91	- 1.78	- 2.04	- 1.26	+ 1.07	- 1.07	+ 1.52
NMB1441	General function prediction only genes	Putative O-methyltransferase	3.68E-04	- 2.04	- 2.23	- 1.95	- 1.11	- 1.09	+ 1.05	+ 1.83
NMB1444	Function unknown genes	Conserved hypothetical protein	6.28E-03	- 1.28	- 1.39	- 1.25	+ 1.53	- 1.08	+ 1.02	+ 1.96
NMB1450	Energy production and conversion genes	Ferredoxin-NADP reductase	3.47E-03	- 1.07	- 1.51	- 1.64	- 1.10	- 1.42	- 1.54	- 1.03
NMB1452	General function prediction only genes	Conserved hypothetical protein	4.66E-03	+ 1.29	- 1.15	- 1.49	+ 1.57	- 1.48	- 1.93	+ 1.21
NMB1458	Energy production and conversion genes	Fumarate hydratase, class II, aerobic	5.43E-03	- 1.06	- 1.77	- 1.21	- 1.56	- 1.67	- 1.15	- 1.47
NMB1459	General function prediction only genes	Conserved hypothetical protein	5.12E-04	- 1.06	- 3.52	- 2.43	- 5.95	- 3.33	- 2.30	- 5.63
NMB1460	Replication recombination and repair	Single-strand binding protein	1.95E-03	+ 2.36	+ 5.95	+ 7.98	+ 3.58	+ 2.52	+ 3.38	+ 1.51
NMB1461	Not in COGs genes	Drug resistance translocase family protein	1.67E-02	+ 1.18	- 1.29	+ 1.16	- 1.38	- 1.52	- 1.02	- 1.63
NMB1462	Cell wall/membrane biogenesis genes	Putative transglycosylase	6.10E-06	- 1.08	- 1.59	+ 1.00	- 4.19	- 1.47	+ 1.09	- 3.87
NMB1463	Not in COGs genes	IS1106A3 transposase	6.65E-03	+ 1.53	+ 3.69	+ 3.98	+ 3.52	+ 2.42	+ 2.60	+ 2.30
NMB1464	replication recombination and repair	Conserved hypothetical protein	2.34E-03	+ 1.47	- 1.11	- 1.04	+ 1.22	- 1.64	- 1.53	- 1.21
NMB1465	Not in COGs genes	Opacity outer membrane protein	1.24E-02	+ 1.24	+ 6.92	+ 3.40	+ 5.63	+ 5.56	+ 2.74	+ 4.53
NMB1467	Inorganic ion transport and metabolism genes	Exopolyphosphatase	1.21E-02	- 1.07	- 1.39	- 1.33	- 1.11	- 1.30	- 1.25	- 1.04
NMB1472	Nucleotide transport and metabolism genes	CipB protein	8.54E-04	- 1.91	- 1.46	- 1.49	+ 1.29	+ 1.31	+ 1.28	+ 2.47
NMB1473	Amino acid transport and metabolism genes	Aminotransferase, class I	1.08E-02	- 1.17	- 1.58	- 1.60	+ 1.29	- 1.35	- 1.37	+ 1.51
NMB1474	General function prediction only genes	Putative 4-oxalocrotonate tautomerase	6.11E-03	+ 1.24	- 1.50	- 1.39	+ 1.01	- 1.86	- 1.72	- 1.23
NMB1480	Not in COGs genes	Hypothetical protein	5.70E-03	- 1.57	- 1.89	- 1.97	- 1.48	- 1.20	- 1.26	+ 1.06
NMB1482	Lipid transport and metabolism genes	Acyl CoA thioester hydrolase family protein	1.39E-02	- 1.06	- 1.44	- 1.34	+ 1.25	- 1.36	- 1.26	+ 1.32
NMB1483	Cell wall/membrane biogenesis genes	Putative lipoprotein NlpD	1.31E-02	- 1.61	- 1.33	- 1.04	- 1.59	+ 1.21	+ 1.54	+ 1.01
NMB1484	General function prediction only genes	Stationary-phase survival protein SurE	4.32E-02	- 1.41	- 1.17	- 1.18	+ 1.30	+ 1.21	+ 1.19	+ 1.84

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1485	General function prediction only genes; Inorganic ion transport and metabolism genes	Conserved hypothetical protein	1.09E-02	+ 1.62	+ 1.32	+ 1.30	+ 1.26	- 1.23	- 1.24	- 1.29
NMB1486	Not in COGs genes	Hypothetical protein	3.32E-02	+ 1.08	- 1.46	- 1.34	+ 1.02	- 1.57	- 1.45	- 1.06
NMB1488	Energy production and conversion genes	Succinate-semialdehyde dehydrogenase (NADP+)	5.97E-03	+ 1.04	+ 1.21	+ 1.28	+ 1.31	+ 1.16	+ 1.23	+ 1.26
NMB1489	Not in COGs genes	Hypothetical protein	1.27E-02	+ 1.19	- 1.34	- 1.22	- 3.07	- 1.60	- 1.46	- 3.67
NMB1490	Not in COGs genes	Hypothetical protein lipoprotein	4.59E-02	+ 1.25	+ 1.78	+ 1.95	+ 2.35	+ 1.42	+ 1.56	+ 1.87
NMB1492	Not in COGs genes	Hypothetical protein	2.19E-02	+ 1.04	+ 1.42	+ 1.41	+ 2.31	+ 1.37	+ 1.36	+ 2.24
NMB1495	Not in COGs genes	Hypothetical protein	2.02E-02	+ 1.30	+ 1.15	+ 1.26	- 1.93	- 1.12	- 1.03	- 2.50
NMB1496	Translation	Conserved hypothetical protein	1.25E-02	+ 1.75	+ 1.15	+ 1.32	- 1.01	- 1.51	- 1.32	- 1.77
NMB1498	Amino acid transport and metabolism genes	Aspartokinase, alpha and beta subunits	8.68E-03	- 1.18	+ 1.24	+ 1.26	+ 1.44	+ 1.46	+ 1.49	+ 1.71
NMB1499	Translation	Ribonuclease PH	3.91E-03	- 1.05	+ 1.24	- 1.34	+ 1.85	+ 1.30	- 1.28	+ 1.94
NMB1500	Signal transduction mechanisms genes	Conserved hypothetical protein	2.48E-04	- 2.05	- 1.17	- 1.12	+ 1.51	+ 1.75	+ 1.83	+ 3.10
NMB1501	Coenzyme transport and metabolism genes	HesA/moeB/hnf family protein	2.06E-02	- 1.21	+ 1.20	- 1.02	+ 1.36	+ 1.44	+ 1.18	+ 1.64
NMB1502	Not in COGs genes	Hypothetical protein	7.96E-04	+ 2.43	+ 1.05	+ 1.13	+ 1.45	- 2.32	- 2.16	- 1.67
NMB1503	Not in COGs genes	Segregation and condensation protein A	7.52E-05	- 1.26	+ 1.26	+ 1.64	+ 1.29	+ 1.59	+ 2.07	+ 1.63
NMB1506	Translation	Arginyl-tRNA synthetase	8.30E-03	+ 1.21	- 1.24	- 1.15	- 1.36	- 1.50	- 1.40	- 1.64
NMB1507	Not in COGs genes	Hypothetical protein	1.36E-03	- 1.44	- 2.27	- 2.07	- 2.36	- 1.58	- 1.44	- 1.64
NMB1511	Carbohydrate transport and metabolism genes	Ribose 5-phosphate isomerase A	3.45E-02	+ 1.42	+ 1.31	+ 1.80	+ 1.77	- 1.08	+ 1.27	+ 1.25
NMB1512	Not in COGs genes	YggB/yacB family protein	2.26E-03	+ 1.90	- 1.38	- 1.29	- 4.39	- 2.63	- 2.46	- 8.35
NMB1515	Not in COGs genes	Putative transporter putative integral membrane protein	1.40E-03	- 1.01	- 1.64	- 1.51	- 1.65	- 1.62	- 1.49	- 1.62
NMB1516	Inorganic ion transport and metabolism genes	Fixs protein putative nitrogen fixation protein	6.95E-03	+ 1.23	- 1.11	- 1.04	+ 2.49	- 1.36	- 1.28	+ 2.02
NMB1517	Not in COGs genes	Conserved hypothetical protein	4.81E-03	- 1.13	- 1.48	- 1.30	+ 1.53	- 1.31	- 1.15	+ 1.73
NMB1518	Energy production and conversion genes	Acetate kinase Code	2.32E-02	- 1.26	+ 1.32	+ 1.69	- 1.06	+ 1.66	+ 2.12	+ 1.18
NMB1523	Not in COGs genes	Hypothetical protein conserved hypothetical protein	5.76E-04	+ 1.60	+ 18.17	+ 12.47	+ 5.93	+ 11.39	+ 7.82	+ 3.72
NMB1525	Not in COGs genes	N/a	1.39E-02	+ 1.04	+ 1.41	+ 1.22	- 1.86	+ 1.36	+ 1.18	- 1.93
NMB1526	Posttranslational modification, protein turnover, chaperones genes	Small major protein B	1.02E-02	- 1.14	- 1.15	- 1.30	+ 1.54	- 1.01	- 1.14	+ 1.75
NMB1527	Cell wall/membrane biogenesis genes	ADP-heptose-LPS heptosyltransferase II	2.80E-03	+ 1.08	- 1.29	- 1.43	+ 1.53	- 1.39	- 1.55	+ 1.41
NMB1529	General function prediction only genes	Hypothetical protein	1.98E-03	- 1.61	- 1.08	+ 1.04	- 1.75	+ 1.49	+ 1.67	- 1.09
NMB1532	Not in COGs genes	Conserved hypothetical protein	4.64E-02	+ 1.16	+ 1.11	+ 1.07	+ 1.49	- 1.05	- 1.09	+ 1.28
NMB1533	Not in COGs genes	H.8 outer membrane protein	1.29E-02	- 1.64	+ 1.32	+ 1.59	+ 1.02	+ 2.16	+ 2.61	+ 1.68
NMB1534	Not in COGs genes	Hypothetical protein conserved hypothetical protein	4.90E-02	- 1.94	- 1.06	+ 1.08	- 2.02	+ 1.82	+ 2.09	- 1.04
NMB1535	Not in COGs genes	Hypothetical protein	2.57E-02	- 1.46	- 1.27	+ 1.02	- 1.19	+ 1.14	+ 1.48	+ 1.22
NMB1538	transcription	RNA polymerase sigma factor rpoD	2.77E-03	- 1.65	- 1.65	- 2.07	- 1.20	+ 1.00	- 1.25	+ 1.37
NMB1539	replication recombination and repair	IS1106 transposase	3.78E-03	+ 1.01	- 2.32	- 2.08	- 2.09	- 2.36	- 2.11	- 2.12
NMB1541	Not in COGs genes	Lactoferrin-binding protein B	1.91E-03	- 1.39	- 1.63	- 1.54	- 2.11	- 1.17	- 1.11	- 1.52

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1542	Not in COGs genes	Hypothetical protein	5.71E-03	+ 1.39	+ 1.22	+ 1.36	- 1.87	- 1.14	- 1.02	- 2.60
NMB1543	replication recombination and repair	Conserved hypothetical protein	3.71E-02	- 1.10	+ 1.39	+ 1.37	- 2.36	+ 1.54	+ 1.51	- 2.14
NMB1544	Not in COGs genes	Hypothetical protein conserved hypothetical protein	2.78E-02	+ 1.05	+ 1.30	+ 1.17	- 3.16	+ 1.24	+ 1.11	- 3.32
NMB1545	Not in COGs genes	Hypothetical protein conserved hypothetical protein	7.28E-03	+ 1.04	+ 1.76	+ 1.70	- 2.34	+ 1.69	+ 1.64	- 2.44
NMB1546	Not in COGs genes	Hypothetical protein	8.70E-04	+ 2.37	+ 3.07	+ 3.08	+ 1.57	+ 1.30	+ 1.30	- 1.51
NMB1547	Not in COGs genes	Hypothetical protein conserved hypothetical protein	1.29E-02	+ 1.25	+ 1.80	+ 1.71	- 1.56	+ 1.44	+ 1.37	- 1.95
NMB1549	Not in COGs genes	Hypothetical protein	8.68E-03	+ 1.45	+ 2.14	+ 2.09	+ 1.53	+ 1.47	+ 1.44	+ 1.06
NMB1552	replication recombination and repair	Pilin gene inverting protein pilvnm-1B	2.92E-02	+ 1.12	- 1.05	- 1.12	- 3.01	- 1.18	- 1.25	- 3.37
NMB1554	Nucleotide transport and metabolism genes	CTP synthase	6.97E-04	- 1.10	+ 1.21	+ 1.22	+ 1.40	+ 1.34	+ 1.34	+ 1.55
NMB1555	Secondary metabolites biosynthesis, transport and catabolism genes	Long-chain-fatty-acid-coa ligase	2.65E-02	+ 1.09	- 1.15	- 1.04	+ 1.35	- 1.24	- 1.13	+ 1.24
NMB1556	translational	Trna (5-methylaminomethyl-2-thiouridylate)-methyltransferase	1.19E-02	+ 1.15	- 1.15	- 1.17	- 1.13	- 1.32	- 1.34	- 1.30
NMB1557	Function unknown genes	Conserved hypothetical protein	1.91E-03	- 1.22	+ 1.09	+ 1.20	+ 2.21	+ 1.33	+ 1.47	+ 2.70
NMB1558	Not in COGs genes	Diacylglycerol kinase	1.61E-02	+ 2.18	- 1.05	- 1.24	- 1.30	- 2.28	- 2.70	- 2.84
NMB1561	Carbohydrate transport and metabolism genes	Transcriptional regulator, deor family	4.20E-03	- 1.23	- 1.36	- 1.44	- 1.23	- 1.10	- 1.17	+ 1.00
NMB1564	Posttranslational modification, protein turnover, chaperones genes	Conserved hypothetical protein	1.95E-03	- 1.28	+ 1.03	- 1.23	+ 1.59	+ 1.32	+ 1.04	+ 2.04
NMB1566	Not in COGs genes	Phosphoribosylglycinamide formyltransferase	3.69E-02	- 1.02	+ 1.04	+ 1.04	- 1.23	+ 1.07	+ 1.06	- 1.20
NMB1567	Posttranslational modification, protein turnover, chaperones genes	Macrophage infectivity potentiator	1.18E-04	+ 1.00	+ 2.76	+ 2.51	+ 1.52	+ 2.76	+ 2.51	+ 1.52
NMB1568	Not in COGs genes	Putative DNA polymerase holoenzyme chi subunit	5.70E-03	+ 1.21	+ 1.11	+ 1.20	- 1.14	- 1.10	- 1.02	- 1.38
NMB1569	Not in COGs genes	Aminopeptidase A	2.23E-03	- 1.55	- 1.07	- 1.12	+ 1.18	+ 1.45	+ 1.38	+ 1.82
NMB1570	Not in COGs genes	Conserved hypothetical protein	2.36E-02	+ 1.11	- 1.25	- 1.29	- 1.05	- 1.38	- 1.43	- 1.17
NMB1575	Function unknown genes	Conserved hypothetical protein	4.07E-02	- 1.14	+ 2.09	+ 1.68	+ 1.17	+ 2.39	+ 1.91	+ 1.34
NMB1580	Function unknown genes	Hypothetical protein conserved hypothetical protein	2.35E-02	+ 1.02	+ 1.35	+ 1.23	- 1.35	+ 1.32	+ 1.20	- 1.38
NMB1581	Not in COGs genes	Histidinol dehydrogenase	1.45E-02	- 1.41	+ 1.12	+ 1.12	+ 1.18	+ 1.58	+ 1.58	+ 1.67
NMB1582	Amino acid transport and metabolism genes	Histidinol-phosphate aminotransferase	2.58E-02	- 1.32	- 1.20	- 1.18	+ 1.12	+ 1.10	+ 1.12	+ 1.47
NMB1583	Not in COGs genes	Imidazoleglycerol-phosphate dehydratase	2.15E-02	- 3.22	- 1.08	- 1.56	- 1.72	+ 2.97	+ 2.07	+ 1.87
NMB1588	Lipid transport and metabolism genes	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase	4.97E-02	+ 1.16	+ 1.26	+ 1.52	+ 1.46	+ 1.09	+ 1.32	+ 1.26
NMB1589	Not in COGs genes	Hypothetical protein conserved hypothetical protein	1.85E-02	- 1.23	- 1.50	- 1.24	- 1.09	- 1.22	- 1.01	+ 1.13
NMB1590	Function unknown genes	Conserved hypothetical protein	2.49E-04	- 1.23	+ 1.97	+ 3.11	+ 3.31	+ 2.43	+ 3.83	+ 4.08
NMB1591	Not in COGs genes	Transcriptional regulator mtra	5.86E-03	+ 1.02	+ 1.83	+ 1.89	+ 1.40	+ 1.79	+ 1.85	+ 1.37
NMB1592	Not in COGs genes	Putative lipoprotein conserved hypothetical protein	3.32E-02	- 1.67	+ 1.02	- 1.10	+ 1.01	+ 1.71	+ 1.52	+ 1.69
NMB1594	Amino acid transport and metabolism genes	Spermidine/putrescine ABC transporter	1.34E-03	- 1.61	- 1.33	- 1.21	+ 1.05	+ 1.21	+ 1.33	+ 1.69

