Functional analysis of the type IV pilus assembly machinery in \textit{Neisseria meningitidis}

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Abstract

Type IV pili (Tfp) are one of the most widespread virulence factors in prokaryotes. Their inherent capacity to mediate an astonishing array of functions differentiates them from other pili and contributes to the pathogenesis of many important human pathogens. Previous intensive efforts by our group in Neisseria meningitidis identified 23 proteins dedicated to Tfp biology, 15 of which are essential for pilus biogenesis. Though these proteins are widely accepted to exert their functions within a large multiprotein complex, the mechanisms governing the biogenesis and functionality of these organelles remained poorly defined. Consequently, the first objective of my project was to perform a large-scale analysis to identify fundamental interactions between 11 Pil proteins from N. meningitidis. To achieve this, we employed the bacterial adenylate cyclase two-hybrid system, which uncovered 20 different binary interactions, many of which are novel and represents the most complex interaction network between Pil proteins reported to date. Significantly, this study revealed that PilE, PilM, PilN and PilO involved in pilus assembly, indeed interact and provided us with a strong foundation to proceed to our main objective, which was to perform a detailed functional analysis of this poorly characterized subcomplex. Using a battery of assays we determined the membrane topology of PilN and PilO, mapped the interaction domains between PilE, PilM, PilN and PilO, and showed that a widely conserved N-terminal motif in PilN is essential for both PilM-PilN interactions and pilus assembly. Furthermore, we established by stability and co-immunoprecipitation studies that PilP (another protein involved in pilus assembly) forms a complex with PilM, PilN and PilO. Finally, we attempted to reconstitute a minimal Tfp assembly machinery in E. coli, however these efforts necessitate further improvements. Taken together, this study has shed light on the molecular mechanisms of Tfp biology and provides a useful blueprint for future studies.
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Thank you everyone!
Declaration of Originality

I certify that the research presented here is my own, except from the work that involved handling of live *N. meningitidis*, which was performed in containment level 3 laboratory by Vladimir Pelicic and Ana Cehovin. I confirm that all the sources of help have been acknowledged and clearly referenced in the text and listed in bibliography.
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Abbreviations

3D  Three-dimensional
aa  Amino acid
amp  Ampicillin
ATP  Adenosine triphosphate
BACTH  Bacterial adenylate cyclase two-hybrid
Bfp  Bundle-forming pilus
bp  Base pairs
BSA  Bovine serum albumin
c-di-GMP  bis-(3’-5’)-cyclic dimeric guanosine monophosphate
CaM  Calmodulin
cAMP  Cyclic adenosine monophosphate
CAP  Catabolite activator protein
CL3  Containment level 3
CTD  C-terminal domain
CyaA  Calmodulin dependent adenylate cyclase
DAPI  4′, 6-diamidino-2-phenylindol
ddH₂O  Double distilled water
DNA  Deoxyribonucleic acid
dNTPs  Deoxyribonucleotide triphosphate
DSE  Donor strand exchange
DSS  Disuccinimidyl suberate
DTT  Dithiothreitol
DUS  DNA uptake sequence
ECL  Enhanced chemi-luminescence
EDTA  Ethylenediaminetetraacetic acid
ELISA  Enzyme-linked immunosorbent assay
EMSA  Electrophoretic mobility shift assay
EPEC  Enteropathogenic *E. coli*
ery  Erythromycin
<table>
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<tr>
<td>g</td>
<td>Grams</td>
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<tr>
<td>GBS</td>
<td>Group B Streptococcus</td>
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<td>GCB</td>
<td>Gonococcal medium base</td>
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<tr>
<td>GSP</td>
<td>General secretory pathway</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>IF</td>
<td>Immuno-fluorescence</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
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<td>ITB</td>
<td>Inoue transformation buffer</td>
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<td>kan</td>
<td>Kanamycin</td>
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<tr>
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<td>Piconewton</td>
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<tr>
<td>rbs</td>
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Red-Gal 6-chloro-4-indolyl-β-D-galactoside
rpm Revolutions per minute
SDS Sodium dodecyl sulphate
SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Spec Spectinomycin
Strep Streptomycin
T18 Amino acids 225-399 of calmodulin dependent adenylate cyclase
T25 Amino acids 1-224 of calmodulin dependent adenylate cyclase
T2S Type II secretion
TAE Tris-Acetate-EDTA buffer
Tcp Toxin co-regulated pilus
TEM Transmission electron microscopy
Tfp Type IV pili
Tfpa Type IVa pili
Tfpb Type IVb pili
TGS Tris glycine sodium dodecyl sulphate buffer
Tris Tris(hydroxymethyl)aminomethane
U/mg Units of enzymatic activity per milligram of bacterial dry weight
UPEC Uropathogenic E. coli
V Volts
WT Wild-type
X-phos 5-bromo-4-chloro-3-indolyl phosphate disodium salt
xg Times gravity (relative centrifugal force)
zip Leucine zipper
<table>
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Chapter 1: Introduction
1.1 Bacterial pili: most widespread bacterial colonization factor

Attachment to surfaces is a vital characteristic of bacteria, that allows different species to colonize distinct niches, varying from the soil to the human body, and thus enables species-specific lifestyle (Pellicic, 2008).

In pathogenic bacteria, adhesion to host cells is the primary and most crucial step in establishing an infection (Proft & Baker, 2009). To achieve this, bacteria express on their surfaces a plethora of adhesive molecules, known as adhesins, which recognize a variety of different elements on host-cell surfaces and within the extracellular matrix and determine the tissue tropism of the pathogens (Soto & Hultgren, 1999). This broad repertoire of adhesins is divided into two groups: the pilus adhesins and the non-pilus adhesins. Pili, also known as fimbriae, are long hair-like, non-flagellar organelles, composed of several hundred, to thousands of small subunits named pilins that protrude from the surface of bacteria. In contrast, non-pilus adhesins are directly associated with the bacterial cell surface (Soto & Hultgren, 1999, Pizarro-Cerda & Cossart, 2006).

The most common mechanism for bacterial adhesion to host surfaces is though pili, which are bacteria’s favourite colonization factor. In the past decades, pili in Gram-negative bacteria have been extensively studied (Telford et al., 2006). Initially they were classified according to their morphological characteristics as seen under the electron microscope. Subsequent genetic and molecular characterization was consistent with this, yet allowed for a more comprehensive insight into the classification of pili based on their individual assembly systems (Busch & Waksman, 2012, Proft & Baker, 2009). Also, pili used to be regarded as a hallmark of Gram-negative bacteria, but recently they have been re-discovered in some Gram-positive bacteria as well. The Gram-positive and Gram-negative pili differ significantly in structure. While in Gram-negative bacteria pilin subunits are connected by non-covalent interaction, in Gram-positive bacteria they are covalently connected (Telford et al., 2006).

Of the many types of pili that have been identified, Type IV pili (Tfp) are the most widespread and these are the focus of my thesis (Pellicic, 2008). The Tfp
characteristics, functions and mechanism of biogenesis will be discussed in detail, following a brief introduction of some of the most extensively characterized pili. These are the Type I and P pili assembled by the chaperone/usher pathway, the curli pili assembled by the extracellular nucleation/precipitation pathway and pili assembled by the Gram-positive bacteria (Proft & Baker, 2009).