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1595	Translation	Alanyl-tRNA synthetase	3.10E-02	-	1.47	-	1.19	1.29	1.23	1.63
NMB1597	Not in cogs genes	Hypothetical protein	3.95E-02	-	1.18	-	1.58	-	1.35	-
NMB1599	Not in cogs genes	Hypothetical protein	3.28E-02	+	1.28	-	1.41	-	1.81	-
NMB1600	Not in cogs genes	Hypothetical protein	2.34E-02	-	1.02	-	1.01	+	1.01	-
NMB1601	Replication recombination and repair	Is1106 transposase	3.42E-02	+	1.61	+	1.50	-	1.08	-
NMB1604	Carbohydrate transport and metabolism genes	Phosphoglycerate mutase	4.57E-03	+	1.24	-	1.85	-	2.30	-
NMB1608	Not in cogs genes	Conserved hypothetical protein	3.93E-02	-	1.52	-	1.02	+	1.55	-
NMB1615	Not in cogs genes	Hypothetical protein	8.76E-03	+	1.81	+	1.78	-	1.02	-
NMB1616	Coenzyme transport and metabolism genes	Phosphomethylpyrimidine kinase	3.86E-02	-	1.33	-	1.16	+	1.14	+
NMB1617	Inorganic ion transport and metabolism genes	Putative tellurite resistance protein	2.29E-03	+	1.13	+	1.08	-	1.05	-
NMB1618	Replication recombination and repair	Ribonuclease hi	1.35E-02	+	1.52	-	1.18	-	1.80	-
NMB1621	Posttranslational modification, protein turnover, chaperones genes	Glutathione peroxidase	7.58E-05	-	1.23	-	1.17	+	1.05	+
NMB1622	Inorganic ion transport and metabolism genes	Nitric oxide reductase	1.68E-06	+	2.29	+	2.04	-	1.12	+
NMB1623	Secondary metabolites biosynthesis, transport and catabolism genes	Major anaerobically induced outer membrane protein	1.99E-03	-	1.17	+	1.07	+	1.25	+
NMB1628	Not in cogs genes	Putative tsbp protein putative cell-surface protein	2.28E-03	+	1.90	+	1.27	-	1.49	-
NMB1629	Not in cogs genes	Hypothetical protein	1.34E-02	+	1.24	+	1.27	-	1.02	-
NMB1635	Not in cogs genes	Hypothetical protein	6.00E-03	+	1.21	+	1.40	+	1.16	-
NMB1638	General function prediction only genes	YhbX/Yhiw/Yjlp/Yjdb family protein	2.39E-02	-	1.11	+	1.06	+	1.18	+
NMB1640	Coenzyme transport and metabolism genes Amino acid transport and metabolism genes	Phosphoserine aminotransferase	1.95E-03	-	1.23	-	1.39	-	1.13	+
NMB1641	Function unknown genes	Conserved hypothetical protein	2.23E-03	-	1.12	+	1.66	+	1.85	+
NMB1642	Transcription	N utilization substance protein a	1.93E-04	-	1.20	+	2.47	+	2.96	+
NMB1643	Translation	Translation initiation factor if2 initiation factor if2	2.34E-03	-	1.12	+	2.32	+	2.61	+
NMB1647	Amino acid transport and metabolism genes	Putative amino acid symporter	2.32E-02	+	1.31	+	1.16	-	1.13	-
NMB1649	Posttranslational modification, protein turnover, chaperones genes	Disulfide bond formation protein B	5.95E-04	-	1.26	+	1.55	-	1.23	+
NMB1650	Transcription	Leucine-responsive regulatory protein	2.76E-06	-	1.01	+	4.57	+	4.62	+
NMB1652	Function unknown genes	Conserved hypothetical protein	2.37E-02	+	1.03	+	1.24	+	1.21	+
NMB1653	Signal transduction mechanisms genes	Conserved hypothetical protein	4.17E-02	+	1.03	+	1.33	+	1.29	-
NMB1656	Not in cogs genes	Hypothetical protein	4.39E-02	+	1.15	+	1.56	+	1.36	-
NMB1660	Transcription	Dna-directed rna polymerase, omega subunit	3.57E-02	-	1.91	+	1.04	+	1.97	+
NMB1661	Nucleotide transport and metabolism genes	Guanylate kinase	1.02E-02	-	1.16	+	1.44	+	1.67	+

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1662	Nucleotide transport and metabolism genes	Adenine phosphoribosyltransferase	5.29E-05	- 1.27	+ 1.14	+ 1.86	+ 1.30	+ 1.45	+ 2.37	+ 1.66
NMB1663	General function prediction only genes	Conserved hypothetical protein	1.91E-03	- 1.48	- 1.89	- 1.62	+ 1.01	- 1.28	- 1.09	+ 1.50
NMB1667	Not in cogs genes	Hypothetical protein	1.09E-03	+ 1.34	+ 1.91	+ 1.89	+ 1.82	+ 1.42	+ 1.41	+ 1.35
NMB1668	Inorganic ion transport and metabolism genes	Hemoglobin receptor	8.17E-03	- 1.17	- 1.42	- 1.35	+ 1.32	- 1.22	- 1.15	+ 1.54
NMB1669	Inorganic ion transport and metabolism genes	Iron-starvation protein piga haem utilisation protein	4.89E-04	- 2.06	- 1.90	- 1.38	+ 1.53	+ 1.09	+ 1.49	+ 3.16
NMB1670	Function unknown genes	Pqia family protein	1.48E-02	+ 1.36	+ 1.13	+ 1.54	+ 1.19	- 1.19	+ 1.13	- 1.14
NMB1674	Amino acid transport and metabolism genes	GDSL lipase family protein	1.24E-02	+ 1.27	+ 1.07	+ 1.07	- 1.82	- 1.19	- 1.18	- 2.31
NMB1675	Not in cogs genes	Hypothetical protein	3.81E-02	+ 1.02	- 1.40	- 1.17	- 1.07	- 1.43	- 1.19	- 1.10
NMB1677	Energy production and conversion genes	Cytochrome c5	3.58E-02	- 1.26	- 1.53	- 1.18	+ 1.05	- 1.22	+ 1.07	+ 1.31
NMB1678	Amino acid transport and metabolism genes	Aromatic-amino-acid aminotransferase	7.52E-05	- 1.70	- 1.16	+ 1.19	- 1.07	+ 1.47	+ 2.02	+ 1.60
NMB1679	Translation	Tma (uracil-5-)-methyltransferase	7.28E-03	- 1.21	- 1.08	- 1.06	+ 1.10	+ 1.13	+ 1.14	+ 1.33
NMB1681	Not in cogs genes	Hypothetical protein conserved hypothetical protein	3.07E-03	- 2.47	- 1.08	- 1.23	- 1.28	+ 2.29	+ 2.01	+ 1.93
NMB1683	General function prediction only genes replication recombination and repair	MutT/nudix family protein	2.77E-03	- 1.61	- 1.08	+ 1.13	- 1.12	+ 1.49	+ 1.81	+ 1.43
NMB1684	Translation	Seryl-tRNA synthetase	8.53E-04	- 1.16	- 1.70	- 1.47	- 1.15	- 1.47	- 1.27	+ 1.01
NMB1685	General function prediction only genes Coenzyme transport and metabolism genes	D-lactate dehydrogenase	7.01E-03	- 1.63	- 2.19	- 2.22	- 1.15	- 1.34	- 1.36	+ 1.42
NMB1687	Replication recombination and repair	Conserved hypothetical protein	1.37E-02	- 1.14	- 1.06	+ 1.14	- 2.06	+ 1.07	+ 1.30	- 1.81
NMB1690	Carbohydrate transport and metabolism genes	Phosphoglucosyltransferase/phosphomannomutase family protein	3.00E-02	+ 1.49	+ 1.31	+ 1.64	+ 1.21	- 1.13	+ 1.10	- 1.24
NMB1691	Coenzyme transport and metabolism genes	Dihydropteroate synthase	3.68E-02	+ 1.29	+ 1.37	+ 1.48	+ 2.21	+ 1.06	+ 1.15	+ 1.71
NMB1695	Not in cogs genes	Hypothetical protein integral membrane protein	2.63E-03	- 1.04	- 1.44	- 1.36	- 1.02	- 1.38	- 1.31	+ 1.03
NMB1698	Lipid transport and metabolism genes	Putative acyltransferase	6.28E-03	+ 1.05	- 2.01	- 1.76	- 3.93	- 2.12	- 1.85	- 4.14
NMB1701	Lipid transport and metabolism genes	Hypothetical protein	6.12E-03	- 1.29	- 1.77	- 1.41	- 1.58	- 1.38	- 1.10	- 1.23
NMB1702	General function prediction only genes	3-oxoacyl-(acyl-carrier-protein) reductase	1.12E-02	- 1.21	- 1.19	- 1.15	- 1.85	+ 1.02	+ 1.05	- 1.53
NMB1706	Not in cogs genes	Hypothetical protein	3.58E-02	+ 1.35	+ 1.54	+ 1.43	+ 1.19	+ 1.14	+ 1.06	- 1.14
NMB1707	General function prediction only genes	Sodium- and chloride-dependent transporter	5.43E-03	+ 1.17	- 1.49	- 1.10	- 1.01	- 1.75	- 1.29	- 1.19
NMB1709	Not in cogs genes	Thymidylate synthase	4.65E-04	- 1.34	- 1.54	- 1.30	- 1.13	- 1.14	+ 1.03	+ 1.19
NMB1710	Amino acid transport and metabolism genes	Glutamate dehydrogenase, NADP-specific	1.06E-03	- 1.33	+ 3.32	+ 2.78	+ 1.45	+ 4.40	+ 3.69	+ 1.92
NMB1711	Transcription	Transcriptional regulator, gntR family	1.93E-04	- 1.15	- 1.44	- 1.49	+ 1.02	- 1.25	- 1.29	+ 1.18
NMB1712	Energy production and conversion genes	L-lactate permease-related protein	1.12E-02	+ 1.10	+ 1.59	+ 1.56	+ 2.35	+ 1.45	+ 1.42	+ 2.14
NMB1715	Defense mechanisms genes	Multiple transferable resistance system protein mtrd	4.71E-03	- 1.35	- 1.62	- 1.34	- 1.85	- 1.20	+ 1.01	- 1.37

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1716	Cell wall/membrane biogenesis genes	Membrane fusion protein	3.98E-04	- 1.56	- 1.11	+ 1.06	- 1.33	+ 1.41	+ 1.65	+ 1.17
NMB1717	Transcription	Transcriptional regulator mtrr	5.02E-03	- 1.03	- 1.77	- 1.69	- 1.12	- 1.71	- 1.64	- 1.08
NMB1719	Coenzyme transport and metabolism genes	Efflux pump component mtrf	1.82E-02	+ 1.57	+ 1.69	+ 1.66	+ 1.77	+ 1.08	+ 1.06	+ 1.13
NMB1720	Replication recombination and repair	Exodeoxyribonuclease v 125 kd polypeptide	3.30E-02	- 1.05	- 1.18	- 1.11	- 1.72	- 1.12	- 1.06	- 1.81
NMB1722	Not in cogs genes	Cytochrome C555	2.93E-03	+ 1.55	- 1.25	- 1.46	- 1.11	- 1.93	- 2.26	- 1.72
NMB1724	Energy production and conversion genes	Cytochrome c oxidase, subunit II	3.70E-03	- 1.23	- 1.39	- 1.42	+ 1.09	- 1.12	- 1.15	+ 1.35
NMB1725	Posttranslational modification, protein turnover, chaperones genes	Cytochrome c oxidase, subunit I	3.24E-03	+ 1.29	- 1.28	- 1.16	+ 1.50	- 1.65	- 1.50	+ 1.16
NMB1728	Intracellular trafficking and secretion genes	Biopolymer transport protein exbd	1.05E-02	- 1.13	+ 1.59	+ 2.01	- 2.61	+ 1.80	+ 2.28	- 2.30
NMB1729	Intracellular trafficking and secretion genes	Biopolymer transport protein exbb	2.88E-03	- 2.27	- 1.78	- 1.10	- 1.78	+ 1.28	+ 2.06	+ 1.28
NMB1732	Inorganic ion transport and metabolism genes	Putative transporter integral membrane protein	2.96E-03	+ 1.70	+ 1.65	+ 1.46	- 1.37	- 1.03	- 1.16	- 2.33
NMB1734	Posttranslational modification, protein turnover, chaperones genes	Glutaredoxin	3.95E-02	+ 1.37	+ 1.42	+ 1.50	+ 2.02	+ 1.04	+ 1.10	+ 1.48
NMB1736	Not in cogs genes	Putative transposase (fragment)	4.09E-02	- 1.20	- 1.23	- 1.04	- 2.07	- 1.03	+ 1.15	- 1.73
NMB1747	Not in cogs genes	Putative tsbp protein	3.16E-03	+ 1.92	+ 1.54	+ 1.45	+ 1.17	- 1.24	- 1.33	- 1.64
NMB1749	Not in cogs genes	Conserved hypothetical protein	1.18E-04	+ 2.25	+ 2.16	+ 2.07	+ 2.00	- 1.04	- 1.09	- 1.12
NMB1750	Replication recombination and repair	Pilin gene inverting protein pivnm-2	2.49E-04	+ 2.62	+ 2.45	+ 2.33	- 1.20	- 1.07	- 1.12	- 3.14
NMB1753	Function unknown genes	Vapd-related protein	8.25E-03	- 1.33	- 1.79	- 1.67	- 2.39	- 1.35	- 1.26	- 1.81
NMB1754	Not in cogs genes	Cryptic plasmid protein A-related protein	3.18E-02	+ 1.65	+ 1.28	+ 1.33	+ 1.11	- 1.29	- 1.24	- 1.49
NMB1755	Not in cogs genes	Hypothetical protein	2.76E-02	- 1.86	- 1.62	- 1.29	- 2.80	+ 1.15	+ 1.44	- 1.50
NMB1757	Not in cogs genes	Hypothetical protein	2.96E-03	+ 1.47	+ 1.52	+ 1.64	- 2.21	+ 1.03	+ 1.11	- 3.26
NMB1758	Not in cogs genes	Hypothetical protein	1.73E-02	+ 1.15	- 1.51	- 1.35	- 2.90	- 1.73	- 1.55	- 3.34
NMB1759	Function unknown genes	Conserved hypothetical protein	7.58E-03	- 1.06	+ 1.02	- 1.09	- 2.38	+ 1.09	- 1.03	- 2.23
NMB1760	Not in cogs genes	Conserved hypothetical protein	1.08E-02	+ 1.47	+ 1.45	+ 1.44	- 2.10	- 1.01	- 1.02	- 3.09
NMB1762	Intracellular trafficking and secretion genes	Putative hemolysin activation protein hecb	1.43E-02	+ 1.23	+ 1.49	+ 1.46	+ 1.20	+ 1.21	+ 1.19	- 1.02
NMB1763	Posttranslational modification, protein turnover, chaperones genes	Putative toxin-activating protein	6.87E-03	+ 1.48	+ 1.46	+ 1.44	- 1.20	- 1.01	- 1.03	- 1.77
NMB1764	Not in cogs genes	Hypothetical protein	1.53E-02	+ 1.48	+ 1.08	+ 1.05	- 1.95	- 1.36	- 1.40	- 2.89
NMB1766	Not in cogs genes	Hypothetical protein	7.14E-03	+ 1.98	+ 1.54	+ 1.43	- 1.62	- 1.29	- 1.38	- 3.22
NMB1769	Not in cogs genes	N/a	3.88E-03	+ 1.70	+ 1.48	+ 1.47	- 1.46	- 1.15	- 1.16	- 2.47
NMB1771	Not in cogs genes	Hypothetical protein	5.25E-03	+ 1.45	+ 1.23	+ 1.22	- 2.05	- 1.18	- 1.19	- 2.98
NMB1772	Intracellular trafficking and secretion genes	Hypothetical protein	2.54E-03	+ 2.39	+ 1.69	+ 1.64	- 1.01	- 1.42	- 1.46	- 2.41