1.1.1 Type I and P pili

Type I and P pili are the best-studied pili. They are assembled by the chaperone/usher pathway, which participates in the assembly of more than thirty pili (Proft & Baker, 2009) (Sauer et al., 2000).

Type I pili are expressed in enteropathogenic *Escherichia coli* (EPEC) and P pili are expressed in uropathogenic *E. coli* (UPEC). The genes involved in their synthesis are grouped into clusters, the *fim* and *pap* clusters (Proft & Baker, 2009). Both pili are formed by two distinct subassemblies: the tip fibrillum and the pilus rod (Busch & Waksman, 2012) (Figure 1.1). In Type I pili, the tip fibrillum is 3 nm wide and it is composed of three minor pilus proteins: FimF, FimG and at the distal end, the FimH adhesin which recognizes mannose-containing glycoprotein receptors expressed in many types of host cells. The pilus rod is 6.9 nm wide and 1-2 µm long and it is formed by 500-3000 copies of the main pilin, FimA, arranged in a right-handed helical array (Figure 1.1A). In the P pilus, the tip fibrillum is 2-3 nm in diameter, flexible and composed of four minor pilus proteins: the adaptor subunit PapK, followed by 5-10 copies of PapE, PapF, and the distal adhesin PapG that binds to the α-D-galactopyranosyl-(1-4)-β-D-galactopyranoside moiety of glycolipids present on uroepithelial cells. More than 1000 copies of the main pilin, PapA, arranged in a right-handed helical array, form the pilus rod that is 6.8 nm wide and up to several micrometers long. Extension of the pilus rod is completed at the outer membrane by PapH, which is the termination subunit. Interestingly, no homologue of PapH has yet been established for the type I pilus system, hence the mechanism that regulates the length of type I pilus is unclear (Busch & Waksman, 2012, Proft & Baker, 2009) (Figure 1.1B).
Two proteins, the chaperone and the usher proteins, are required to assemble the pilin subunits described above into a pilus (Busch & Waksman, 2012). All pilin subunits contain an immunoglobulin (Ig)-like domain that lacks the seventh C-terminal β-strand. This causes the formation of a deep groove on their surface and renders them unstable. Therefore, once they are secreted in the periplasm by the Sec secretion system, they interact with the periplasmic chaperones, FimC in Type I pili and PapD in P pili, that stabilize them and prevent their premature aggregation or polymerization in the periplasm (Busch & Waksman, 2012). The chaperones consist of two Ig-like domains, which form a boomerang-like structure. They interact with the pilins by inserting one of their own strands, the G1 strand, present on the N-terminal domain, thereby completing the missing β-strand in pilins and thus reconstituting the incomplete Ig-like domain of the pilins. This mechanism is known as ‘donor strand complementation’ (Sauer et al., 1999). Subsequently, the chaperone-pilin complexes are delivered to the usher, FimD in Type I pili and PapC in P pili, which itself forms an outer membrane β-barrel channel and serves as an assembly platform. Pilus assembly occurs by a ‘donor strand exchange’ (DSE) mechanism. Pilins have an N-terminal extension of about 15 residues with a conserved motif of alternating hydrophobic residues, which is similar to the motif present on the G1 strand of the chaperone. Therefore, the G1 strand of the chaperone complementing the pilin’s groove is replaced by the N-terminal extension of another pilin subunit (Munera et al., 2007, Busch & Waksman, 2012). Recently, a crystal structure of the FimD usher in complex with FimC-FimF-FimG-FimH provided structural evidence supporting a model for the catalytic ability of the usher for subunits polymerization and nascent pilus translocation (Geibel et al., 2013). The usher consists of five functional domains: an N-terminal domain (NTD), a large central pore that in the resting state is obstructed by a plug domain, and two carboxy-terminal domains (CTD1 and CTD2) (Geibel et al., 2013). In this proposed model, at the base of the pilus fibre the chaperone-subunit complexes reside at the usher’s CTDs, whilst incoming subunits are recruited to the NTD. These are subsequently brought into an ideal position to undergo DSE with the subunit bound at the CTDs. Upon DSE, the chaperone is displaced from the penultimate subunit and, since it has no binding affinity for the CTD on its own, it dissociates from the
complex. To reset the assembly machinery, the incoming chaperone-subunit complex bound to the NTD is transferred to the CTD site, concomitantly pushing the penultimate subunit into the channel. The NTD is then free to participate in another cycle of pilus elongation (Phan et al., 2011, Geibel et al., 2013).

![Figure 1.1 Schematic presentation of the assembly of various pili in Gram-negative bacteria.](image)

(A) Assembly of Type I pili by the chaperone/usher pathway, letters denote Fim proteins. (B) Assembly of P pili by the chaperone/usher pathway, letters denote Pap proteins. (C) Assembly of curli pili by the nucleation/precipitation pathway, letters denote Csg proteins. Adapted from Busch & Waksman (2012) and Barnhart & Chapman (2006). OM, outer membrane.

Moreover, in recent years advancements in antibiotic discovery have not surpassed the growing issue of antibiotic resistance in bacteria, so there is a pressing need to find new ways to fight infection. Understanding of the Type I and P pili assembly has led to the design of new drugs and also vaccines. Receptor binding studies have unveiled a novel class of high affinity inhibitors of the FimH adhesin, which could be used as anti-adhesive drugs to prevent urinary tract infections by UPEC (Bouckaert et
al., 2005). Moreover, a family of bicyclic 2-pyridones, termed pilicides, target the interaction between the chaperone-subunit complexes and the NTD of the usher, which is required for pilus biogenesis (Pinkner et al., 2006). Hence, by blocking the functions of chaperone and usher, they inhibit pilus formation. Importantly, it has been shown in vitro, that pilicides could reduce biofilm formation and adherence to cultured bladder cells by 90% (Pinkner et al., 2006). Moreover, it was demonstrated in both murine and primate models that FimH has an efficacy as a vaccine (Langermann et al., 1997, Langermann et al., 2000).

1.1.2 Curli

Curli are assembled by the extracellular nucleation/precipitation pathway and are expressed by enteric bacteria, such as E. coli and Salmonella species (Barnhart & Chapman, 2006). What is remarkable about curli is that they share biochemical and structural properties with the eukaryotic amyloid fibers associated with neurodegenerative diseases such as Alzheimer’s disease (Chapman et al., 2002). These amyloid fibers are formed by proteins that adopt the distinct β-sheet-rich amyloid fold, and within the fibers the individual β-strands are perpendicular to the fiber axis (Blanco et al., 2012). As eukaryotic amyloid fibers, they are non-branching and resistant to both protease digestions and 1% sodium dodecylsulphate (SDS) (Collinson et al., 1991, Barnhart & Chapman, 2006, Blanco et al., 2012).

The genes involved in the biogenesis of curli are organized into two operons csgBA and cgsDEFG (Hammar et al., 1995). The csgBA operon encodes the major subunit protein CsgA (the curlin) and the nucleator protein CsgB. The csgDEFG operon encodes four proteins required for the assembly of the pili. All proteins apart from CsgD, which is transcriptional regulator of the csgBA operon, have Sec-dependent signal sequences for translocation into the periplasm via the Sec system (Barnhart & Chapman, 2006).

The mechanism of curli assembly is unique and distinctive from that of Type I and P pili. Soluble CsgA are secreted into the extracellular space via a multimeric pore formed by CsgG, which is an outer membrane lipoprotein (Robinson et al., 2006)
CsgB is also secreted from the cell and it nucleates CsgA into an insoluble coiled amyloid fiber (Hammer et al., 2007) (Figure 1.1C). Additionally, CsgG is required for the stability of CsgA and CsgB (Robinson et al., 2006). CsgE and CsgF are periplasmic proteins that interact with CsgG in the outer membrane, however their exact role is not completely understood (Robinson et al., 2006). CsgE has been shown to gate the outer membrane GsgG pore, and it is proposed to be a CsgA-specific chaperone, while CsgF is required for CsgB surface exposure (Nenninger et al., 2009, Nenninger et al., 2011).

Curli adhere to many host proteins including the extracellular matrix proteins fibronectin and laminin, and the proteins involved in blood clotting plasminogen and tissue type plasminogen activator. The latter delays blood clotting and promotes spreading of the bacteria deeper in tissues (Olsen et al., 1989, Sjobring et al., 1994).

Attempts have also been made to design curli inhibitors. A class of curli inhibitors termed as curlicides, has been discovered which share a common structural scaffold (ring fused 2-pyridones) with the previously described pilicides (Cegelski et al., 2009). Remarkably, two curlicides, FN075 and BibC6, have been shown to exhibit a dual mode of action by acting as curlicides and pilicides. This was determined as both compounds blocked CsgA polymerization in vitro and UPEC curli formation in vivo, and they also retained pilicide activity, as assessed by their ability to block formation of type I pili-dependent biofilms (Cegelski et al., 2009). Nonetheless, a subsequent study signified the importance of cross-reactivity of curlicides with different amyloidogenic proteins, as it showed that FN075 cross-reacts with the human α-synuclein protein, and exerts opposite effects on the CsgA and α-synuclein (Horvath et al., 2012). Whilst FN075 inhibits the polymerization of CsgA, in vitro it was shown to accelerate the formation of α-synuclein oligomers, which are associated with Parkinson’s disease (Horvath et al., 2012). Furthermore, fluorescent curlicides have been synthesized to facilitate studies for the interactions between these compounds and the curli assembly systems (Chorell et al., 2012).
1.1.3 Pili in Gram-positive bacteria

Pili in Gram-positive bacteria were first detected by electron microscopy in *Corynebacterium renale* in 1968, however they remained unstudied until recently when they were detected in other species including *Corynebacterium diphtheriae, Streptococcus pyogenes, Streptococcus agalactiae* and *Streptococcus pneumoniae* (Yanagawa et al., 1968, Ton-That & Schneewind, 2003, Mora et al., 2005, Rosini et al., 2006, Barocchi et al., 2006).

Using electron microscopy, two types of pili have been identified in Gram-positive bacteria. In *Streptococcus gordonii* and *Streptococcus oralis*, short, thin rods have been observed that extend between 70-500 nm in length. Conversely, in the oral pathogens *Corynebacterium* species and the pathogenic streptococci, much longer flexible pili, up to 3 µm in length, have been observed (Telford et al., 2006).

Ton-That and Scheewind, working on *C. diphtheriae*, were the first to provide insights into the assembly mechanism of the long Gram-positive pili (Ton-That & Schneewind, 2003). This study showed that, in contrast to the assembly mechanism of Gram-negative pili, adjacent pilin subunits within the pili are connected by covalent interactions and both the polymerization of pilin subunits and the attachment of the pilus to the cell wall are catalyzed by dedicated sortase enzymes. Also, it showed that pili are composed of three subunits and immunogold electron microscopy revealed that one these subunits is the main pilin, as it is uniformly distributed along the pilus, while the other two are ancillary proteins (Ton-That & Schneewind, 2003).

Pilin subunits of most species share three conserved amino acid sequences, whose role in the assembly mechanism will be described below: 1) An LPXTG amino-acid motif (Ton-That & Schneewind, 2003). 2) A sequence known as E-box, due to the presence of a highly conserved glutamic-acid residue (Ton-That et al., 2004, Telford et al., 2006). 3) An WXXXVXYPKN, amino-acid motif with a conserved lysine (K) residue (X denotes any amino acid) (Ton-That & Schneewind, 2003, Telford et al., 2006).
The assembly process is mediated by a transpeptidase sortase in four steps. In the first step, the three pilins are translocated by the Sec-dependent secretion system across the cell membrane, however they remain attached to the cell membrane due to a C-terminal membrane-spanning domain (Telford et al., 2006). The second step is the sortase-dependent reaction, in which the membrane-anchored proteins are cleaved at their LPXTG-motif, between the threonine (T) and glycine (G) residues. This reaction leads to the formation of an acyl-enzyme intermediate, in which a covalent thioester bond is formed between the thiol group of the cysteine residue in the sortase catalytic pocket and the carboxyl group of the pilin threonine residue (Telford et al., 2006). In *C. diphtheriae*, it has been demonstrated that the specificity of one of the ancillary subunits for the sortases is partly determined by the E-box, as substitution of the conserved glutamic acid within the E-box prevented the incorporation of the ancillary protein into the pilus (Ton-That et al., 2004). However, such a role of the E-box in the main pilin or in the pilins of other species has not been shown. The third step is the oligomerization of the pilin subunits. This involves the nucleophilic attack of the thioester bond that links the pilin subunit with the sortase, by the ε-amino group of the lysine residue within the WXXXVVYPKN motif of another subunit (Telford et al., 2006). This step is repeated and the length of the pilus depends on the abundance of pilins coupled to the sortases. The final fourth step is the anchoring of the oligomerized pilus to the cell wall. This requires the nucleophilic attack of the thioester bond between the sortase and the pilin subunit by the amino group of the pentapeptide of lipid II, which is the precursor of peptidoglycan, and leads to the formation of an amide bond connecting the elongated pilus to the cell wall (Ton-That & Schneewind, 2004, Telford et al., 2006).

Interestingly, in streptococci species all the genes encoding pilus proteins are contained within a pathogenicity island. This indicates that the capacity of Gram-positive bacteria to express pili might have been acquired by horizontal gene transfer (Telford et al., 2006).

Significantly, attempts to produce pilus-based vaccines against Gram-positive pathogens seem more successful than attempts performed against Gram-negative structures. In a study, 3 components of the Group B Streptococcus (GBS) pili along
with another conserved GBS protein were used to immunize mice and surprisingly this combination elicited protection in mice against a large panel of GBS strains (Maione et al., 2005). Moreover, a synthetic protein has been designed which carries six different pilin variants of GBS. This chimeric protein was shown to induce a strong protection in mice against a panel of GBS strains, expressing different pilin variants (Nuccitelli et al., 2011).

Intriguingly, aside from covalent pili, some Gram-positive species such as Clostridium perfringens and Ruminococcus albus have been found to express Tfp, and these are described in more depth in the following section (Varga et al., 2006, Rakotoarivonina et al., 2002).