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1777	Not in cogs genes	Hypothetical protein conservative hypothetical protein	6.91E-03	+ 1.49	+ 1.30	+ 1.24	- 1.19	- 1.15	- 1.20	- 1.77
NMB1778	Not in cogs genes	Hypothetical protein conservative hypothetical protein	5.25E-03	+ 1.92	- 1.24	- 1.27	+ 1.13	- 2.38	- 2.44	- 1.70
NMB1781	Not in cogs genes	Hypothetical protein conserved hypothetical protein	2.44E-02	- 3.90	- 4.53	- 4.80	- 7.67	- 1.16	- 1.23	- 1.96
NMB1782	Not in cogs genes	Hypothetical protein	2.47E-02	+ 1.56	+ 1.14	+ 1.23	- 1.74	- 1.37	- 1.28	- 2.73
NMB1783	Not in cogs genes	Putative secretion protein (pseudogene)	9.05E-03	- 1.84	- 2.54	- 2.33	- 2.32	- 1.39	- 1.27	- 1.26
NMB1785	Not in cogs genes	Hypothetical protein putative lipoprotein	3.88E-02	+ 1.56	+ 1.13	+ 1.09	- 1.03	- 1.38	- 1.43	- 1.61
NMB1786	Function unknown genes	Hypothetical protein conserved hypothetical protein	2.21E-02	+ 1.57	+ 1.62	+ 1.56	- 1.03	+ 1.03	- 1.00	- 1.62
NMB1787	Amino acid transport and metabolism genes	N-acetyl-gamma-glutamyl-phosphate reductase	1.20E-02	+ 1.59	- 1.22	- 1.27	- 2.42	- 1.95	- 2.02	- 3.84
NMB1788	Replication recombination and repair transcription	ATP-dependent dna helicase recg	4.64E-02	+ 1.46	+ 1.40	+ 1.51	+ 1.01	- 1.05	+ 1.03	- 1.45
NMB1789	Intracellular trafficking and secretion genes	Protein-export protein secb	4.36E-05	- 2.81	+ 1.03	+ 1.01	+ 1.17	+ 2.90	+ 2.83	+ 3.30
NMB1790	Posttranslational modification, protein turnover, chaperones genes	Glutaredoxin 3	1.18E-04	- 2.84	- 1.17	- 1.21	- 1.03	+ 2.43	+ 2.34	+ 2.75
NMB1793	Not in cogs genes	Putative two-component system sensor kinase	4.31E-03	+ 1.13	+ 1.42	+ 1.50	- 2.96	+ 1.25	+ 1.32	- 3.36
NMB1794	Energy production and conversion genes	Citrate transporter	2.00E-04	+ 1.28	- 1.60	- 1.56	+ 1.16	- 2.05	- 2.00	- 1.11
NMB1795	Not in cogs genes	Hypothetical protein	1.18E-04	+ 3.45	+ 1.21	+ 1.60	- 3.33	- 2.85	- 2.16	- 11.51
NMB1796	General function prediction only genes	Conserved hypothetical protein	6.65E-04	- 2.46	+ 2.17	+ 2.05	+ 1.64	+ 5.34	+ 5.04	+ 4.03
NMB1797	Cell wall/membrane biogenesis genes	Penicillin-binding protein 3	1.95E-03	- 1.12	+ 1.91	+ 2.78	+ 1.05	+ 2.13	+ 3.11	+ 1.17
NMB1801	Cell wall/membrane biogenesis genes	Htrb/msbb family protein	1.39E-03	+ 1.00	- 1.58	- 1.40	- 1.16	- 1.58	- 1.40	- 1.16
NMB1802	Posttranslational modification, protein turnover, chaperones genes	O-sialoglycoprotein endopeptidase	2.72E-02	- 1.71	- 1.45	- 1.45	- 1.15	+ 1.18	+ 1.18	+ 1.49
NMB1803	Posttranslational modification, protein turnover, chaperones genes	Putative cytochrome c-type biogenesis protein	1.18E-04	+ 1.24	- 1.47	- 1.39	- 1.22	- 1.82	- 1.72	- 1.51
NMB1804	Posttranslational modification, protein turnover, chaperones genes	Putative cytochrome c-type biogenesis protein	4.86E-03	+ 1.26	- 1.24	- 1.30	- 1.03	- 1.56	- 1.64	- 1.29
NMB1805	Energy production and conversion genes	Cytochrome c4	2.89E-03	- 1.30	- 2.07	- 2.05	- 1.34	- 1.60	- 1.58	- 1.03
NMB1806	General function prediction only genes	Conserved hypothetical protein	2.53E-03	- 1.58	- 1.33	- 1.27	- 1.09	+ 1.19	+ 1.24	+ 1.44
NMB1807	Cell wall/membrane biogenesis genes	Penicillin-binding protein 1	1.15E-03	- 1.33	- 1.60	- 1.56	+ 1.28	- 1.20	- 1.17	+ 1.70
NMB1809	Fimbrial assembly-piln protein	Piln protein type IV pilus assembly protein piln	8.53E-04	- 1.88	- 1.39	- 1.59	- 1.17	+ 1.35	+ 1.18	+ 1.60
NMB1810	Intracellular trafficking and secretion genes Cell motility genes	Pilo protein type IV pilus assembly protein pilo	1.36E-02	- 2.25	- 1.24	- 1.62	- 1.74	+ 1.82	+ 1.39	+ 1.29
NMB1811	Not in cogs genes	Pilp protein type IV pilus assembly protein pilp	2.72E-03	- 2.21	- 1.31	- 1.51	- 1.02	+ 1.69	+ 1.47	+ 2.17
NMB1814	Amino acid transport and metabolism genes	3-dehydroquinase synthase	9.50E-04	+ 1.41	+ 1.61	+ 2.05	+ 1.11	+ 1.14	+ 1.45	- 1.27
NMB1816	Not in cogs genes	Conserved hypothetical protein	1.26E-02	+ 1.04	- 1.72	- 1.07	- 2.68	- 1.80	- 1.12	- 2.79

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1819	Not in cogs genes	Hypothetical protein	8.68E-03	+ 1.31	+ 1.63	+ 1.74	+ 1.13	+ 1.25	+ 1.33	- 1.16
NMB1822	Carbohydrate transport and metabolism genes Cell wall/membrane biogenesis genes	Pilin glycosylation protein pgld	3.68E-02	+ 1.36	+ 1.81	+ 1.46	+ 1.21	+ 1.33	+ 1.08	- 1.12
NMB1823	Amino acid transport and metabolism genes	Valine-pyruvate aminotransferase	4.00E-02	+ 1.26	+ 1.60	+ 1.49	- 1.00	+ 1.27	+ 1.18	- 1.26
NMB1824	General function prediction only genes	Conserved hypothetical protein	2.82E-02	+ 1.30	+ 1.74	+ 1.58	+ 1.20	+ 1.35	+ 1.22	- 1.08
NMB1825	Not in cogs genes	Hypothetical protein	1.34E-03	+ 2.11	+ 1.28	+ 1.39	+ 1.53	- 1.65	- 1.52	- 1.38
NMB1826	Function unknown genes	Conserved hypothetical protein	1.35E-02	+ 1.21	- 1.22	- 1.31	- 1.12	- 1.48	- 1.59	- 1.37
NMB1827	Replication recombination and repair	Dna polymerase III, alpha subunit	8.66E-04	+ 2.28	- 1.26	+ 1.09	- 2.27	- 2.86	- 2.10	- 5.15
NMB1831	Not in cogs genes	Lyb protein	1.34E-02	- 1.29	- 1.51	- 1.10	- 1.03	- 1.17	+ 1.18	+ 1.26
NMB1832	Intracellular trafficking and secretion genes, cell wall/membrane biogenesis genes	Signal peptidase II	6.80E-04	- 1.08	- 1.35	- 1.17	+ 1.16	- 1.25	- 1.08	+ 1.26
NMB1833	Translation	Isoleucyl-tRNA synthetase	2.30E-02	+ 1.47	+ 1.26	+ 1.34	+ 1.67	- 1.17	- 1.10	+ 1.14
NMB1834	Coenzyme transport and metabolism genes	Riboflavin kinase/FMN adenylyltransferase	1.87E-02	- 1.05	+ 1.14	+ 1.30	+ 1.32	+ 1.19	+ 1.36	+ 1.39
NMB1837	Not in cogs genes	Hypothetical protein	1.01E-02	- 1.60	+ 1.68	+ 1.31	+ 1.28	+ 2.68	+ 2.09	+ 2.04
NMB1839	Nucleotide transport and metabolism genes	Formate-tetrahydrofolate ligase	9.72E-03	+ 1.13	+ 1.37	+ 1.71	+ 1.93	+ 1.21	+ 1.52	+ 1.71
NMB1840	Function unknown genes	Conserved hypothetical protein integral membrane protein	2.07E-02	+ 1.05	- 1.48	- 1.27	- 1.35	- 1.56	- 1.33	- 1.42
NMB1841	Cell wall/membrane biogenesis genes translation	Mannose-1-phosphate guanylttransferase-related protein	4.25E-02	- 1.58	- 1.08	+ 1.17	- 1.29	+ 1.46	+ 1.86	+ 1.23
NMB1842	General function prediction only genes	Putative 4-hydroxyphenylacetate 3-hydroxylase, small subunit	2.38E-03	+ 1.53	- 1.67	- 1.37	- 2.33	- 2.56	- 2.10	- 3.58
NMB1844	Not in cogs genes	Hypothetical protein	4.89E-02	- 1.29	- 1.11	- 1.16	- 1.54	+ 1.17	+ 1.11	- 1.19
NMB1845	Energy production and conversion genes; posttranslational modification	Thioredoxin	3.82E-02	+ 1.30	- 1.10	- 1.01	+ 1.09	- 1.43	- 1.31	- 1.19
NMB1846	Cell cycle control, mitosis and meiosis genes	Mrp/NBP35 family protein	1.26E-02	+ 1.73	+ 1.27	+ 1.75	- 1.42	- 1.36	+ 1.01	- 2.46
NMB1848	Not in COG - hypothetical protein	Hypothetical protein	3.98E-04	- 3.04	- 2.13	- 1.93	- 2.85	+ 1.43	+ 1.58	+ 1.07
NMB1852	Function unknown genes	Conserved hypothetical protein	1.99E-03	+ 1.38	- 1.14	- 1.03	- 2.04	- 1.57	- 1.42	- 2.82
NMB1854	Not in cogs genes	Hypothetical protein	4.08E-03	- 1.64	+ 1.42	+ 1.17	- 2.22	+ 2.34	+ 1.92	- 1.35
NMB1857	General function prediction only genes	Modulator of drug activity B	6.01E-03	+ 1.17	+ 2.64	+ 1.67	+ 2.28	+ 2.27	+ 1.43	+ 1.96
NMB1858	Not in cogs genes	Hypothetical protein conserved hypothetical protein	1.83E-03	- 1.30	+ 4.08	+ 2.93	+ 4.87	+ 5.32	+ 3.83	+ 6.36
NMB1859	Not in cogs genes	S-adenosylmethionine:trna ribosyltransferase-isomerase	2.55E-02	- 1.61	- 1.35	- 1.00	- 1.13	+ 1.19	+ 1.60	+ 1.42
NMB1860	Lipid transport and metabolism genes	Acetyl-coa carboxylase, biotin carboxy	9.59E-03	- 1.56	- 1.34	- 1.21	- 2.61	+ 1.16	+ 1.29	- 1.67
NMB1863	RNA processing and modification genes	Oligoribonuclease	7.55E-03	+ 1.05	- 1.22	- 1.24	+ 1.00	- 1.28	- 1.30	- 1.05
NMB1865	Not in cogs genes	Hypothetical protein	1.96E-02	- 1.56	- 1.41	- 1.08	- 1.44	+ 1.11	+ 1.44	+ 1.08
NMB1868	Replication recombination and repair	Integrase/recombinase xerc	3.22E-02	- 1.19	- 1.10	+ 1.16	- 1.23	+ 1.08	+ 1.37	- 1.04

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1870	Not in cogs genes	Hypothetical protein lipoprotein	2.79E-02	+ 1.26	+ 1.19	+ 1.15	+ 1.35	- 1.06	- 1.10	+ 1.07
NMB1871	Posttranslational modification, protein turnover, chaperones genes	Conserved hypothetical protein	2.46E-02	+ 1.31	+ 1.69	+ 2.26	+ 1.41	+ 1.29	+ 1.73	+ 1.08
NMB1872	General function prediction only genes	Putative ribosomal-protein-alanine acetyltransferase	1.20E-02	+ 1.17	+ 1.70	+ 2.31	+ 1.25	+ 1.46	+ 1.97	+ 1.07
NMB1876	Amino acid transport and metabolism genes	N-acetylglutamate synthase	5.90E-03	- 1.78	- 1.11	- 1.10	- 2.21	+ 1.60	+ 1.62	- 1.24
NMB1879	Not in cogs genes	Hypothetical protein arac-family transcriptional regulator	3.75E-02	- 1.37	- 1.46	- 1.33	- 2.01	- 1.06	+ 1.03	- 1.47
NMB1880	Inorganic ion transport and metabolism genes	Putative ABC transporter, periplasmic solute-binding protein	2.86E-02	- 1.10	+ 1.58	+ 1.43	- 1.19	+ 1.73	+ 1.56	- 1.08
NMB1881	Hypothetical protein-mannose-1-phosphate guanylyltransferase	Conserved hypothetical protein	4.89E-02	- 1.39	- 1.61	- 1.57	- 2.90	- 1.16	- 1.13	- 2.08
NMB1882	Inorganic ion transport and metabolism genes	TonB-dependent receptor ferric siderophore receptor protein	7.01E-03	- 1.06	+ 1.14	+ 1.46	- 1.76	+ 1.21	+ 1.55	- 1.65
NMB1883	Not in cogs genes	Hypothetical protein	5.29E-05	- 1.64	- 2.94	- 2.65	- 2.07	- 1.80	- 1.62	- 1.27
NMB1884	Inorganic ion transport and metabolism genes	Conserved hypothetical protein	1.00E-03	+ 1.35	- 1.47	- 1.30	+ 1.48	- 1.98	- 1.75	+ 1.10
NMB1885	Posttranslational modification, protein turnover, chaperones genes	Protein-L-isopartate O-methyltransferase	1.50E-03	+ 1.08	- 1.58	- 1.65	+ 1.79	- 1.72	- 1.78	+ 1.65
NMB1886	Signal transduction mechanisms genes	Conserved hypothetical protein	1.19E-02	- 1.21	- 1.63	- 1.57	+ 1.20	- 1.34	- 1.30	+ 1.45
NMB1870	Not in cogs genes	Hypothetical protein lipoprotein	2.78E-02	+ 1.26	+ 1.19	+ 1.15	+ 1.35	- 1.06	- 1.10	+ 1.07
NMB1871	Posttranslational modification, protein turnover, chaperones genes	Conserved hypothetical protein	2.46E-02	+ 1.31	+ 1.69	+ 2.26	+ 1.41	+ 1.29	+ 1.73	+ 1.08
NMB1872	General function prediction only genes	Putative ribosomal-protein-alanine acetyltransferase	1.20E-02	+ 1.17	+ 1.70	+ 2.31	+ 1.25	+ 1.46	+ 1.97	+ 1.07
NMB1876	Amino acid transport and metabolism genes	N-acetylglutamate synthase	5.90E-03	- 1.78	- 1.11	- 1.10	- 2.21	+ 1.60	+ 1.62	- 1.24
NMB1879	Not in cogs genes	Hypothetical protein arac-family transcriptional regulator	3.75E-02	- 1.37	- 1.46	- 1.33	- 2.01	- 1.06	+ 1.03	- 1.47
NMB1880	Inorganic ion transport and metabolism genes	Putative ABC transporter, periplasmic solute-binding protein	2.86E-02	- 1.10	+ 1.58	+ 1.43	- 1.19	+ 1.73	+ 1.56	- 1.08
NMB1881	Hypothetical protein-mannose-1-phosphate guanylyltransferase	Conserved hypothetical protein	4.89E-02	- 1.39	- 1.61	- 1.57	- 2.90	- 1.16	- 1.13	- 2.08
NMB1882	Inorganic ion transport and metabolism genes	TonB-dependent receptor ferric siderophore receptor protein	7.01E-03	- 1.06	+ 1.14	+ 1.46	- 1.76	+ 1.21	+ 1.55	- 1.65
NMB1883	Not in cogs genes	Hypothetical protein	5.29E-05	- 1.64	- 2.94	- 2.65	- 2.07	- 1.80	- 1.62	- 1.27
NMB1884	Inorganic ion transport and metabolism genes	Conserved hypothetical protein	1.00E-03	+ 1.35	- 1.47	- 1.30	+ 1.48	- 1.98	- 1.75	+ 1.10
NMB1885	Posttranslational modification, protein turnover, chaperones genes	Protein-L-isopartate O-methyltransferase	1.50E-03	+ 1.08	- 1.58	- 1.65	+ 1.79	- 1.72	- 1.78	+ 1.65
NMB1886	Signal transduction mechanisms genes	Conserved hypothetical protein	1.19E-02	- 1.21	- 1.63	- 1.57	+ 1.20	- 1.34	- 1.30	+ 1.45
NMB1887	Carbohydrate transport and metabolism genes	Triosephosphate isomerase	1.36E-02	- 1.38	- 1.26	- 1.22	+ 1.02	+ 1.09	+ 1.13	+ 1.41
NMB1888	Not in cogs genes	Protein-export membrane protein secg	8.73E-04	- 2.06	+ 1.22	+ 1.19	- 1.11	+ 2.51	+ 2.46	+ 1.85
NMB1889	Not in cogs genes	Hypothetical protein	1.40E-02	+ 1.16	- 1.12	+ 1.09	- 1.58	- 1.29	- 1.06	- 1.83

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1890	Function unknown genes	Conserved hypothetical protein	2.43E-03	- 1.98	- 1.65	- 1.64	- 1.34	+ 1.20	+ 1.21	+ 1.48
NMB1891	Transcription	Helix-turn-helix family protein	2.23E-03	- 1.04	+ 1.02	+ 1.10	+ 2.56	+ 1.05	+ 1.14	+ 2.65
NMB1893	Not in cogs genes	Conserved hypothetical protein	9.85E-04	- 1.52	- 1.98	- 1.89	- 1.52	- 1.30	- 1.24	- 1.00
NMB1894	Hypothetical protein	Leucyl-trna synthetase, truncation	2.29E-03	+ 1.78	+ 1.31	+ 1.74	+ 1.13	- 1.36	- 1.02	- 1.58
NMB1895	Hypothetical protein	Dna adenine methylase, truncation	3.54E-03	+ 2.06	+ 1.89	+ 1.78	+ 1.00	- 1.10	- 1.16	- 2.06
NMB1896	Not in cogs genes	Type II restriction enzyme dpmi	1.41E-02	+ 1.42	+ 1.68	+ 1.66	- 1.34	+ 1.18	+ 1.17	- 1.91
NMB1898	Translation	Lipo protein	2.32E-02	+ 1.40	- 1.49	- 1.23	- 1.94	- 2.08	- 1.72	- 2.70
NMB1902	Replication recombination and repair	Dna polymerase iii, beta subunit	1.45E-02	- 1.28	- 1.78	- 1.40	- 1.13	- 1.39	- 1.09	+ 1.13
NMB1903	Replication recombination and repair	Chromosomal replication initiator protein dnaa	2.89E-02	+ 1.23	+ 1.07	+ 1.15	+ 1.52	- 1.15	- 1.07	+ 1.24
NMB1904	Not in cogs genes	50S ribosomal protein l34	5.98E-05	- 2.59	+ 1.47	+ 2.03	+ 1.14	+ 3.80	+ 5.26	+ 2.94
NMB1905	Translation	Ribonuclease p protein component	9.79E-04	+ 1.51	+ 2.43	+ 2.28	+ 1.39	+ 1.61	+ 1.51	- 1.09
NMB1906	Not in cogs genes	Conserved hypothetical protein	3.93E-02	+ 1.02	+ 1.77	+ 1.62	+ 1.04	+ 1.73	+ 1.58	+ 1.02
NMB1908	General function prediction only genes	Conserved hypothetical protein hypothetical protein	1.95E-03	- 1.35	- 1.34	- 1.51	+ 1.71	+ 1.01	- 1.12	+ 2.31
NMB1909	Cell cycle control, mitosis and meiosis genes	Maf/ycef/yhde family protein	4.57E-03	- 1.65	- 1.31	- 1.17	+ 1.94	+ 1.26	+ 1.41	+ 3.19
NMB1910	General function prediction only genes	Conserved hypothetical protein	4.46E-04	- 2.17	+ 3.83	+ 2.65	+ 3.38	+ 8.30	+ 5.74	+ 7.33
NMB1911	Translation	50s ribosomal protein l32	7.52E-05	- 1.84	+ 4.46	+ 2.97	+ 2.14	+ 8.19	+ 5.45	+ 3.94
NMB1914	Not in cogs genes	Hypothetical protein conserved hypothetical protein	5.66E-04	+ 1.76	+ 1.41	+ 1.14	- 1.45	- 1.25	- 1.55	- 2.55
NMB1915	Not in cogs genes	Hypothetical protein conserved hypothetical protein	4.56E-02	+ 1.13	+ 1.25	+ 1.32	- 1.29	+ 1.11	+ 1.17	- 1.45
NMB1918	Lipid transport and metabolism genes	Malonyl coa-acyl carrier protein transacylase	4.75E-02	- 1.13	+ 1.10	+ 1.15	- 1.34	+ 1.25	+ 1.30	- 1.18
NMB1919	Defense mechanisms genes	ABC transporter, ATP-binding protein	2.47E-02	+ 1.03	- 1.20	+ 1.13	- 1.60	- 1.24	+ 1.10	- 1.65
NMB1923	Function unknown genes	Conserved hypothetical protein	6.56E-03	- 1.51	+ 1.90	+ 1.38	+ 1.78	+ 2.87	+ 2.08	+ 2.69
NMB1924	Carbohydrate transport and metabolism genes	Inositol monophosphatase family protein	6.21E-04	+ 1.39	- 1.39	- 1.50	+ 1.25	- 1.92	- 2.08	- 1.11
NMB1925	Function unknown genes	Conserved hypothetical protein	7.95E-04	+ 1.14	- 1.69	- 1.32	- 2.78	- 1.92	- 1.51	- 3.16
NMB1926	Cell wall/membrane biogenesis genes	Lacto-N-neotetraose biosynthesis glycosyl transferase lgtc	1.11E-03	+ 1.13	+ 1.04	+ 1.03	- 1.91	- 1.08	- 1.10	- 2.15
NMB1927	Not in cogs genes	Lacto-N-neotetraose biosynthesis glycosyl transferase-related protein	4.46E-03	- 1.07	- 2.08	- 1.77	- 1.20	- 1.93	- 1.65	- 1.11
NMB1928	Cell wall/membrane biogenesis genes	Lacto-N-neotetraose biosynthesis glycosyl transferase lgtb	4.91E-04	+ 1.15	- 1.20	- 1.22	- 1.29	- 1.38	- 1.40	- 1.48
NMB1929	Cell wall/membrane biogenesis genes	Lacto-N-neotetraose biosynthesis glycosyl transferase lgtc	4.65E-04	+ 1.05	- 1.37	- 1.42	- 1.14	- 1.43	- 1.49	- 1.20
NMB1931	Not in cogs genes	Hypothetical protein conserved hypothetical protein	3.33E-03	- 1.13	- 1.23	- 1.30	+ 1.24	- 1.08	- 1.15	+ 1.40
NMB1941	Energy production and conversion genes	Hypothetical protein ATP synthase I	2.56E-02	+ 1.82	- 1.10	- 1.04	+ 1.70	- 2.00	- 1.88	- 1.07

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1944	Transcription	ParB family protein putative chromosome segregation proteins	1.31E-02	- 1.04	+ 1.26	+ 1.53	+ 1.53	+ 1.31	+ 1.59	+ 1.59
NMB1945	Coenzyme transport and metabolism genes	3-octaprenyl-4-hydroxybenzoate carboxylase	6.19E-04	+ 1.34	- 1.74	- 1.60	- 2.23	- 2.34	- 2.15	- 3.00
NMB1946	Inorganic ion transport and metabolism genes	Outer membrane lipoprotein	1.15E-02	- 1.38	+ 1.05	+ 1.29	+ 1.20	+ 1.45	+ 1.78	+ 1.66
NMB1949	Cell wall/membrane biogenesis genes	Putative soluble lytic murein transglycosylase	4.31E-02	+ 1.22	+ 1.16	+ 1.77	- 1.09	- 1.05	+ 1.45	- 1.33
NMB1950	Translation	30S ribosomal protein s21	2.46E-04	- 1.39	+ 3.68	+ 2.76	+ 2.66	+ 5.12	+ 3.84	+ 3.71
NMB1953	Posttranslational modification, protein turnover, chaperones genes	Stringent starvation protein A	2.34E-03	- 2.29	- 1.15	- 1.22	+ 1.38	+ 2.00	+ 1.88	+ 3.16
NMB1955	Not in cogs genes	Cadmium resistance protein	1.40E-02	+ 1.14	- 1.48	- 1.01	- 2.60	- 1.70	- 1.16	- 2.98
NMB1956	Translation	50S ribosomal protein l31	3.41E-04	- 2.34	+ 1.67	+ 2.08	+ 1.49	+ 3.91	+ 4.85	+ 3.48
NMB1959	General function prediction only genes	Conserved hypothetical protein	3.59E-02	+ 1.20	+ 2.50	+ 1.65	- 1.27	+ 2.08	+ 1.37	- 1.53
NMB1961	Not in cogs genes	VacJ-related protein	3.95E-02	+ 1.06	- 1.30	+ 1.01	- 1.70	- 1.38	- 1.05	- 1.80
NMB1962	General function prediction only genes	Hypothetical protein conserved hypothetical protein	2.38E-02	- 1.87	+ 1.00	+ 1.22	- 1.21	+ 1.88	+ 2.29	+ 1.55
NMB1963	Secondary metabolites biosynthesis, transport and catabolism genes	Conserved hypothetical protein periplasmic transport protein	4.71E-03	- 1.02	+ 1.77	+ 2.30	+ 1.18	+ 1.81	+ 2.36	+ 1.21
NMB1965	Not in cogs genes	Conserved hypothetical protein putative ABC transport system permease protein	2.18E-02	+ 1.02	+ 1.12	+ 1.47	+ 1.19	+ 1.09	+ 1.43	+ 1.16
NMB1967	Not in cogs genes	Transcriptional regulator, arac family	4.76E-03	- 1.57	- 1.41	- 1.29	- 1.15	+ 1.11	+ 1.22	+ 1.37
NMB1969	Not in cogs genes	Putative serotype-1-specific antigen autotransporter serine protease	3.67E-02	- 1.49	- 1.47	- 1.50	- 1.50	+ 1.01	- 1.01	- 1.01
NMB1971	Not in cogs genes	Conserved hypothetical protein	6.96E-03	+ 1.19	- 1.75	- 3.17	- 2.53	- 2.08	- 3.76	- 3.00
NMB1972	Posttranslational modification, protein turnover, chaperones genes	Chaperonin, 60 kDa	8.45E-03	- 1.39	+ 1.87	+ 1.52	+ 1.98	+ 2.60	+ 2.11	+ 2.75
NMB1973	Posttranslational modification, protein turnover, chaperones genes	Chaperonin, 10 kDa	2.08E-02	+ 1.44	+ 1.85	+ 2.40	+ 3.20	+ 1.28	+ 1.66	+ 2.22
NMB1976	Amino acid transport and metabolism genes	Diaminopimelate decarboxylase	2.08E-03	+ 1.03	- 1.40	- 1.28	- 1.04	- 1.44	- 1.32	- 1.07
NMB1977	Not in cogs genes	Hypothetical protein lipoprotein	9.19E-04	- 1.57	- 2.23	- 2.03	- 1.30	- 1.42	- 1.30	+ 1.20
NMB1978	Not in cogs genes	Cyay protein	3.91E-03	- 1.25	+ 1.00	- 1.07	+ 2.01	+ 1.25	+ 1.16	+ 2.51
NMB1979	Not in cogs genes	Conserved hypothetical protein; hypothetical inner membrane protein	7.91E-04	+ 1.41	+ 1.74	+ 1.95	- 1.11	+ 1.24	+ 1.39	- 1.56
NMB1980	Function unknown genes	Conserved hypothetical protein	1.54E-02	+ 1.26	+ 1.58	+ 1.29	+ 1.03	+ 1.26	+ 1.03	- 1.22
NMB1981	Not in cogs genes	Conserved hypothetical protein	9.03E-04	- 1.10	- 1.55	- 1.45	+ 1.15	- 1.42	- 1.32	+ 1.26
NMB1982	Replication recombination and repair	Dna polymerase i	2.68E-02	+ 1.51	+ 1.28	+ 1.46	- 1.03	- 1.18	- 1.04	- 1.56
NMB1984	Not in cogs genes	IS1106A3 transposase	2.86E-03	- 2.31	+ 1.67	+ 1.09	- 1.02	+ 3.86	+ 2.52	+ 2.27
NMB1988	Inorganic ion transport and metabolism genes	Iron-regulated outer membrane protein frpb	5.86E-03	- 1.53	- 1.33	- 1.21	- 1.24	+ 1.15	+ 1.27	+ 1.24
NMB1989	Inorganic ion transport and metabolism genes	Iron(III) ABC transporter, periplasmic-binding protein	2.72E-03	+ 1.85	- 1.31	- 1.12	- 1.94	- 2.42	- 2.06	- 3.59

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB1990	Inorganic ion transport and metabolism genes	Iron(III) ABC transporter, permease protein	2.48E-04	+ 1.21	- 2.81	- 1.94	- 4.17	- 3.41	- 2.36	- 5.06
NMB1995	Amino acid transport and metabolism genes	Nitrogen regulatory protein P-II	4.21E-03	+ 1.31	- 1.08	- 1.13	+ 2.62	- 1.41	- 1.48	+ 2.00
NMB1998	Intracellular trafficking and secretion genes Cell wall/membrane biogenesis genes	Serine-type peptidase serine-type peptidase (pseudogene)	3.29E-02	- 1.58	- 1.39	- 1.35	- 1.47	+ 1.13	+ 1.17	+ 1.07
NMB2000	Not in cogs genes	Conserved hypothetical protein	3.68E-04	- 1.72	+ 1.01	- 1.11	+ 1.54	+ 1.74	+ 1.55	+ 2.64
NMB2001	Cell wall/membrane biogenesis genes	Conserved hypothetical protein, outer membrane protein precursor	3.00E-02	+ 1.44	+ 1.11	+ 1.56	- 1.05	- 1.29	+ 1.09	- 1.51
NMB2003	General function prediction only genes	Conserved hypothetical protein	7.91E-04	+ 1.25	- 1.91	- 1.63	- 1.41	- 2.38	- 2.03	- 1.76
NMB2005	Not in cogs genes	Glutamate N-acetyltransferase/amino-acid acetyltransferase	1.19E-02	+ 1.33	+ 1.02	+ 1.38	- 1.98	- 1.29	+ 1.04	- 2.63
NMB2008	Not in cogs genes	ABC transporter, ATP-binding protein-related protein	1.29E-02	+ 1.42	+ 1.15	+ 1.05	- 2.06	- 1.24	- 1.35	- 2.93
NMB2011	ATP-dependent RNA helicase hrpA truncation	ATP-dependent RNA helicase hrpA, truncation	1.57E-02	+ 1.32	+ 1.91	+ 1.59	+ 1.37	+ 1.44	+ 1.20	+ 1.04
NMB2015	Not in cogs genes	Hypothetical protein hypothetical protein NMA0425	8.75E-03	+ 2.23	+ 1.07	+ 1.29	- 1.22	- 2.07	- 1.72	- 2.71
NMB2016	Intracellular trafficking and secretion genes Cell motility genes	Type IV pilin-related protein	1.54E-02	+ 1.30	+ 1.11	+ 1.23	- 1.81	- 1.17	- 1.06	- 2.35
NMB2019	Coenzyme transport and metabolism genes	Lipopolysaccharide core biosynthesis protein kdtb	4.31E-02	+ 1.01	+ 1.01	+ 1.05	+ 1.29	- 1.01	+ 1.04	+ 1.28
NMB2020	General function prediction only genes	Conserved hypothetical protein integral membrane protein	3.07E-03	- 1.27	+ 1.39	+ 1.69	+ 1.96	+ 1.76	+ 2.14	+ 2.49
NMB2021	Not in cogs genes	Conserved hypothetical protein	2.19E-02	- 1.07	+ 1.11	+ 1.21	+ 1.79	+ 1.19	+ 1.29	+ 1.91
NMB2022	Function unknown genes	Conserved hypothetical protein	6.52E-03	- 1.09	+ 1.63	+ 2.03	+ 1.62	+ 1.77	+ 2.21	+ 1.77
NMB2023	Not in cogs genes	Conserved hypothetical protein	2.80E-03	+ 1.25	+ 1.46	+ 1.93	+ 1.64	+ 1.16	+ 1.54	+ 1.31
NMB2027	Amino acid transport and metabolism genes, Carbohydrate transport and metabolism genes	Glucanate permease	6.30E-03	+ 1.01	+ 1.27	+ 1.40	+ 1.15	+ 1.26	+ 1.39	+ 1.14
NMB2033	Amino acid transport and metabolism genes	Putative histidinol-phosphatase	1.48E-02	+ 1.06	- 1.60	- 1.08	- 1.67	- 1.69	- 1.14	- 1.77
NMB2037	Signal transduction mechanisms genes	Hypothetical protein	1.87E-02	+ 1.71	+ 1.26	+ 1.34	+ 1.32	- 1.35	- 1.27	- 1.29
NMB2038	Signal transduction mechanisms genes	Penik-related protein	3.25E-02	- 1.01	- 1.20	+ 1.11	- 1.38	- 1.19	+ 1.12	- 1.36
NMB2039	Cell wall/membrane biogenesis genes	Major outer membrane protein PIB	7.52E-05	- 4.61	+ 3.15	+ 2.05	+ 3.63	+ 14.52	+ 9.45	+ 16.75
NMB2041	Not in cogs genes	Thiamin pyrophosphokinase-related protein	2.22E-04	+ 1.38	- 1.23	- 1.18	- 1.08	- 1.70	- 1.62	- 1.49
NMB2044	Carbohydrate transport and metabolism genes	Phosphoenolpyruvate-protein phosphotransferase	8.09E-03	+ 1.78	+ 1.39	+ 1.66	+ 1.01	- 1.28	- 1.07	- 1.77
NMB2047	Nucleotide transport and metabolism genes	Putative hypoxanthine-guanine phosphoribosyltransferase	1.82E-02	- 1.00	- 1.41	- 1.29	+ 1.00	- 1.41	- 1.29	+ 1.00
NMB2048	Replication recombination and repair	Dna ligase	1.16E-02	- 1.22	- 1.91	- 1.60	- 2.35	- 1.56	- 1.31	- 1.92
NMB2050	Function unknown genes	Conserved hypothetical protein	1.03E-06	- 1.39	- 2.04	- 2.22	+ 1.41	- 1.46	- 1.59	+ 1.96
NMB2053	Energy production and conversion genes	Ubiquinol-cytochrome c reductase, iron-sulfur subunit	1.07E-03	- 1.22	- 3.41	- 2.26	- 1.64	- 2.80	- 1.86	- 1.34
NMB2054	Function unknown genes	Conserved hypothetical protein	1.29E-04	- 1.20	- 1.80	- 1.72	- 1.11	- 1.50	- 1.43	+ 1.08