1.2 Type IV pili

This section provides a comprehensive review of Type IV pili (Tfp), on which my PhD is based. The prevalence, morphological characteristics, functions and mechanism of biogenesis of Type IV pili are discussed.

1.2.1 Tfp prevalence

Type IV pili are the most widespread pili known, likely to be expressed in more than 150 different species. This is based either on direct observation of filaments on the surface of bacteria, the observation of twitching motility - a form of flagella-independent translocation exclusively mediated by Tfp, or the identification of genes involved in Tfp biology in countless genome sequencing projects. Most of the Tfp expressing bacteria belong to Proteobacteria, but Tfp are likely to be present in 13/30 other phyla in the Bacteria domain (Acidobacteria, Actinobacteria, Aquificae, Caldiserica, Cyanobacteria, Defferribacteres, Deinococcus Thermus, Dictyoglomi, Fibrobacteres, Firmicutes, Gemmatimonadetes, Nitrospira and Thermodesulfobacteria). Consequently, they are the only pili known to be present in both Gram-negative and Gram-positive bacteria (Mattick, 2002, Pelicic, 2008).

Additionally, bacterial species use machineries that bear extensive parallels to the Tfp biogenesis machinery, namely the terminal branch of the general secretory
pathway (GSP) also known as the Type II secretion system, which is a conserved system in Gram-negative bacteria. This system is dedicated to the secretion of folded proteins, using a pilus-like structure termed pseudopilus, through the outer membrane into the extracellular milieu (Douzi et al., 2012). This system and its similarities to Tfp system will be discussed in section 1.2.4.2. Secondly, a competence pseudopilus, which is involved in the uptake of free DNA from the extracellular milieu to be used for generating genetic diversity, DNA repair or as a source of food (Chen & Dubnau, 2004). Therefore, these systems represent variations of the common theme of transport of macromolecules across membranes in prokaryotes, and are very likely to be evolutionary related.

Remarkably, motility structures similar to Tfp have been described in another domain of life, the Archaea. These swimming organelles, recently named archaella share many common features with Tfp and genetic analysis revealed that the archaellum-encoding loci contain many gene homologues of Tfp genes (Jarrell & Albers, 2012). This suggests that Tfp genes were present in a common ancestor to the two domains: the Bacteria and Archaea, in which they probably encoded a macromolecule transport machinery.

1.2.2 Morphological and molecular characteristics of Tfp

The typical morphological characteristics of Tfp, as determined by electron microscopy, were originally employed to classify them into a specific pilus type (type IV) that gave them their name, and are still used in their identification. Tfp are helical structures composed of thousand copies of the major subunit, the pilin, which form extremely thin (5-8 nm in width), several micrometers long, flexible filaments that often aggregate laterally to form highly distinctive bundles (Figure 1.2) (Pelicic, 2008).
Figure 1.2 Tfp morphology in *N. meningitidis*.


The Tfp pilin subunits have been extensively studied in many systems, and their structures have established a common basis for Tfp structure and assembly. Though they are extremely variable in sequence and in length, they all display a conserved N-terminal motif that has recently been named ‘class III signal peptide’ (Figure 1.3) (Szabo *et al.*, 2007). Pilins are synthesized as precursors, referred to as prepilins, which have a hydrophilic N-terminal leader peptide that invariably ends with a glycine residue (Strom *et al.*, 1993b) (Figure 1.3A). This is then cleaved by a dedicated prepilin peptidase prior to assembly (Nunn & Lory, 1991). All mature pilins have a conserved N-terminal hydrophobic domain of approximately 20-25 residues, in which the N-terminal residue is methylated by the prepilin peptidase and the fifth residue is almost always a charged residue, a conserved glutamate (Strom *et al.*, 1993b, Craig *et al.*, 2004) (Figure 1.3A).

The lengths of both the mature protein and the leader peptide of the prepilin precursor have been used to classify Tfp into two subtypes, type IVa (Tfpa) and the type IVb (Tfpb) (Pelicic, 2008). This classification is consistent with the differences
between their biogenesis machineries, as explained below. Tfpa pilins have short leader peptides of less than 10 residues and a length of 150-160 residues. In contrast, Tfpb pilins possess longer leader peptides of 15-30 residues and are also longer in length, 180-200 residues (Craig et al., 2004). However, an exception to this are the Flp (fimbrial low molecular weight protein) pilins encoded by the tad locus (tight adherence), which are found in a few organisms including the Actinobacillus actinomycetemcomitans. These Flp pilins are classified as Tfpb based on phylogenetic analysis; however, they are significantly shorter, consisting of only 40-50 residues (Tomich et al., 2007). Also, for Tfpa the N-methylated N-terminal residue is phenylalanine, whilst for Tfpb this residue varies (Craig et al., 2004). Additionally, beyond the class III signal peptide, the Tfpa share minimal sequence similarity to Tfpb (Craig et al., 2004). The widespread nature of Tfp is mostly attributed to Tfpa, which have a broad host range (Pellicic, 2008). Tfpa have been most extensively studied in three model species: the human pathogens Neisseria gonorrhoeae and Neisseria meningitidis, and the opportunistic pathogen Pseudomonas aeruginosa. In contrast, Tfpb are found in a small subset of genera, exclusively in bacteria that colonize the human intestine and they have been most extensively studied in Vibrio cholerae (producing toxin co-regulated pilus, Tcp) and EPEC (producing bundle-forming pilus, Bfp) (Craig et al., 2004, Pellicic, 2008).

The atomic-resolution 3D structures of several Tfpa and Tfpb pilins reveal a common ‘lollipop’ architecture. Some of the structures solved are the full-length Tfpa pilins; PilE from N. gonorrhoeae (Parge et al., 1995, Craig et al., 2006), PAK from P. aeruginosa strain K (Craig et al., 2003), and FimA from Dichelobacter nodosus (Hartung et al., 2011), the N-terminally truncated Tfpa pilin K122-4 from P. aeruginosa strain K122-4 (Keizer et al., 2001, Audette et al., 2004), and the N-terminally truncated Tfpb pilins; BfpA from EPEC (Ramboarina et al., 2005) and TcpA from V. cholerae (Craig et al., 2003). This ‘lollipop’ architecture consists of an N-terminal α-helical spine, α1, attached to a globular C-terminal head domain (Craig et al., 2004) (Figure 1.3B). The N-terminal half of the α-helix, α1-N, protrudes from the protein and is primarily hydrophobic. The C-terminal half, α1-C, is amphipathic and is embedded in the globular domain against an anti-parallel four to five stranded β-
The structures of *P. aeruginosa* and *N. gonorrhoeae* pilins, showed that α1 has an S-shaped curve caused by the helix-perturbing residues at positions 22 (proline) and 42 (proline for *P. aeruginosa* and glycine for *N. gonorrhoeae*), which introduce two kinks in the helix (Craig et al., 2003). Superposition of these pilins over α1-C, revealed flexibility in α1 at the point it protrudes from the globular head domain (Craig et al., 2003). Therefore, the N-terminal α-helix provides both a flexible anchor for the globular domain and a large hydrophobic surface for interactions between the pilin subunits (Craig et al., 2004).