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB2055	Transcription	Transcriptional regulator, lysr family	3.85E-02	- 1.37	- 1.32	+	- 1.11	+	+	+
NMB2056	Not in cogs genes	30S ribosomal protein S9	3.77E-04	- 1.69	+	+	+	+	+	+
NMB2057	Not in cogs genes	50S ribosomal protein L13	1.18E-04	- 2.04	+	+	+	+	+	+
NMB2058	Function unknown genes	Conserved hypothetical protein	1.95E-03	+	- 1.89	-	+	-	-	+
NMB2059	Not in cogs genes	Hypothetical protein conserved hypothetical protein	4.25E-02	- 1.51	- 1.09	-	- 1.13	+	+	+
NMB2062	Coenzyme transport and metabolism genes	Thif protein 5-(beta-hydroxyethylthiazole monophosphate synthesis protein	2.78E-02	- 1.02	+	+	- 1.38	+	+	-
NMB2067	Nucleotide transport and metabolism genes	Probable hydroxymethylpyrimidine transporter cyfx	2.24E-02	+	+	+	+	-	+	-
NMB2069	Coenzyme transport and metabolism genes	Thiamin-phosphate pyrophosphorylase	9.03E-04	+	+	+	- 2.00	-	+	-
NMB2070	Coenzyme transport and metabolism genes	Thiamine biosynthesis protein this	3.31E-02	+	+	+	+	-	-	-
NMB2071	Coenzyme transport and metabolism genes	Thig protein thiamine biosynthesis protein	8.56E-03	+	+	+	+	+	+	-
NMB2072	Not in cogs genes	Hypothetical protein	3.69E-02	+	+	+	+	+	+	-
NMB2074	Not in cogs genes	Hypothetical protein	3.52E-03	- 1.04	+	+	+	+	+	+
NMB2076	Cell wall/membrane biogenesis genes	Aut protein transferase	4.68E-02	+	+	+	+	+	+	-
NMB2078	Not in cogs genes	Conserved hypothetical protein	2.75E-02	+	+	+	- 1.88	-	-	-
NMB2080	Not in cogs genes	Hypothetical protein	2.51E-04	+	- 1.99	-	- 2.93	-	-	-
NMB2081	Not in cogs genes	Hypothetical protein	2.48E-04	- 1.38	- 1.86	-	+	-	-	+
NMB2082	Replication recombination and repair	Exodeoxyribonuclease	1.30E-03	+	- 1.04	+	- 1.06	-	-	-
NMB2083	Translation	CysteinyI-trna synthetase	3.16E-02	+	+	+	- 1.05	-	-	-
NMB2085	General function prediction only genes	Hypothetical protein	3.98E-04	- 1.79	- 1.37	-	- 4.12	+	+	-
NMB2090	Carbohydrate transport and metabolism genes	Phosphoheptose isomerase	2.77E-03	- 2.05	+	+	- 1.25	+	+	+
NMB2091	General function prediction only genes	Putative hemolysin	4.93E-02	+	+	+	+	+	+	+
NMB2092	Not in cogs genes	Periplasmic protein	1.07E-02	+	+	+	+	+	+	+
NMB2094	Transcription	conserved hypothetical protein	1.45E-02	- 2.03	+	-	+	+	+	+
NMB2095	Not in cogs genes	Putative adhesin complex protein	3.98E-04	- 2.94	+	+	+	+	+	+
NMB2097	Not in cogs genes	Hypothetical protein	5.98E-04	+	+	-	+	-	-	-
NMB2099	Not in cogs genes	Conserved hypothetical protein	2.29E-02	+	+	+	+	+	+	+
NMB2101	Not in cogs genes	30S ribosomal protein S2	8.98E-04	- 1.29	+	+	+	+	+	+
NMB2102	Not in cogs genes	Elongation factor TS (EF-TS)	6.56E-03	+	+	+	+	+	+	+
NMB2103	Nucleotide transport and metabolism genes	Uridylate kinase	1.55E-02	+	- 1.14	-	- 1.21	-	-	-
NMB2105	Not in cogs genes	MafB protein adhesin putative mafB-like protein	2.94E-02	+	+	+	- 1.10	-	-	-

Table B - continued. Complete list of differentially expressed genes.

Gene-ID	COG	Description (KEGG)	p-value	4 h vs 3 h- DMEM	24 h vs 3 h- DMEM	96 h vs 3 h- DMEM	21 d vs 3 h- DMEM	24 h vs 4 h	96 h vs 4 h	21 d vs 4 h
NMB2106	Not in cogs genes	Conserved hypothetical protein	3.69E-02	- 1.11	- 1.38	- 1.77	- 3.86	- 1.25	- 1.60	- 3.49
NMB2110	Not in cogs genes	Conserved hypothetical protein	7.14E-03	+ 1.37	+ 1.35	+ 1.24	- 1.59	- 1.01	- 1.10	- 2.18
NMB2111	Not in cogs genes	MafB-related protein	5.61E-04	+ 2.03	+ 1.72	+ 1.65	- 1.40	- 1.18	- 1.23	- 2.84
NMB2113	Not in cogs genes	Hypothetical protein	2.00E-02	+ 1.15	+ 1.42	+ 1.06	- 2.15	+ 1.23	- 1.09	- 2.48
NMB2115	Not in cogs genes	Hypothetical protein	1.34E-02	+ 1.78	+ 1.23	+ 1.18	- 1.22	- 1.45	- 1.51	- 2.17
NMB2118	Not in cogs genes	Hypothetical protein	3.31E-03	+ 1.71	- 1.01	+ 1.13	- 1.11	- 1.72	- 1.51	- 1.89
NMB2119	Not in cogs genes	MafB-related protein	2.14E-02	+ 1.46	+ 1.58	+ 1.38	- 1.25	+ 1.09	- 1.05	- 1.83
NMB2120	Not in cogs genes	Hypothetical protein	1.55E-02	+ 1.53	+ 1.10	+ 1.03	- 1.78	- 1.38	- 1.48	- 2.72
NMB2123	Not in cogs genes	Hypothetical protein; conserved hypothetical protein	1.45E-02	+ 1.02	+ 1.02	- 1.03	- 3.29	- 1.00	- 1.05	- 3.35
NMB2124	Not in cogs genes	Hypothetical protein; conserved hypothetical protein	6.71E-03	+ 1.62	- 1.05	- 1.02	- 1.87	- 1.70	- 1.64	- 3.02
NMB2125	Not in cogs genes	Hypothetical protein	8.49E-03	+ 1.32	- 1.35	- 1.24	- 2.06	- 1.78	- 1.64	- 2.72
NMB2126	Not in cogs genes	IS1016 transposase	2.76E-02	+ 1.42	- 1.23	+ 1.00	- 1.77	- 1.74	- 1.41	- 2.50
NMB2129	Amino acid transport and metabolism genes	Argininosuccinate synthase	3.07E-03	- 1.34	- 1.63	- 1.48	- 1.29	- 1.22	- 1.11	+ 1.04
NMB2131	Not in cogs genes	Hypothetical protein; integral membrane protein	4.89E-04	+ 1.14	- 1.98	- 1.51	- 1.26	- 2.26	- 1.71	- 1.43
NMB2132	Not in cogs genes	Transferrin-binding protein-related protein	3.86E-02	- 1.28	- 1.36	- 1.50	- 1.33	- 1.06	- 1.17	- 1.04
NMB2137	Not in cogs genes	Hypothetical protein; conserved hypothetical protein	1.17E-03	- 7.62	+ 3.35	+ 1.27	+ 3.35	+ 25.53	+ 9.64	+ 25.53
NMB2138	Translation	Peptide chain release factor 2	7.72E-03	- 1.14	- 1.27	- 1.46	- 1.02	- 1.12	- 1.28	+ 1.12
NMB2139	General function prediction only genes	Conserved hypothetical protein; lipoprotein	2.41E-02	+ 1.01	+ 1.29	+ 1.26	+ 1.19	+ 1.28	+ 1.25	+ 1.18
NMB2140	Function unknown genes	Conserved hypothetical protein; integral membrane protein	9.58E-03	+ 1.59	+ 2.32	+ 1.83	+ 1.32	+ 1.46	+ 1.16	- 1.20
NMB2141	Not in cogs genes	Hypothetical protein; periplasmic protein	2.14E-05	- 3.65	+ 1.59	- 1.15	- 1.05	+ 5.80	+ 3.16	+ 3.47
NMB2144	Transcription	Putative sigma factor	2.94E-02	- 1.22	- 1.70	- 1.74	- 1.99	- 1.40	- 1.43	- 1.64
NMB2145	Not in cogs genes	Conserved hypothetical protein	8.03E-04	+ 1.93	+ 1.96	+ 1.90	- 1.28	+ 1.02	- 1.01	- 2.47
NMB2146	Not in cogs genes	Hypothetical protein	3.59E-02	- 1.59	- 1.39	- 1.76	- 3.78	+ 1.14	- 1.11	- 2.39
NMB2147	Not in cogs genes	Hypothetical protein	1.71E-02	- 1.14	+ 1.09	+ 1.19	- 2.43	+ 1.24	+ 1.35	- 2.14
NMB2149	Not in cogs genes	Hypothetical protein	4.76E-03	+ 1.08	- 1.48	+ 1.03	- 2.70	- 1.60	- 1.04	- 2.92
NMB2150	Translation	Conserved hypothetical protein; site	6.80E-04	+ 1.66	+ 1.38	+ 1.22	- 1.03	- 1.20	- 1.36	- 1.71
NMB2151	Nucleotide transport and metabolism genes	Phosphoribosylamine-glycine ligase	1.54E-02	- 1.15	- 1.38	- 1.49	+ 1.02	- 1.20	- 1.30	+ 1.17
NMB2152	Not in cogs genes	Conserved hypothetical protein	2.03E-02	+ 1.75	+ 1.54	+ 1.45	- 1.55	- 1.14	- 1.21	- 2.72
NMB2159	Carbohydrate transport and metabolism genes	Glyceraldehyde 3-phosphate dehydrogenase	2.19E-02	- 1.63	+ 1.51	+ 2.15	+ 1.14	+ 2.47	+ 3.51	+ 1.86
NMB2160	Replication recombination and repair	Dna mismatch repair protein muts	7.65E-03	- 1.16	- 1.22	+ 1.06	- 1.62	- 1.05	+ 1.23	- 1.39

Table C: Common and unique identified genes at 24 h and 96 h vs 3 h-DMEM.as shown in Figure 5.4.
Expression profiles of each genes are shown in Table B.

145 common DOWN-regulated genes at 24 h and 96 h vs 3 h-DMEM					257 common UP-regulated genes at 24 h and 96 h vs 3 h-DMEM							
NMB0001	NMB0582	NMB0918	NMB1500	NMB1909	NMB0006	NMB0151	NMB0343	NMB0634	NMB0831	NMB1153	NMB1545	NMB1888
NMB0005	NMB0615	NMB0957	NMB1507	NMB1924	NMB0016	NMB0152	NMB0344	NMB0641	NMB0840	NMB1154	NMB1546	NMB1891
NMB0034	NMB0619	NMB0963	NMB1512	NMB1925	NMB0018	NMB0153	NMB0345	NMB0648	NMB0857	NMB1156	NMB1549	NMB1904
NMB0036	NMB0620	NMB0982	NMB1516	NMB1927	NMB0027	NMB0154	NMB0346	NMB0658	NMB0870	NMB1215	NMB1557	NMB1905
NMB0041	NMB0649	NMB1062	NMB1517	NMB1945	NMB0046	NMB0155	NMB0347	NMB0659	NMB0875	NMB1230	NMB1567	NMB1910
NMB0043	NMB0650	NMB1063	NMB1527	NMB1953	NMB0075	NMB0156	NMB0348	NMB0665	NMB0876	NMB1231	NMB1590	NMB1911
NMB0047	NMB0669	NMB1064	NMB1538	NMB1971	NMB0093	NMB0157	NMB0362	NMB0677	NMB0883	NMB1290	NMB1622	NMB1914
NMB0053	NMB0672	NMB1070	NMB1539	NMB1977	NMB0098	NMB0158	NMB0382	NMB0689	NMB0900	NMB1301	NMB1635	NMB1923
NMB0064	NMB0691	NMB1076	NMB1541	NMB1989	NMB0102	NMB0159	NMB0403	NMB0712	NMB0937	NMB1311	NMB1641	NMB1926
NMB0068	NMB0703	NMB1107	NMB1604	NMB1990	NMB0124	NMB0160	NMB0430	NMB0722	NMB0941	NMB1313	NMB1642	NMB1950
NMB0069	NMB0709	NMB1125	NMB1669	NMB1995	NMB0125	NMB0161	NMB0449	NMB0723	NMB0942	NMB1320	NMB1643	NMB1956
NMB0070	NMB0717	NMB1126	NMB1681	NMB2003	NMB0127	NMB0162	NMB0450	NMB0724	NMB0946	NMB1321	NMB1650	NMB1963
NMB0085	NMB0730	NMB1138	NMB1685	NMB2050	NMB0128	NMB0163	NMB0463	NMB0725	NMB0969	NMB1322	NMB1662	NMB1972
NMB0202	NMB0773	NMB1205	NMB1698	NMB2053	NMB0129	NMB0164	NMB0466	NMB0731	NMB0983	NMB1323	NMB1710	NMB1979
NMB0217	NMB0779	NMB1207	NMB1729	NMB2058	NMB0130	NMB0165	NMB0467	NMB0744	NMB0994	NMB1335	NMB1732	NMB2015
NMB0261	NMB0783	NMB1211	NMB1753	NMB2080	NMB0131	NMB0166	NMB0471	NMB0745	NMB0995	NMB1341	NMB1749	NMB2020
NMB0265	NMB0801	NMB1221	NMB1778	NMB2081	NMB0132	NMB0167	NMB0491	NMB0746	NMB1035	NMB1343	NMB1750	NMB2022
NMB0283	NMB0810	NMB1247	NMB1783	NMB2085	NMB0133	NMB0168	NMB0506	NMB0748	NMB1055	NMB1355	NMB1757	NMB2039
NMB0288	NMB0847	NMB1296	NMB1790	NMB2124	NMB0136	NMB0169	NMB0550	NMB0753	NMB1056	NMB1368	NMB1766	NMB2056
NMB0292	NMB0848	NMB1317	NMB1794	NMB2125	NMB0137	NMB0196	NMB0554	NMB0754	NMB1074	NMB1370	NMB1771	NMB2057
NMB0308	NMB0858	NMB1337	NMB1805	NMB2131	NMB0138	NMB0200	NMB0579	NMB0758	NMB1091	NMB1378	NMB1772	NMB2069
NMB0339	NMB0861	NMB1352	NMB1807		NMB0140	NMB0201	NMB0580	NMB0762	NMB1092	NMB1379	NMB1789	NMB2090
NMB0378	NMB0864	NMB1360	NMB1811		NMB0142	NMB0220	NMB0589	NMB0763	NMB1094	NMB1380	NMB1795	NMB2095
NMB0396	NMB0865	NMB1387	NMB1842		NMB0143	NMB0225	NMB0590	NMB0787	NMB1096	NMB1381	NMB1796	NMB2101
NMB0419	NMB0866	NMB1404	NMB1852		NMB0144	NMB0252	NMB0591	NMB0788	NMB1098	NMB1427	NMB1797	NMB2102
NMB0460	NMB0886	NMB1437	NMB1860		NMB0145	NMB0297	NMB0592	NMB0789	NMB1108	NMB1429	NMB1814	NMB2110
NMB0477	NMB0896	NMB1441	NMB1876		NMB0146	NMB0298	NMB0595	NMB0790	NMB1111	NMB1460	NMB1825	NMB2137
NMB0520	NMB0898	NMB1444	NMB1883		NMB0147	NMB0316	NMB0604	NMB0791	NMB1114	NMB1502	NMB1854	NMB2140
NMB0526	NMB0912	NMB1452	NMB1884		NMB0148	NMB0321	NMB0605	NMB0792	NMB1116	NMB1503	NMB1857	NMB2145
NMB0543	NMB0914	NMB1459	NMB1885		NMB0149	NMB0324	NMB0609	NMB0817	NMB1135	NMB1523	NMB1858	
	NMB0917	NMB1472	NMB1908		NMB0150	NMB0325	NMB0625	NMB0822	NMB1136	NMB1542	NMB1882	
Unique up- and down-regulated genes at 24 h and 96 h vs 3 h-DMEM												
17 genes DOWN-regulated at 24 h vs 3 h-DMEM and UP-regulated at 96h vs 3 h-DMEM					10 genes UP-regulated at 24 h vs 3 h-DMEM and DOWN-regulated genes at 96 h vs 3 h-DMEM							
			NMB0087						NMB0076			
			NMB0268						NMB0350			
			NMB0322						NMB0968			
			NMB0330						NMB1366			
			NMB0546						NMB1499			
			NMB0627						NMB1564			
			NMB0681						NMB1759			
			NMB0749						NMB1978			
			NMB0814						NMB2000			
			NMB0862						NMB2141			
			NMB0897									
			NMB1245									
			NMB1462									
			NMB1623									
			NMB1678									
			NMB1827									
			NMB2149									

Table D: Common and unique genes at 4 h vs 3 h-DMEM, 24 – 96 h vs 3 h-DMEM and 21 days vs 3 h-DMEM. Gene lists of Venn diagrams in Figure 5.5. Expression profiles of each gene are shown in Table B.