Flanking the anti-parallel β-sheet of the globular domain, there are two regions that vary extensively in sequence, length and structure (Craig et al., 2004) (Figure 1.3B). The first region is the α/β loop, which is formed by the hydrophobic packing of the α1-C against the antiparallel β-sheet. The second region is the disulphide-bonded loop, known as the D-region, which is bound to the β-sheet by a pair of conserved C-terminal cysteines, forming a disulphide bridge (Craig et al., 2004). A study performed in the TcpA showed that the first 2/3 of the D-region are essential for pilin folding, whilst the last third is involved in pilus-pilus interactions (Kirn et al., 2000). Further evidence of the importance of the D-region came from another study in which treatment of both Tfpa and Tfpb with a reducing agent led to complete dissociation of the filaments as observed by TEM (Li et al., 2012). This indicated that the D-region disulphide bond is essential for both subunit and filament stability. Also, interestingly the length of the D-region is longer in Tfpb than in Tfpa (Craig et al., 2004).
Figure 1.3 Type IV pilins conserved sequence motif and structure.

(A) The conserved N-terminal motif, also known as ‘class III signal peptide’ found in all type IV pilins. The arrow after glycine (G) indicates the cleavage site by the prepilin peptidase. (B) Structure of full-length *N. gonorrhoeae* pilin. The N-terminal half of the α-helix, α1-N, protrudes from the protein, while the C-terminal half, α1-C, is embedded in the globular domain of an anti-parallel four stranded β-sheet. Flanking the anti-parallel β-sheet of the globular domain, there are two regions that show extensive variation: the α/β loop (coloured green) and the D-region (coloured magenta). The disulphides are coloured cyan. The α/β loop of the *Neisseria* pilins has two unusual post-translational modifications: an O-glycosylated serine at position 63 (coloured orange) and a phosphorylated serine at position 68 (coloured red). From Craig et al. (2004).
Although many pilin structures have been solved, no high-resolution Tfp 3D structures are available yet. Models based on both electron microscopy of TfpA (N. gonorrhoeae) and TfpB (EPEC, V. cholerae) and hydrogen/deuterium exchange mass spectrometry of TfpB (V. cholerae), however, agree that pilins within a filament are arranged in a helical fashion (Craig & Li, 2008) (Figure 1.4). This helical organization is facilitated via extensive hydrophobic interactions among the α1-N domains of the pilins, which are located in the core of the pilus, whilst the globular domains are more loosely packed and exposed on the surface. However, the number of pilin subunits per helical turn, the rise from one subunit to the next and the rotation between the subunits can vary considerably between Tfp of different species. Also, the D-region and α/β loop are predicted to be optimally exposed, providing the distinct surface chemistry and defining the multiple functions of the pilus (Craig et al., 2004, Craig et al., 2006, Craig & Li, 2008).

The Tfp quaternary structure is also affected by force. This was demonstrated in a study in which N. gonorrhoeae Tfp subjected to 100 pN force using optical and magnetic tweezers, transitioned reversibly into a new conformation (Biais et al., 2010). This new quaternary structure was three times longer and 40% narrower than the original structure and exposed hidden epitopes that were previously buried, which if found to be conserved and immunogenic, they might be potential vaccine candidates. It is hypothesized that this structural polymorphism provides a means for bacteria to remain attached to the host cells while encountering intermittent forces in the environment (Biais et al., 2010).

A recent study has shown that upon heating the subunits from 20°C to 80°C the TfpB pilin subunits (V. cholerae) are more stable than TfpA subunits (N. gonorrhoea) due to differences in protein folds within the C-terminal globular domain between the two pilin types (Li et al., 2012). However, interestingly pilin stability is not synonymous with pilus filament stability, as TfpA pili were shown to be more resistant than TfpB pili to proteolysis, thermal and chemical denaturation, remaining intact with up to 8 M urea (Li et al., 2012). This stability is mainly attributed to tighter hydrophobic interactions between TfpA pilin subunits and it may be an adaptation of the TfpA pili to harsher environmental conditions (Li et al., 2012).
1.2.3 Tfp mediate a vast array of diverse functions

As stated above, pathogenic bacteria employ pili to mediate adhesion to host surfaces. Therefore, Tfp are key virulence factors in many human pathogens, including *N. meningitidis*, *N. gonorrhoeae*, and *V. cholerae*. Moreover, due to their widespread nature, they facilitate adhesion to a variety of biotic and abiotic surfaces. As shown in *N. meningitidis*, Tfp adhesion is a two-step process (Pujol *et al.*, 1999). In the first step, termed localized adherence, adhesion of bacteria on cells leads to the formation of 3D microcolonies (or aggregates), which are likely to be equivalent to the bacterial aggregates observed in liquid culture. In the second step, termed diffuse adherence, aggregates disappear and bacteria spread over most of the cell
surface, where they adhere intimately and firmly (Pujol et al., 1999). Bacterial aggregation in liquid culture, which is promoted by inter-bacterial interactions, is another Tfp-mediated property. Adhesion and aggregation are closely linked Tfp-mediated properties, as in Tfp-expressing bacteria a reduced Tfp propensity to form bacterial aggregates is usually accompanied by a reduced adherence/colonization (Helaine et al., 2005). Therefore, it is not clear whether Tfp adhere by directly interacting with the cells or by promoting the formation of aggregates on the cells.

The significance of Tfp as a virulence factor is not only due to their wide distribution but also because of their functional versatility. Apart from bacterial adhesion described above which is mediated by most pili, Tfp mediate remarkably diverse functions including a unique form of flagella-independent bacterial translocation over surfaces in the presence of humidity, known as twitching motility (Mattick, 2002). This term was derived from the observation of cells moving in a jerky manner. Twitching motility appears to be important for the rapid colonization of surfaces under high nutrient availability (Mattick, 2002). It is enabled by the striking ability of Tfp to retract, which was experimentally demonstrated in a study using laser tweezers (Merz et al., 2000). Retraction, a process of pilus depolymerization at the inner membrane, is powered by a cytoplasmic ATPase (PilT, in N. meningitidis), which is dispensable for Tfp biogenesis (Merz et al., 2000). Impressively, retraction by a single pilus generates significant mechanical force of up to 100 pN, making Tfp one of the strongest molecular motors analyzed to date (Maier et al., 2002). Moreover, it has been shown that Tfp bundles containing 8-10 Tfp filaments, act as coordinated retractable units, generating remarkable mechanical force in the nanonewton range (Biais et al., 2008). It should be noted, that pilus retraction has only been directly demonstrated in Tfpa-expressing bacteria, and it is still unknown whether Tfpb can retract, as many bacteria including V. cholerae lack the gene encoding the ATPase that powers retraction. Nevertheless, EPEC possess an ATPase, BfpF, which when disrupted leads to hyperpiliation, increased aggregation and adhesion, and reduced infectivity, all of which are typical phenotypes controlled by retraction (Bieber et al., 1998). Interestingly, it has been demonstrated that in N. gonorrhoeae and Myxococcus xanthus retraction occurs at two discrete speed
modes, low and high. In *N. gonorrhoeae*, oxygen depletion was shown to trigger a reversible switch between high to low speed mode (Kurre & Maier, 2012). This is associated with the biological function of oxygen as an energy source, since it is essential for ATP synthesis by oxidative phosphorylation, thus reduced oxygen concentration correlates with a decrease in energy consumption and a reduction of pilus retraction speed (Kurre & Maier, 2012).