55 common UP-regulated genes at 4h, 24-96, 21 days vs 3 h-DMEM	112 common UP-regulated genes at 24-96 h and 21 days vs 3 h-DMEM	22 UP-regulated genes at 4 h and 21 days vs 3 h-DMEM	45 UP-regulated genes at 4 h and 24-96 h vs 3 h-DMEM	49 unique UP-regulated genes at 4 h vs 3 h-DMEM	34 unique UP-regulated genes at 24-96 h vs 3 h-DMEM	37 unique UP-regulated genes at 21 days vs 3 h-DMEM
NMB0006	NMB0018	NMB0064	NMB0016	NMB0001	NMB0125	NMB0217
NMB0027	NMB0124	NMB0087	NMB0093	NMB0005	NMB0138	NMB0322
NMB0046	NMB0127	NMB0261	NMB0098	NMB0036	NMB0144	NMB0330
NMB0075	NMB0128	NMB0308	NMB0132	NMB0043	NMB0150	NMB0339
NMB0102	NMB0129	NMB0378	NMB0133	NMB0047	NMB0151	NMB0619
NMB0225	NMB0130	NMB0574	NMB0196	NMB0265	NMB0154	NMB0681
NMB0252	NMB0131	NMB0896	NMB0200	NMB0283	NMB0156	NMB0703
NMB0297	NMB0136	NMB0897	NMB0201	NMB0288	NMB0157	NMB0709
NMB0346	NMB0137	NMB0898	NMB0316	NMB0292	NMB0158	NMB0773
NMB0362	NMB0140	NMB0914	NMB0347	NMB0396	NMB0159	NMB0801
NMB0430	NMB0142	NMB0917	NMB0449	NMB0419	NMB0160	NMB0810
NMB0466	NMB0143	NMB0918	NMB0450	NMB0520	NMB0161	NMB0848
NMB0467	NMB0145	NMB1452	NMB0491	NMB0526	NMB0162	NMB0957
NMB0580	NMB0146	NMB1516	NMB0506	NMB0615	NMB0163	NMB0963
NMB0604	NMB0147	NMB1527	NMB0579	NMB0627	NMB0164	NMB1063
NMB0658	NMB0148	NMB1778	NMB0625	NMB0669	NMB0348	NMB1125
NMB0659	NMB0149	NMB1794	NMB0665	NMB0672	NMB0403	NMB1126
NMB0677	NMB0152	NMB1884	NMB0725	NMB0691	NMB0471	NMB1207
NMB0689	NMB0153	NMB1885	NMB0731	NMB0717	NMB0589	NMB1247
NMB0753	NMB0155	NMB1924	NMB0746	NMB0730	NMB0648	NMB1366
NMB0758	NMB0165	NMB1995	NMB0817	NMB0779	NMB0745	NMB1387
NMB0788	NMB0166	NMB2058	NMB0831	NMB0862	NMB0792	NMB1444
NMB0789	NMB0167		NMB0870	NMB0912	NMB0857	NMB1472
NMB0941	NMB0168		NMB0883	NMB0968	NMB0900	NMB1499
NMB0942	NMB0169		NMB0969	NMB1062	NMB1035	NMB1500
NMB1074	NMB0220		NMB1311	NMB1064	NMB1056	NMB1517
NMB1096	NMB0325		NMB1545	NMB1360	NMB1370	NMB1807
NMB1098	NMB0343		NMB1635	NMB1404	NMB1854	NMB1908
NMB1108	NMB0344		NMB1732	NMB1512	NMB1882	NMB1909
NMB1111	NMB0345		NMB1750	NMB1539	NMB1888	NMB1953
NMB1116	NMB0382		NMB1757	NMB1604	NMB2090	NMB1978
NMB1135	NMB0463		NMB1766	NMB1698		NMB2000
NMB1153	NMB0550		NMB1771	NMB1827		NMB2050
NMB1154	NMB0554		NMB1772	NMB1842		NMB2081
NMB1156	NMB0590		NMB1795	NMB1852		
NMB1215	NMB0591		NMB1914	NMB1925		
NMB1368	NMB0592		NMB1926	NMB1945		
NMB1381	NMB0595		NMB1979	NMB1971		
NMB1460	NMB0605		NMB2015	NMB1989		
NMB1502	NMB0609		NMB2069	NMB1990		
NMB1523	NMB0634		NMB2110	NMB2003		
NMB1546	NMB0641		NMB2145	NMB2080		
NMB1549	NMB0712			NMB2124		
NMB1567	NMB0722			NMB2125		
NMB1622	NMB0723			NMB2131		
NMB1749	NMB0724			NMB2149		
NMB1814	NMB0744					
NMB1825	NMB0748					
NMB1857	NMB0754					
NMB1905	NMB0762					
NMB2102	NMB0763					
NMB2140	NMB0787					
	NMB0790					
	NMB0791					
	NMB0822					
	NMB0840					

Table D - continued. Common and unique UP-regulated genes at all time points

55 common UP-regulated genes at 4h, 24-96, 21 days vs 3 h-DMEM	112 common UP-regulated genes at 24-96 h and 21 days vs 3 h-DMEM	22 UP-regulated genes at 4 h and 21days vs 3 h-DMEM	45 UP-regulated genes at 4 h and 24-96 h vs 3 h-DMEM	49 unique UP-regulated genes at 4 h vs 3 h-DMEM	34 unique UP-regulated genes at 24-96 h vs 3 h-DMEM	37 unique UP-regulated genes at 21 days vs 3 h-DMEM
	NMB0875					
	NMB0876					
	NMB0937					
	NMB0946					
	NMB0983					
	NMB0994					
	NMB0995					
	NMB1055					
	NMB1230					
	NMB1231					
	NMB1301					
	NMB1313					
	NMB1320					
	NMB1321					
	NMB1322					
	NMB1323					
	NMB1335					
	NMB1341					
	NMB1355					
	NMB1378					
	NMB1379					
	NMB1380					
	NMB1429					
	NMB1503					
	NMB1557					
	NMB1590					
	NMB1641					
	NMB1642					
	NMB1643					
	NMB1650					
	NMB1662					
	NMB1710					
	NMB1789					
	NMB1797					
	NMB1858					
	NMB1891					
	NMB1904					
	NMB1910					
	NMB1911					
	NMB1923					
	NMB1950					
	NMB1956					
	NMB1963					
	NMB1972					
	NMB2020					
	NMB2022					
	NMB2039					
	NMB2056					
	NMB2056					
	NMB2057					
	NMB2095					
	NMB2101					
	NMB2137					

Table E: Common genes of 4 h vs 3 h-DMEM and 180 min vs 180 min data of Grifantini *et al.* (2002). Expression profiles of each gene are shown in Table B
47 common down-regulated genes

Gene ID	COG	Description (KEGG)
NMB0018	Intracellular trafficking and secretion genes; Cell motility genes	Pilin PIIe
NMB0124	Translation	Translation elongation factor Tu
NMB0129	Not in COGs genes	Hypothetical protein
NMB0131	Translation	50S ribosomal protein L7/L12
NMB0138	Translation	Elongation factor G
NMB0140	Translation	30S ribosomal protein S10
NMB0153	Translation	50S ribosomal protein L24
NMB0154	Translation	50S ribosomal protein L5
NMB0155	Translation	30S ribosomal protein S14
NMB0156	Not in COGs genes	30S ribosomal protein S8
NMB0157	Translation	50S ribosomal protein L6
NMB0158	Translation	50S ribosomal protein L18
NMB0159	Translation	30S ribosomal protein S5
NMB0160	Translation	50S ribosomal protein L30
NMB0161	Translation	50S ribosomal protein L15
NMB0162	Intracellular trafficking and secretion genes	Preprotein translocase SecY subunit
NMB0165	Translation	30S ribosomal protein S13
NMB0166	Translation	30S ribosomal protein S11
NMB0167	Translation	30S ribosomal protein S4
NMB0168	Transcription	DNA-directed RNA polymerase, alpha subunit
NMB0298	Not in COGs genes	Hypothetical protein
NMB0321	Translation	50S ribosomal protein L28
NMB0550	Posttranslational modification, protein turnover, chaperones genes	Thiol disulfide interchange protein DsbC
NMB0554	Posttranslational modification, protein turnover, chaperones genes	DnaK protein
NMB0609	Not in COGs genes	30S ribosomal protein S15
NMB0712	Transcription	RNA polymerase sigma-32 factor
NMB0722	Translation	50S ribosomal protein L35
NMB0723	Not in COGs genes	50S ribosomal protein L20
NMB0754	Not in COGs genes	Hypothetical protein
NMB0791	Posttranslational modification, protein turnover, chaperones genes	Peptidyl-prolyl cis-trans isomerase
NMB0875	Nucleotide transport and metabolism genes; Amino acid transport and metabolism genes	Ribose-phosphate pyrophosphokinase
NMB0876	Translation	50S ribosomal protein L25
NMB0937	Translation	Elongation factor P
NMB1205	Not in COGs genes	Hypothetical protein
NMB1207	Inorganic ion transport and metabolism genes	Bacterioferritin A
NMB1211	Not in COGs genes	Hypothetical protein
NMB1313	Posttranslational modification, protein turnover, chaperones genes	Trigger factor
NMB1320	Translation	50S ribosomal protein L9
NMB1322	Replication recombination and repair	Primosomal replication protein n, putative
NMB1323	Translation	30S ribosomal protein S6
NMB1811	Not in COGs genes	PIIP protein
NMB1972	Posttranslational modification, protein turnover, chaperones genes	Chaperonin, 60 kDa
NMB2056	Not in COGs genes	30S ribosomal protein S9
NMB2057	Not in COGs genes	50S ribosomal protein L13
NMB2101	Not in COGs genes	30S ribosomal protein S2
NMB2137	Not in COGs genes	Hypothetical protein
NMB2141	Not in COGs genes	Hypothetical protein

Table E - continued: Common genes of 4 h vs 3 h-DMEM and 180 min vs 180 min data of Grifantini *et al.* (2002). Expression profiles of each gene are shown in Table B.

19 common up-regulated genes

Gene ID	COG	Description (KEGG)
NMB0102	Not in COGs genes	Hypothetical protein
NMB0292	General function prediction only genes	Conserved hypothetical protein
NMB0362	Not in COGs genes	Hypothetical protein
NMB0419	General function prediction only genes	Conserved hypothetical protein
NMB0615	Not in COGs genes	Ammonium transporter AmtB, putative
NMB0659	Not in COGs genes	Hypothetical protein
NMB0665	Coenzyme transport and metabolism genes	Oxygen-independent coprophorphyrinogen III oxidase family protein
NMB0689	Transcription	Transcription elongation factor GreB
NMB0788	Amino acid transport and metabolism genes	Amino acid ABC transporter, permease protein
NMB0789	Amino acid transport and metabolism genes	Amino acid ABC transporter, ATP-binding protein
NMB0968	Not in COGs genes	Hypothetical protein
NMB1153	Inorganic ion transport and metabolism genes	Sulfate adenylyltransferase, subunit 1
NMB1381	Function unknown genes	HesB/YadR/YfhF family protein
NMB1794	Energy production and conversion genes	Citrate transporter
NMB1795	Not in COGs genes	Hypothetical protein
NMB1857	General function prediction only genes	Modulator of drug activity B
NMB1971	Not in COGs genes	Conserved hypothetical protein
NMB1990	Inorganic ion transport and metabolism genes	Iron ABC transporter, permease protein
NMB2069	Coenzyme transport and metabolism genes	Thiamin-phosphate pyrophosphorylase

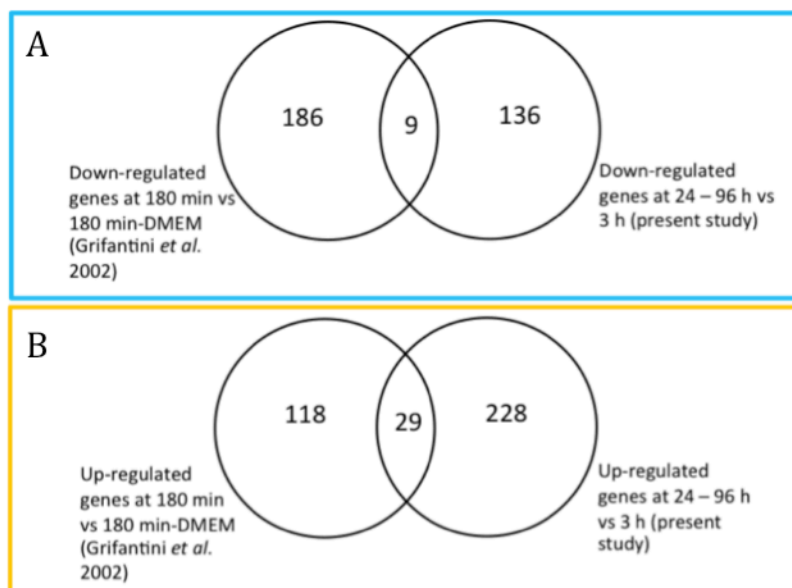


Figure A. Venn diagrams of 24 h – 96 h vs 3 h-DMEM of the present study and 180 min vs 180 min-DMEM of Grifantini *et al.* (2002). Both data sets were filtered on $fc \geq 2.0$, $p \leq 0.01$ and grouped into down-regulated genes (panel A) and up-regulated genes (panel B). A total of 199 genes were found to be down-regulated by Grifantini *et al.* (2002) of which 9 genes were also found to be down-regulated in the present study (of 145). Of the 147 up-regulated genes identified by Grifantini *et al.* (2002), 29 genes were also found to be up-regulated in the present study (of 257). Lists of the common genes are shown in Table F below.

Table F: 29 common up- and 9 common down-regulated genes at 180 min vs 180 min DMEM (Grifantini *et al.* 2002) and at 24 h–96 h vs 3 h DMEM (present study). Both data sets were filtered on $fc \geq 2.0$, $p \leq 0.01$.