Another important property of Tfp is DNA uptake during natural transformation, which contributes to the virulence of bacteria as it promotes genetic adaptability by introducing new traits such as antibiotic resistance and metabolic properties. This Tfp property makes many Tfp-expressing species including *N. meningitidis*, *N. gonorrhoeae*, *Thermus thermophilus* and *Haemophilus influenzae* competent. However, it is important to mention that not all bacteria harbouring Tfp are naturally competent, and in many competent species Tfp have not been detected, which suggests that short competence pseudopili like the ones employed by *B. subtilis*, described in 1.2.1, are used instead (Chen & Dubnau, 2004). Competence is a multistage process that involves: the uptake of DNA into the periplasm, its translocation across the inner membrane, and integration into the genome by homologous recombination (Aas *et al.*, 2002). Therefore, Tfp are involved in the first step at which bacteria bind free DNA and transport it though the outer membrane in Gram-negative species or the thick layer of peptidoglycan in Gram-positive species (Cehovin *et al.*, 2013). Like twitching motility, this process also depends on the unique ability of Tfp to retract, which is powered by the cytoplasmic ATPase PilT, suggesting that on retraction Tfp pull DNA in the periplasm (Wolfgang *et al.*, 1998a). Transformation of *Neisseria* species requires the presence of a specific 10-12 base pair sequence element, called the DNA uptake sequence (DUS), which is widely distributed in their genomes, thus they favour uptake of homotypic DNA (Elkins *et al.*, 1991). Significantly, a study in *N. meningitidis* identified a minor (low-abundance) Tfp pilin, ComP, as the DUS-specific DNA receptor. This protein has been shown to bind DNA via an electropositive stripe that is predicted to be exposed on the filament surface and also to display a marked preference for DUS (Cehovin *et al.*, 2013).
Examples of other important Tfp functions are their contribution to biofilm formation and also in *Geobacter sulfurreducens* Tfp act as nanowires involved in electron transfer (Reguera *et al.*, 2005, Pelicic, 2008).

1.2.4 Tfp biogenesis

One of the fundamental questions of Tfp biology is: how do bacteria assemble Tfp? My PhD concentrates on this conundrum by studying the biogenesis of Tfpa and more specifically the Tfpa assembly machinery components. During the course of this project, the field of Tfp biology has been highly dynamic and significant progress has been made. Therefore, regarding the area of Tfpa biogenesis I studied, the purpose of this introduction is to provide the background information that was available at the start of the project. More recent advances that became apparent during the last four years and relate significantly to my work will be alluded to in the context of the main Discussion (Chapter 6).

1.2.4.1 Tfp biogenesis involves complex and conserved multi-protein machineries

In the last 15 years, systematic genetic studies in human pathogens have identified a large set of proteins, between 10 and 18 proteins, dedicated to the biogenesis of both Tfpa (in *P. aeruginosa* and *N. meningitidis*) and Tfpb (in EPEC, *V. cholerae*) (Alm & Mattick, 1997, Carbonnelle *et al.*, 2005, Ramer *et al.*, 2002, Kirn *et al.*, 2003) (Figure 1.5). These studies led to three important general findings on Tfp biogenesis machineries, discussed below. Unfortunately, in each system different nomenclature is used, thus unless otherwise stated I will use the *N. meningitidis* nomenclature for the sake of simplicity.
Figure 1.5 Schematic presentation of all the Pil proteins essential for the biogenesis of Tfp in *N. meningitidis*, which is resolved into three dynamic steps.

Tfp biogenesis in *N. meningitidis* involves 15 proteins: PilC1/2, PilD, PilE, PilF, PilG, PilH, PilI, PilJ, PilK, PilM, PilN, PilO, PilP, PilQ and PilW. The major pilin is PilE, which is assembled into Tfp, coloured in red. Six more Pil proteins, apart from PilE, are predicted to be dedicated in assembly, these are: PilD, PilF, PilM, PilN, PilO and PilP, coloured in blue. The ATPase PilT, coloured in yellow, is not involved in Tfp biogenesis but it powers retraction. A set of 7 proteins: PilC1/2, PilG, PilH, PilI, PilJ, PilK and PilW, is involved in counter-retraction, coloured in gray. Tfp emerge on the cell surface through a pore formed by 12 subunits of a secretin protein, PilQ, coloured in green.
Tfp biogenesis machineries are complex and composed of many proteins

The first finding of the systematic genetic studies was that even though Tfp appear to be relatively simple polymers of mainly a single protein, the biogenesis system is utterly complex due to the large number of proteins required.

In a clinical isolate of *N. meningitidis*, which is heavily piliated and presents all Tfp linked phenotypes probably all of the essential proteins for Tfp biogenesis were identified, including the core proteins described below (PilE, PilD, PilF, PilG and PilQ) (Table 1.1) (Carbonnelle *et al.*, 2005) (Figure 1.5). These were identified by searching for non-aggregative mutants in a collection of defined mutants that contained non-polar transposon insertions into most of the non-essential genes (Carbonnelle *et al.*, 2005). This phenotype was tested because it was the most easily assayable on a genomic-scale. The analysis identified 39 mutants, which had mutations in 12 different genes. Piliation of the mutants was next assessed by immuno-fluorescence (IF) microscopy using a monoclonal antibody specific to the Tfp fibers, which showed that 11 of the identified genes were involved in Tfp biogenesis as their corresponding mutants were non-piliated (*pilD, pilE, pilF, pilG, pilH, pilI, pilJ, pilK, pilM, pilW* and *pilQ*) (Carbonnelle *et al.*, 2005) (Figure 1.5). Subsequently, three more proteins were identified to be involved in Tfp biogenesis, as their corresponding genes were part of the same operon as two of the biogenesis genes (*pilM* and *pilQ*) found in the systematic screen. This was confirmed since corresponding non-polar mutants, were non-piliated. Therefore in total, 15 proteins (PilC1/2, PilD, PilE, PilF, PilG, PilH, PilI, PilJ, PilK, PilM, PilN, PilO, PilP, PilQ and PilW) were identified to be essential for Tfp biogenesis, including PilC, which was not studied using this mutagenesis approach because it is encoded by two alleles (*pilC1/pilC2*) in the genome of this strain (Carbonnelle *et al.*, 2005) (Figure 1.5).

The systems are further complicated by additional proteins, which are dispensable for Tfp biogenesis but fine-tune various Tfp functions. In *N. meningitidis*, there are seven additional proteins, three minor pilins (ComP, PilV and PilX), three traffic ATPases (PilT, PilT2 and PilU) and PilZ (Brown *et al.*, 2010).
**Tfp biogenesis machineries comprise a conserved set of genes**

The second finding was that the biogenesis of Tfp in all systems requires a conserved set of proteins, referred to as ‘core’, in contrast to the non-conserved proteins (Pellicic, 2008). These are: 1) several proteins apart from the major pilin, with class III signal peptides, 2) a specific inner-membrane peptidase, 3) a traffic ATPase that powers pilus assembly, 4) a polytopic inner membrane protein of unknown function and 5) a secretin, which is an integral outer membrane protein required for the emergence of pili on the bacterial surface, although absent in piliated Gram-positive species. These ‘core proteins’ and a few other Tfp proteins have homologues in the evolutionary related T2S machinery of Gram-negative bacteria (Pellicic, 2008).

**Distribution and organization of Tfp biogenesis genes**

The third finding was significant differences in the Tfpa and Tfpb systems that are consistent with the Tfpa and Tfpb classification. The genes encoding the Tfpa biogenesis proteins are scattered throughout the genome of Gram-negative bacteria, whereas Tfpa genes of Gram-positive bacteria are clustered (Pellicic, 2008). Also, the same Tfpa genes or gene clusters are almost always flanked by the same genes, which are mainly housekeeping genes. For instance, the pilMNOPQ operon is always flanked by ponA and aroK and the pilW gene is always flanked by ispG and yfgB (Pellicic, 2008). Moreover, all the pilin-like genes essential for Tfpa biogenesis (pilH, pilI, pilJ, pilK) are clustered as well. Strikingly, all these Tfp genes are extremely conserved in all species expressing Tfpa even if they are phylogenetically distant, indicating that Tfpa are a homogeneous group (Pellicic, 2008). In addition, even if these genes are scattered throughout the genome, the same genes or clusters are always found in the same genomic location in more than 150 species (Pellicic, 2008).

In contrast, the genes of Tfpb biogenesis are fewer (10-12) and always clustered in a single operon. Strikingly, apart from the ‘core proteins’, there is no sequence conservation in the rest of the proteins (Pellicic, 2008). These indicate that Tfpb pili are considerably less homogeneous than Tfpa pili. The only known exception to this is the Flp family, in which the flp genes are conserved ‘en bloc’ in *A. actinomycetemcomitans* and *Caulobacter crescentus* (Tomich et al., 2007). Also,
whilst this organization into a single operon made genetic studies difficult due to the possibility of polar mutants, it allowed for the transfer of Tfpb biogenesis genes into surrogate hosts, leading to the synthesis of pili and confirming that these genes are both required and sufficient for the Tfpb biogenesis (Sohel et al., 1996, Stone et al., 1996). Such an experiment has not yet been successful for Tfpa pili. Furthermore, this organization suggested that the Tfpb biogenesis genes are encoded by pathogenicity islands, as it is the case for *V. cholerae* (Karaolis et al., 1999). The *V. cholerae* tcp pathogenicity island has been reported to be a filamentous bacteriophage, however this is not generally supported by other studies (Karaolis et al., 1999, Faruque et al., 2003). In addition, this potential motility of the *V. cholerae* pathogenicity island did not lead to a wide distribution of Tfpb pili, unlike the Tfpa (Pelicic, 2008).

In conclusion, these findings show that even though two subtypes, Tfpa and Tfpb, share a common origin they separated and evolved independently long ago. Apart from the core proteins shared by both subtypes, they do not share any non-core Pil proteins, which in *N. meningitidis* account for the 40% of the proteins essential for the biogenesis (PilC, PilM, PilN, PilO, PilP and PilW) (Pelicic, 2008). Also, all of the Tfpb non-core proteins are not shared among the species that express Tfpb, apart from the Flp non-core proteins (Pelicic, 2008). Despite these differences, it is likely that the mechanism of Tfp biogenesis is uniform and different proteins perform similar functions. This is supported by recent structural data, which show that different non-core proteins can have similar 3D structures. For example, despite a lack of sequence similarity, the 3D crystal structure of the cytoplasmic region of BfpC (N-BfpC), which is a bitopic inner membrane protein involved in the Tfpb biogenesis of EPEC, resembles both PilM, a cytoplasmic Tfpa biogenesis protein, and the cytoplasmic N-terminal domain of GspL, which is a bitopic inner membrane involved in T2S (Yamagata et al., 2012). This cytoplasmic globular domain of the protein consists of two similar domains, each composed of an α-helix-β-sheet combination. BfpC together with core integral protein BfpE, has been shown to recruit the core ATPase BfpD to the inner membrane. Similarly, in T2S, GspL has been shown to interact with the core ATPase GspE, thus BfpC and GspL are functional homologues.
as well. This structural evidence further demonstrates how similar the mechanism is of Tfpa and Tfpb biogenesis and T2S (Yamagata et al., 2012).

1.2.4.2 The evolutionary related Type II secretion system (T2S)

T2S is a conserved system in Gram-negative bacteria involved in the release of folded proteins, including several virulence factors, enzymes and toxins, into the surrounding environment. This system involves a set of 12 to 16 different proteins and as mentioned previously it is evolutionary related to Tfp, having many protein homologues including the core proteins (Table 1.1) (Douzi et al., 2012).

Exoproteins are secreted into the extracellular milieu by a two-step process. Firstly they are exported into the periplasm. Proteins that require cytoplasmic folding are exported by the Tat export pathway, while unfolded precursors are transported by the Sec export system. In the second step, the folded exoproteins localized in the periplasm are translocated across the outer membrane by a supramolecular complex called the secreton, made of many Gsp proteins, which spans both the inner and the outer membranes (Douzi et al., 2012) (Figure 1.6).

The secreton comprises three subcomplexes: 1) An inner membrane platform (IMP) composed of the cytoplasmic traffic ATPase, GspE together with four other inner-membrane proteins: GspC, GspF, GspL and GspM (Douzi et al., 2012) (Figure 1.6). 2) The pseudopilus, which is a pilus-like structure formed by five proteins with class III signal peptides. These are GspG, GspH, GspI, GspJ and GspK and are all processed by the prepilin peptidase, GspO (Nunn & Lory, 1992) (Figure 1.6). The most abundant pseudopilin, the major pilin, is GspG, while the rest are minor pseudopilins. In agreement with this, it was shown that when overproduced GspG is able to assemble into a long filament structure, named hyperpseudopilus. This structure protrudes out of the cell and resembles Tfp, and its length depends on the cellular levels of GspG (Sauvonnet et al., 2000, Durand et al., 2003, Vignon et al., 2003). In contrast, the four minor pseudopilins do not form a hyperpseudopilus when overproduced, but it is proposed that they are involved in the initiation step of the fibre formation and also in controlling its length (Durand et al., 2005, Cisneros et al., 2012a). 3) The outer-membrane protein secretin, GspD, which forms a large gated
channel of 12-15 subunits, to allow the passage of the proteins to the extracellular medium (Douzi et al., 2012) (Figure 1.6).

**Figure 1.6 Model of the T2S pathway in Gram-negative bacteria.**

The secreton is divided into three subcomplexes. The IMP is composed of the traffic ATPase GspE and GspC, GspF, GspL and GspM. The pseudopilus, is mostly composed of GspG, and capped by the minor pseudopilins GspH, GspI, GspJ and GspK. All pseudopilins are processed by the prepilin peptidase, GspO. The secretin GspD forms a dodecameric pore in the OM, through which the pseudopilus pushes the exoproteins into the extracellular milieu. OM, outer membrane. IM, inner membrane. Adapted from Douzi et al. (2012).