29 common up-regulated genes

Gene ID	COG	Description (KEGG)
NMB0102	Not in COGs genes	Hypothetical protein
NMB0130	Translation	50S ribosomal protein L10
NMB0149	Translation	50S ribosomal protein L16
NMB0164	Not in COGs genes	50S ribosomal protein L36
NMB0169	Translation	50S ribosomal protein L17
NMB0362	Not in COGs genes	Hypothetical protein
NMB0382	Cell wall/membrane biogenesis genes	Outer membrane protein class 4
NMB0634	Inorganic ion transport and metabolism genes	Iron ABC transporter, periplasmic binding protein
NMB0659	Not in COGs genes	Hypothetical protein
NMB0665	Coenzyme transport and metabolism genes	Oxygen-independent Coprophorphyrinogen III oxidase family protein
NMB0689	Transcription	Transcription elongation factor GreB
NMB0763	Amino acid transport and metabolism genes	Cysteine synthase
NMB0787	Amino acid transport and metabolism genes; Signal transduction mechanisms genes	Amino acid ABC transporter, periplasmic amino acid-binding protein
NMB0788	Amino acid transport and metabolism genes	Amino acid ABC transporter, permease protein
NMB0789	Amino acid transport and metabolism genes	Amino acid ABC transporter, ATP-binding protein
NMB0968	Not in COGs genes	Hypothetical protein
NMB0994	Lipid transport and metabolism genes	Acyl-CoA dehydrogenase family protein
NMB0995	Function unknown genes	Macrophage infectivity potentiator-related protein
NMB1153	Inorganic ion transport and metabolism genes	Sulfate adenylyltransferase, subunit 1
NMB1366	Energy production and conversion genes; Posttranslational modification, protein turnover, chaperones genes	Thioredoxin
NMB1370	Not in COGs genes	Hypothetical protein
NMB1379	Amino acid transport and metabolism genes	NifS protein
NMB1380	Energy production and conversion genes	NifU protein
NMB1381	Function unknown genes	HesB/YadR/YfhF family protein
NMB1710	Amino acid transport and metabolism genes	Glutamate dehydrogenase, NADP-specific
NMB1795	Not in COGs genes	Hypothetical protein
NMB1857	General function prediction only genes	Modulator of drug activity B
NMB2069	Coenzyme transport and metabolism genes	Thiamine-phosphate pyrophosphorylase
NMB2095	Not in COGs genes	Adhesin complex protein, putative

9 common down-regulated genes

NMB0001	Not in COGs genes	Acetyltransferase, putative
NMB0378	Inorganic ion transport and metabolism genes	Phosphate permease, putative
NMB0396	Coenzyme transport and metabolism genes	Nicotinate-nucleotide Pyrophosphorylase
NMB1205	Not in COGs genes	Hypothetical protein
NMB1207	Inorganic ion transport and metabolism genes	Bacterioferritin A
NMB1211	Not in COGs genes	Hypothetical protein
NMB1811	Not in COGs genes	PilP protein
NMB1924	Carbohydrate transport and metabolism genes	Inositol monophosphatase family protein
NMB1977	Not in COGs genes	Hypothetical protein

Table G: Common differentially expressed meningococcal genes at 4 h vs 3 h-DMEM with 16HBE14 cells (present study), 6 h vs 6 h-DMEM with Hep-2 and HBMEC cells (Dietrich *et al.*, 2003). Genes differentially expressed in the same direction are printed bold. Data not available are indicated with n/a.

Gene ID	COG	Description (KEGG)	4 h vs 3 h-DMEM 16HBE14 cells (present study)	6 h vs 6 h-DMEM Hep-2 cells (Dietrich <i>et al.</i> , 2003)	6 h vs 6 h-DMEM HBMEC cells (Dietrich <i>et al.</i> , 2003)
NMB0069	Cell wall/membrane biogenesis genes	Polysialic acid capsule biosynthesis protein SiaB	down	up	n/a
NMB0070	Cell wall/membrane biogenesis genes	Polysialic acid capsule biosynthesis protein SynX	down	up	n/a
NMB0093	Not in COGs genes	Hypothetical protein	up	n/a	down
NMB0124	Translation	Translation elongation factor Tu	down	up	n/a
NMB0129	Not in COGs genes	Hypothetical protein	down	up	n/a
NMB0143	Not in COGs genes	50S ribosomal protein L4	down	n/a	up
NMB0144	Translation	50S ribosomal protein L23	down	n/a	up
NMB0148	Translation	30S ribosomal protein S3	down	n/a	up
NMB0149	Translation	50S ribosomal protein L16	down	n/a	up
NMB0150	Not in COGs genes	50S ribosomal protein L29	down	n/a	up
NMB0159	Translation	30s ribosomal protein S5	down	n/a	up
NMB0159	Translation	30s ribosomal protein S5	down	up	n/a
NMB0160	Translation	50S ribosomal protein L30	down	n/a	up
NMB0161	Translation	50S ribosomal protein L15	down	n/a	up
NMB0163	Translation	Translation initiation factor IF-1	down	n/a	up
NMB0200	Function unknown genes	Hypothetical protein	up	n/a	up
NMB0200	Function unknown genes	Hypothetical protein	up	up	n/a
NMB0328	Not in COGs genes	Hypothetical protein	up	up	n/a
NMB0429	Not in COGs genes	Hypothetical protein	up	down	n/a
NMB0442	Not in COGs genes	Opacity protein, authentic frameshift	up	up	n/a
NMB0475	Not in COGs genes	Hypothetical protein	down	up	n/a
NMB0546	General function prediction only genes	Alcohol dehydrogenase, propanol-preferring	down	n/a	down
NMB0584	Not in COGs genes	FrpC operon protein	down	up	n/a
NMB0744	Not in COGs genes	Hypothetical protein	down	n/a	up
NMB0744	Not in COGs genes	Hypothetical protein	down	up	n/a
NMB0880	Inorganic ion transport and metabolism genes	Sulfate ABC transporter, permease protein	down	up	n/a
NMB0886	Intracellular trafficking and secretion genes; Cell motility genes	Fimbrial protein FimT	down	up	n/a
NMB0942	Translation	50S ribosomal protein L31, putative	up	n/a	up
NMB1017	Inorganic ion transport and metabolism genes	Sulfate ABC transporter, periplasmic sulfate-binding protein	down	up	n/a
NMB1330	Not in COGs genes	Hypothetical protein	up	n/a	up
NMB1412	Not in COGs genes	FrpC operon protein	down	up	n/a
NMB1429	Cell wall/membrane biogenesis genes	Outer membrane protein PorA	down	up	n/a
NMB1809	Intracellular trafficking and secretion genes; Cell motility genes	PilN protein	down	up	n/a
NMB1810	Intracellular trafficking and secretion genes; Cell motility genes	pilO protein	down	up	n/a
NMB1888	Not in COGs genes	Protein-export membrane protein SecE	down	up	n/a
NMB2000	Not in COGs genes	Conserved hypothetical protein	down	down	n/a

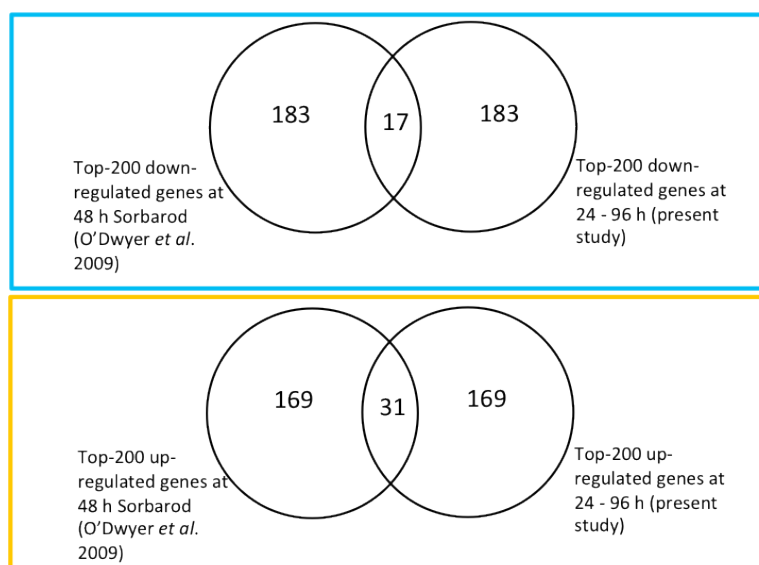


Figure B. Venn diagrams of 24 h – 96 h of the present study and of 48 h Sorbarod (O'Dwyer *et al.* 2009). Both data sets were normalized to the median spot intensity and grouped into down-regulated genes (blue margin) and up-regulated genes (yellow margin) and the top 200 genes of each list compared to each other. A total of 17 genes were found to be down-regulated and 31 up-regulated in both studies. Lists of the common genes are shown in Table H below.

Table H. 31 common up-regulated and 17 common down-regulated genes at 48 h Sorbarod (O'Dwyer *et al.* 2009) and 24 h-96 h host cell interaction (present study). Data were normalized to the mean spot intensity and the top 200 genes of each list compared.

31 common up-regulated genes

Gene ID	COG	Description (KEGG)
NMB0124	Translation	Translation elongation factor Tu
NMB0131	Translation	50S ribosomal protein L7/L12
NMB0320	Not in COGs genes	Hypothetical protein
NMB0321	Translation	50S ribosomal protein L28
NMB0322	Translation	50S ribosomal protein L33
NMB0324	Not in COGs genes	50S ribosomal protein L27
NMB0382	Cell wall/membrane biogenesis genes	Outer membrane protein class 4
NMB0631	General function prediction only genes; Energy production and conversion genes	Phosphate acetyltransferase Pta, putative
NMB0729	Not in COGs genes	Integration host factor, alpha subunit
NMB0748	General function prediction only genes	Host factor-I
NMB0818	Not in COGs genes	Hypothetical protein
NMB0827	Not in COGs genes	Type II restriction enzyme-related protein
NMB0956	Energy production and conversion genes	2-oxoglutarate dehydrogenase, E2 Component, dihydrolipoamide Succinyltransferase
NMB0959	Energy production and conversion genes	Succinyl-CoA synthetase, beta subunit

Table H – continued. 31 common up-regulated and 17 common down-regulated genes at 48 h Sorbarod (O'Dwyer *et al.*, 2009) and 24 h-96 h host cell interaction (present study).

<i>31 common up-regulated genes - continued</i>		
Gene ID	COG	Description (KEGG)
NMB1285	Carbohydrate transport and metabolism genes	Enolase
NMB1437	Function unknown genes	Conserved hypothetical protein
NMB1438	Energy production and conversion genes	Conserved hypothetical protein
NMB1468	Not in COGs genes	Hypothetical protein
NMB1534	Not in COGs genes	Hypothetical protein
NMB1594	Amino acid transport and metabolism genes	Spermidine/putrescine ABC transporter, Periplasmic spermidine/putrescine-binding protein
NMB1724	Energy production and conversion genes	Cytochrome c oxidase, subunit II
NMB1888	Not in COGs genes	Protein-export membrane protein SecG
NMB1910	General function prediction only genes	Conserved hypothetical protein
NMB1911	Translation	50S ribosomal protein L32
NMB1934	Energy production and conversion genes	ATP synthase F1, beta subunit
NMB1935	Energy production and conversion genes	ATP synthase F1, gamma subunit
NMB1936	Energy production and conversion genes	ATP synthase F1, alpha subunit
NMB1937	Energy production and conversion genes	ATP synthase F1, delta subunit
NMB1956	Translation	50S ribosomal protein L31
NMB2051	Energy production and conversion genes	Ubiquinol--cytochrome c reductase, cytochrome c1
NMB2056	Not in COGs genes	30S ribosomal protein S9
<i>17 common down-regulated genes - continued</i>		
Gene ID	COG	Description (KEGG)
NMB0014	Not in COGs genes	3-deoxy-D-manno-octulosonic-acid transferase
NMB0189	Inorganic ion transport and metabolism genes	Hypothetical protein
NMB0225	Inorganic ion transport and metabolism genes	Transposase, IS30 family
NMB0367	Not in COGs genes	Hypothetical protein
NMB0372	Not in COGs genes	Hypothetical protein
NMB0493	Replication recombination and repair	Hemagglutinin/hemolysin-related protein
NMB0860	Signal transduction mechanisms genes	Hypothetical protein
NMB1114	Not in COGs genes	Conserved hypothetical protein
NMB1399	Replication recombination and repair	IS1106 transposase
NMB1544	Function unknown genes	Hypothetical protein
NMB1606	Not in COGs genes	Sensor histidine kinase
NMB1634	Intracellular trafficking and secretion genes	Conserved hypothetical protein
NMB1760	Not in COGs genes	Conserved hypothetical protein
NMB1878	Not in COGs genes	Transcriptional regulator, AraC family
NMB1882	Replication recombination and repair	TonB-dependent receptor
NMB1990	Not in COGs genes	Iron ABC transporter, permease protein
NMB2080	Cell wall/membrane biogenesis genes	Hypothetical protein

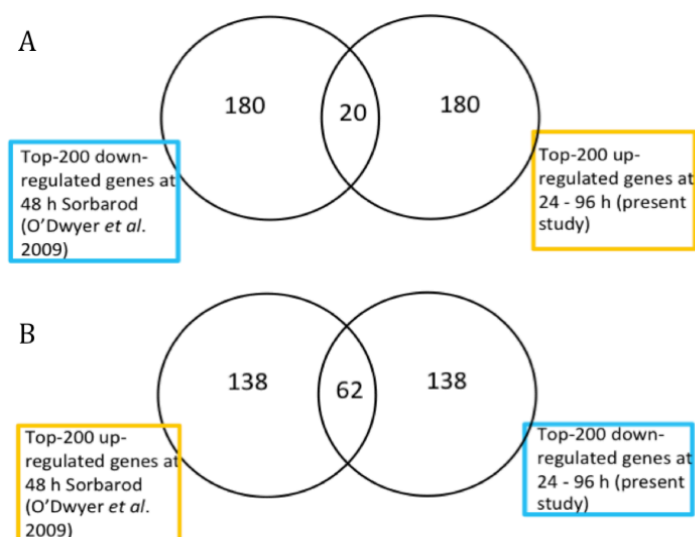


Figure b. Venn diagrams of 24 h – 96 h of the present study and of 48 h Sorbarod (O'Dwyer *et al.*, 2009). Both data sets were normalized to the median spot intensity and grouped into down-regulated genes (blue margin) and up-regulated genes (yellow margin) and the top 200 up-regulated genes of one study compared to the top 200 down-regulated genes of the other study. A total of 20 genes were found to be down-regulated in the Sorbarod and up-regulated in the present study. Sixtytwo genes were up-regulated in the Sorbarod and down-regulated in the present study. Lists of the common genes are shown in Table h below.

Table h: 62 and 20 common genes when the top 200 up-regulated genes of O'Dwyer *et al.* (2009) were compared to the top 200 down-regulated genes at 24 h-96 h of the present study and vice versa (see Venn diagram Figure b).

62 common genes (up-regulated in O'Dwyer *et al.*, 2009 and down-regulated in the present study)

Gene ID	COG	Description (KEGG)
NMB0125	Intracellular trafficking and secretion genes	Preprotein translocase SecE subunit
NMB0127	Not in COGs genes	50S ribosomal protein L11
NMB0129	Not in COGs genes	Hypothetical protein
NMB0136	Not in COGs genes	30S ribosomal protein S12
NMB0137	Translation	30S ribosomal protein S7
NMB0138	Translation	Elongation factor G
NMB0140	Translation	30S ribosomal protein S10
NMB0142	Not in COGs genes	50S ribosomal protein L3
NMB0143	Not in COGs genes	50S ribosomal protein L4
NMB0144	Translation	50S ribosomal protein L23
NMB0145	Translation	50S ribosomal protein L2
NMB0146	Translation	30S ribosomal protein S19
NMB0147	Not in COGs genes	50S ribosomal protein L22
NMB0148	Translation	30S ribosomal protein S3
NMB0149	Translation	50S ribosomal protein L16
NMB0150	Not in COGs genes	50S ribosomal protein L29
NMB0151	Translation	30S ribosomal protein S17
NMB0152	Not in COGs genes	50S ribosomal protein L14
NMB0153	Translation	50S ribosomal protein L24
NMB0154	Translation	50S ribosomal protein L5
NMB0155	Translation	30S ribosomal protein S14
NMB0156	Not in COGs genes	30S ribosomal protein S8
NMB0157	Translation	50S ribosomal protein L6
NMB0158	Translation	50S ribosomal protein L18
NMB0159	Translation	30S ribosomal protein S5

NMB0160	Translation	50S ribosomal protein L30
NMB0161	Translation	50S ribosomal protein L15
NMB0162	Intracellular trafficking and secretion genes	Preprotein translocase SecY subunit

Table h – continued. 62 and 20 common genes when the top 200 up-regulated genes of O'Dwyer *et al.* (2009) were compared to the top 200 down-regulated genes at 24 h-96 h of the present study and vice versa (see Venn diagram Figure b).

<i>62 common genes (up-regulated in O'Dwyer et al., 2009 and down-regulated in the present study) - continued</i>		
Gene ID	COG	Description (KEGG)
NMB0163	Translation	Translation initiation factor IF-1
NMB0164	Not in COGs genes	50S ribosomal protein L36
NMB0165	Translation	30S ribosomal protein S13
NMB0166	Translation	30S ribosomal protein S11
NMB0167	Translation	30S ribosomal protein S4
NMB0168	Transcription	DNA-directed RNA polymerase, alpha subunit
NMB0298	Not in COGs genes	Hypothetical protein
NMB0343	Not in COGs genes	Conserved hypothetical protein
NMB0546	General function prediction only genes	Alcohol dehydrogenase, propanol-preferring
NMB0590	Not in COGs genes	tRNA -methyltransferase
NMB0591	Translation	16S rRNA processing protein RimM
NMB0604	General function prediction only genes	Alcohol dehydrogenase, zinc-containing
	Amino acid transport and metabolism genes	
NMB0721	Translation	Translation initiation factor 3
NMB0722	translation	50S ribosomal protein L35
NMB0920	Not in COGs genes	Isocitrate dehydrogenase
NMB1301	Translation	30S ribosomal protein S1
NMB1321	Not in COGs genes	30S ribosomal protein S18
NMB1322	Replication recombination and repair	Primosomal replication protein n, putative
NMB1323	Translation	30S ribosomal protein S6
NMB1429	Cell wall/membrane biogenesis genes	Outer membrane protein PorA
NMB1557	Function unknown genes	Conserved hypothetical protein
NMB1575	Function unknown genes	Conserved hypothetical protein
NMB1576	Amino acid transport and metabolism genes	Acetolactate synthase III, small subunit
NMB1660	Transcription	DNA-directed RNA polymerase, omega subunit
NMB1666	Cell cycle control, mitosis and meiosis genes	Hypothetical protein
NMB1812	Intracellular trafficking and secretion genes	PilQ protein
NMB1860	Lipid transport and metabolism genes	Acetyl-CoA carboxylase, biotin carboxyl carrier protein
NMB1950	Translation	30S ribosomal protein S21
NMB1963	Secondary metabolites biosynthesis, transport and catabolism genes	Conserved hypothetical protein
NMB2057	Not in COGs genes	50S ribosomal protein L13
NMB2091	General function prediction only genes	Hemolysin, putative
NMB2102	Not in COGs genes	Elongation factor TS
NMB2137	Not in COGs genes	Hypothetical protein
NMB2159	Carbohydrate transport and metabolism genes	Glyceraldehyde 3-phosphate dehydrogenase
<i>20 common genes (down-regulated in O'Dwyer et al. 2009 and up-regulated in the present study)</i>		
Gene ID	COG	Description (KEGG)
NMB0063	Cell wall/membrane biogenesis genes	dTDP-D-glucose 4,6-dehydratase
NMB0209	Inorganic ion transport and metabolism genes	Glutathione-regulated potassium-efflux system protein
NMB0293	Inorganic ion transport and metabolism genes	TonB-dependent receptor, putative
NMB0316	Function unknown genes	Conserved hypothetical protein
NMB0459	Function unknown genes	Conserved hypothetical protein
NMB0460	Not in COGs genes	Transferrin-binding protein 2
NMB0463	Translation	30S ribosomal protein S20
NMB0482	Not in COGs genes	Hypothetical protein
NMB0483	Not in COGs genes	Hypothetical protein

NMB0497	Intracellular trafficking and secretion genes	Hemagglutinin/hemolysin-related protein
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Table h – continued: 62 and 20 common genes when the top 200 up-regulated genes of O'Dwyer *et al.* (2009) were compared to the top 200 down-regulated genes at 24 h-96 h of the present study and vice versa (see Venn diagram Figure b).

20 common genes (down-regulated in O'Dwyer et al. 2009 and up-regulated in the present study) – continued

Gene ID	COG	Description (KEGG)
NMB0511	Intracellular trafficking and secretion genes	Hypothetical protein
NMB0819	Not in COGs genes	Hypothetical protein
NMB1112	Not in COGs genes	Conserved hypothetical protein
NMB1203	Posttranslational modification, protein turnover, chaperones genes	Protein-Pil uridylyltransferase
NMB1396	Replication recombination and repair	A/G-specific adenine glycosylase
NMB1602	Replication recombination and repair	Transposase, putative
NMB1719	Coenzyme transport and metabolism genes	Efflux pump component MtrF
NMB1737	Intracellular trafficking and secretion genes ;Cell wall/membrane biogenesis genes	Secretion protein, putative
NMB1738	Cell wall/membrane biogenesis genes	Secretion protein, putative
NMB1971	Not in COGs genes	Conserved hypothetical protein

Table I: 33 common up- and 16 common down-regulated genes in 90 min blood infection (Hedman, 2009) and at 24 h-96 h host cell interaction (present study) both datasets were normalized to the median

33 common up-regulated genes

Gene ID	COG	Description (KEGG)
NMB0056	Signal transduction mechanisms genes	DnaK suppressor protein
NMB0068	Cell wall/membrane biogenesis genes	Polysialic acid capsule biosynthesis protein SiaC
NMB0092	Not in COGs genes	Hypothetical protein
NMB0124	Translation	Translation elongation factor Tu
NMB0131	Translation	50S ribosomal protein L7/L12
NMB0320	Not in COGs genes	Hypothetical protein
NMB0321	Translation	50S ribosomal protein L28
NMB0322	Translation	50S ribosomal protein L33
NMB0324	Not in COGs genes	50S ribosomal protein L27
NMB0340	Amino acid transport and metabolism genes	Lactoylglutathione lyase
NMB0382	Cell wall/membrane biogenesis genes	Outer membrane protein class 4
NMB0449	Not in COGs genes	Hypothetical protein
NMB0463	Translation	30S ribosomal protein S20
NMB0748	General function prediction only genes	Host factor-I
NMB0827	Not in COGs genes	Type II restriction enzyme-related protein
NMB1438	Energy production and conversion genes	Conserved hypothetical protein
NMB1468	Not in COGs genes	Hypothetical protein
NMB1534	Not in COGs genes	Hypothetical protein
NMB1594	Amino acid transport and metabolism genes	Spermidine/putrescine ABC transporter, periplasmic spermidine/putrescine-binding protein PilP protein
NMB1811	Not in COGs genes	Protein-export membrane protein SecE
NMB1888	Not in COGs genes	Conserved hypothetical protein
NMB1910	General function prediction only genes	50S ribosomal protein L32
NMB1911	Translation	ATP synthase F1, epsilon subunit
NMB1933	Not in COGs genes	ATP synthase F1, beta subunit
NMB1934	Energy production and conversion genes	ATP synthase F1, gamma subunit
NMB1935	Energy production and conversion genes	ATP synthase F1, alpha subunit
NMB1936	Energy production and conversion genes	ATP synthase F1, delta subunit
NMB1937	Energy production and conversion genes	ATP synthase F1, delta subunit

NMB1940	Not in COGs genes	ATP synthase F0, A subunit
NMB1956	Translation	50S ribosomal protein L31
NMB1985	Intracellular trafficking and secretion genes; cell wall/membrane biogenesis genes	Adhesion and penetration protein

Table I – continued.

Gene ID	COG	Description (KEGG)
NMB2051	Energy production and conversion genes	Ubiquinol-cytochrome c reductase, cytochrome c1
NMB2056	Not in COGs genes	30S ribosomal protein S9
<i>16 common down-regulated genes</i>		
Gene ID	COG	Description (KEGG)
NMB0016	Not in COGs genes	Hypothetical protein
NMB0099	Not in COGs genes	Hypothetical protein
NMB0166	Translation	30S ribosomal protein S11
NMB0225	Replication recombination and repair	Transposase, IS30 family
NMB0372	Not in COGs genes	Hypothetical protein
NMB0493	Intracellular trafficking and secretion genes	Hemagglutinin/hemolysin-related protein
NMB0504	Not in COGs genes	Hypothetical protein
NMB0583	Replication recombination and repair	IS1016C2 transposase
NMB0860	Not in COGs genes	Hypothetical protein
NMB1399	Replication recombination and repair	IS1106 transposase
NMB1542	Not in COGs genes	Hypothetical protein
NMB1544	Not in COGs genes	Hypothetical protein
NMB1552	Replication recombination and repair	Pilin gene inverting protein PivNM-1A
NMB1634	Replication recombination and repair	Conserved hypothetical protein
NMB1878	Not in COGs genes	Transcriptional regulator, AraC family
NMB2092	Not in COGs genes	Hypothetical protein

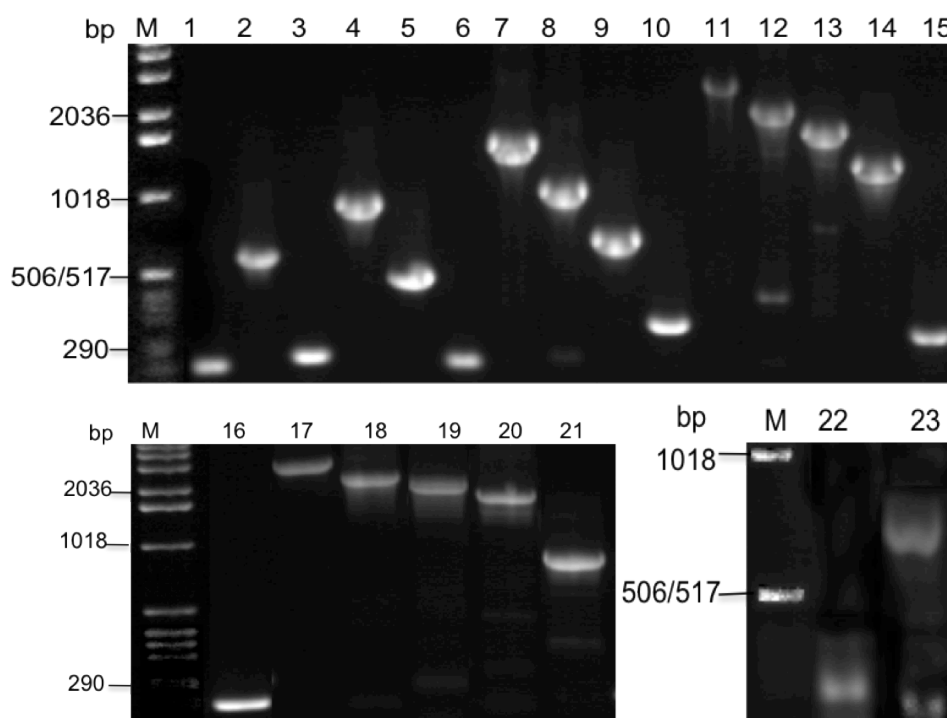


Figure C. Control experiment to confirm that rt-primers generate a PCR product (sequences of primers are shown in Materials and Methods, Table 2.9). Template was MC58 wild type RNA. **Lane 1:** NMB0342 rt-r- and rt-f-primer (148 bp), **lane 2** NMB0343-rt-r-primer and NMB0342-rt-f-primer (612 bp), **lane 3:** NMB0343-rt-r- and NMB0343-rt-f-primer (170 bp), **lane 4:** NMB0344-rt-r-primer and NMB0342-rt-f-primer (928 bp), **lane 5:** NMB0344-rt-r-primer and NMB0343-rt-f-primer (486 bp), **lane 6:** NMB0344-rt-r-primer and NMB0344-rt-f-primer (155 bp), **lane 7:** NMB0345-rt-r-primer and NMB0342-rt-f-primer (1471 bp), **lane 8:** NMB0345-rt-r-primer and NMB0343-rt-f-primer (1029 bp), **lane 9:** NMB0345-rt-r-primer and NMB0344-rt-f-primer (698 bp), **lane 10:** NMB0345-rt-r-primer and NMB0345-rt-f-primer (292 bp), **lane 11:** NMB0346-rt-r-primer and NMB0342-rt-f-primer (2438 bp), **lane 12:** NMB0346-rt-r-primer and NMB0343-rt-f-primer (1996 bp), **lane 13:** NMB0346-rt-r-primer and NMB0344-rt-f-primer (1665 bp), **lane 14:** NMB0346-rt-r-primer and NMB0345-rt-f-primer (1259 bp), **lane 15:** NMB0346-rt-r-primer and NMB0346-rt-f-primer (267 bp), **lane 16:** NMB0347-rt-r-primer and NMB0347-rt-f-primer (139 bp), **lane 17:** NMB0347-rt-r-primer and NMB0342-rt-f-primer (3011 bp), **lane 18:** NMB0347-rt-r-primer and NMB0343-rt-f-primer (2569 bp), **lane 19:** NMB0347-rt-r-primer and NMB0344-rt-f-primer (2238 bp), **lane 20:** NMB0347-rt-r-primer and NMB0345-rt-f-primer (1832 bp), **lane 21:** NMB0347-rt-r-primer and NMB0346-rt-f-primer (840 bp), **lane 22:** NMB0348-rt-r-primer and NMB0348-rt-f-primer (290 bp), **lane 23:** NMB0348-rt-r-primer and NMB0347-rt-f-primer (873 bp).

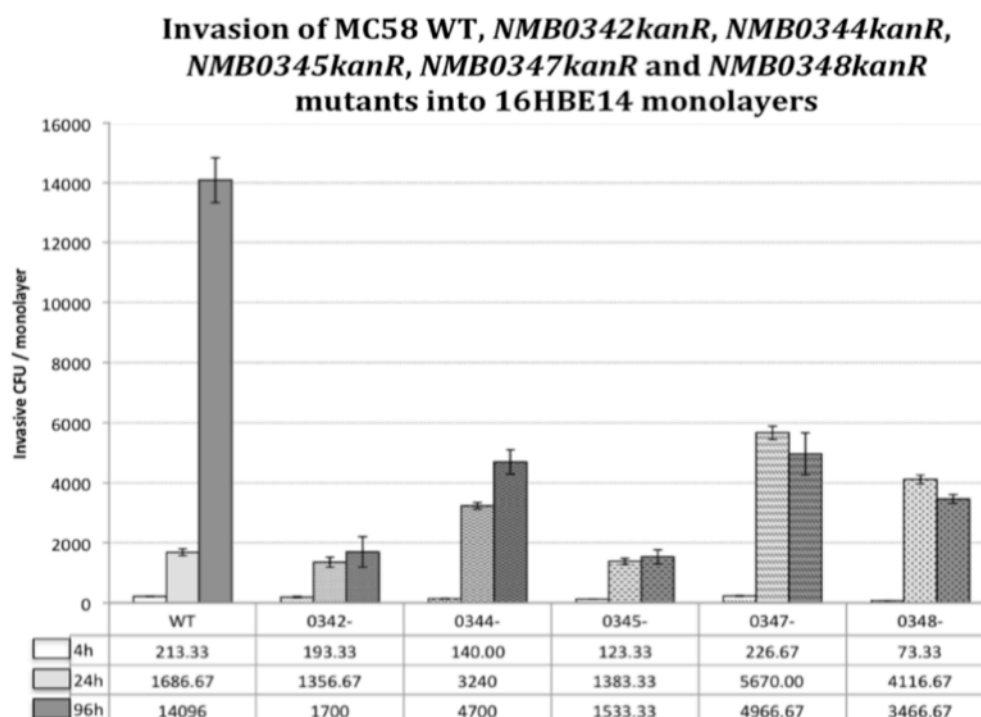


Figure D: Invasive CFU of MC58 wild type, *NMB0342kanR*, *NMB0344kanR*, *NMB0345kanR*, *NMB0347kanR* and *NMB0348kanR* mutants into 16HBE14 cells. Error bars represent the SEM. Experiments were performed in triplicate.

Table J. pSORT analysis of NMB0342-NMB0348 using the deduced aa sequence of each protein.

	NMB0342	NMB0343 and NMB0344	NMB0345	NMB0346	NMB0347	NMB0348
CMSVM-	Cytoplasmic	Unknown	Unknown	Unknown	Unknown	Unknown
Membrane	Unknown	Unknown	Unknown	Cytoplasmic	Cytoplasmic	Cytoplasmic
CytoSVM-	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
ECSVM	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
ModHMM	Cytoplasmic	Unknown	Unknown	Unknown	Unknown	Unknown
Membrane	5 internal helices found	Unknown	Unknown	Unknown	Unknown	Unknown
Motif-	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
OMPMotif	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
OMSVM	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
PPSVM	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Profile	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
SCL-BLAST	Cytoplasmic membrane, probable intracellular septation protein	Unknown	Outer Membrane, Outer membrane integral membrane protein	Unknown	Unknown	Cytoplasmic
SCL-BLASTe	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Signal-	Non-Cytoplasmic, Signal peptide detected	Unknown	Non-Cytoplasmic	Non-Cytoplasmic	Unknown	Unknown
Localization Scores						
Cytoplasmic	0.00	2.00	0.00	0.00	8.96	9.97
Cytoplasmic	10.00	2.00	0.01	4.90	0.51	0.01
Membrane						
Periplasmic	0.00	2.00	0.03	2.50	0.26	0.01
OuterMembrane	0.00	2.00	9.93	0.10	0.01	0.00
Extracellular	0.00	2.00	0.03	2.50	0.26	0.00
Final Prediction	Cytoplasmic Membrane	Unknown	Outer membrane 9.93	Unknown, This protein may have multiple localization sites	Cytoplasmic, 8.96	Cytoplasmic, 9.97