So far, the exact mechanism via which the proteins are exported in not known, but briefly it is proposed that ATP hydrolysis by GspE promotes the assembly of pseudopilus, which pushes the exoproteins through the secretin channel (Filloux, 2004) (Figure 1.6). The architectural similarity to Tfp denotes that T2SS functions by a similar mechanism and allows important parallels to be drawn between the two systems.
1.2.4.3 Molecular mechanism of Tfp biogenesis

The molecular mechanisms underlying Tfp biogenesis are still to be elucidated largely because of the complexity of a system, involving so many proteins. It is, however, generally acknowledged that they exert their action within a large multiprotein complex, similar to the T2S. Therefore, a comprehensive understanding of the Tfp multiprotein machinery would require the characterization of underlying interactions between the Pil proteins.

Systematic studies aiming to identify interactions between Tfp proteins have been performed only in EPEC that express Tfpb (Bfp). In the first study, a phenotypic characterization of in-frame mutations of each of the 14 bfp operon genes (bfpA, bfpG, bfpB, bfpC, bfpU, bfpD, bfpE, bfpF, bfpP, bfpH, bfpI, bfpJ, bfpK and bfpL) was conducted and it was found that 12 genes (all apart from bfpF which encodes an ATPase that potentially mediates retraction and bfpH) are essential for the biogenesis of Bfp. Subsequently, interactions were identified by determining the stability of every Bfp protein (except of the prepilin peptidase BfpP, and BfpH) using Western blotting, in mutants containing in-frame deletions of each of the 14 bfp genes (Ramer et al., 2002). The principle of this experiment is that proteins within a complex stabilize each other; therefore absence of one protein might lead to the instability of its interacting partners. The possibility that lower protein expression levels were due to polar effects was eliminated because for the majority of deletions, proteins encoded by genes downstream of a mutation were observed at wild-type (WT) levels. In addition, complemented mutants were created which restored the protein expression of all proteins to WT levels. In this study the localization of the Bfp proteins was also determined by analyzing cell fractions of inner membrane, outer membrane, periplasm and cytoplasm using Western blotting and finally it was shown that the prepilin peptidase BfpP is required for cleavage of the major pilin BfpA as well as the pilin-like proteins BfpI, BfpJ and BfpK. Based on the results of the subcellular localization analysis and the interaction analysis, four subcellular protein ensembles were proposed: 1) the outer membrane complex consisting of the secretin protein BfpB, and BfpG and BfpU. 2) A periplasmic subassembly defined by BfpU which interacts with the outer membrane
subassembly complex and the inner membrane pilin-like proteins BfpI, BfpJ and BfpK. 3) A subassembly that resides on the periplasmic face of the inner membrane consisting of BfpC, BfpU, BfpI, BfpJ, BfpK and BfpL. 4) The integral membrane protein BfpE, is a distinct entity as its absence reduced the levels of all proteins apart from the main pilin BfpA and BfpC. Interestingly, the levels of BfpA were not affected by any of the bfp mutations and it was found mostly associated with the inner membrane. The core ATPase BfpD, the retraction ATPase BfpF and the prepilin peptidase BfpP were not localized in this study (Ramer et al., 2002).

In the second study, the secretin BfpB was replaced with a hexahistidine-tagged version in EPEC, and subsequently the Bfp expressing cells were incubated with a membrane permeable, disulphide cleavable cross-linker. The chemically cross-linked complex was purified by affinity chromatography and proteins in the complex were identified by Western blotting (Hwang et al., 2003). This showed that 10 out of the 14 Bfp proteins form a macromolecular complex spanning the periplasmic space. These are the major pilin BfpA, the secretin BfpB, the core ATPase BfpD, the core integral protein BfpE, the retraction ATPase BfpF, the minor pilin BfpJ, BfpC, BfpG, BfpL, and BfpU (Hwang et al., 2003). Figure 1.7 displays most of the key interactions identified in these two studies, based on all of the core proteins and a few non-core proteins.
Figure 1.7 Schematic presentation of the biogenesis machinery of Bfp (Tfpb) pili in EPEC.

This figure displays the key definite interactions identified from studies of Bfp proteins.

Unfortunately, the results of these two studies could not be extrapolated to Tfpα-expressing bacteria, owing to the significant differences between these two systems. Nonetheless, a picture of the organization of the Tfpα biogenesis machinery has emerged from a genetic analysis in *N. meningitidis* which has shown that Tfp biogenesis can be resolved into clearly distinct steps: prepilin transport and processing, pilus assembly, functional maturation, counteraction of PilT-mediated pilus retraction, and emergence of Tfp on the surface (Carbonnelle *et al.*, 2006).
1.2.4.3.1 Prepilin transport and processing

PilE is synthesized as prepilin, and is translocated by the Sec machinery, via the signal recognition protein (SRP) route, into the inner membrane where it remains as a bitopic protein with its charged leader peptide in the cytoplasm, the α1-N helix in the membrane, and the C-terminal globular head in the periplasm (Arts et al., 2007b, Francetic et al., 2007). Subsequently, the polytopic bifunctional enzyme prepilin peptidase, PilD, cleaves the leader peptide and methylates the N-terminal amino acid of the mature pilin (Nunn & Lory, 1991, Strom et al., 1993b). However, it was shown that the two activities of the PilD are uncoupled and whilst cleavage of the leader peptide is a necessary step for the assembly of the pilus, methylation is not required and its function is still to be determined (Pepe & Lory, 1998). A mutational analysis of P. aeruginosa pilin subunits revealed that the only prepilin residue necessary for cleavage of the prepilin leader peptide is the invariant glycine at -1, while the conserved glutamate at +5 was found to be dispensable for processing yet essential for pilus assembly (Strom & Lory, 1991). However, efficient processing of the prepilin of the R64 thin pilus (Tfpb) of E. coli, which is required for conjugation in liquid culture, was shown to require more residues apart from the conserved glutamate (Horiuchi & Komano, 1998).

Studies to identify the catalytic site of PilD initially focused on two pairs of cysteines in the largest cytoplasmic domain of the enzyme which are conserved in many prepilin peptidases. However, mutational analysis of the cysteines failed to completely eliminate the enzyme’s activity (Strom et al., 1993a). A later study showed that only two conserved aspartate residues, in the predicted two smaller cytoplasmic domains, are absolutely required for the protease activity, as substitution of those residues by a number of amino acids caused a complete loss of activity (LaPointe & Taylor, 2000). Therefore, prepilin peptidases represent a novel class of aspartate proteases (LaPointe & Taylor, 2000).

The only atomic-resolution structure in the GXGD family of proteases (a common moniker for the aspartate proteases) is of FlaK, a preflagellin peptidase from Methanococcus maripaludis and it provides a framework for understanding this
family of proteases (Hu et al., 2011) (Figure 1.8). This showed that FlaK contains two compactly folded domains: a membrane-spanning domain consisting of six α-helices (α1-6) and a soluble cytoplasmic domain consisting of four anti-parallel β-strands formed between α5 and α6 (β1-4) (Hu et al., 2011) (Figure 1.8). The protein is tilted in the membrane to direct charged amino acids away from the hydrophobic environment and also to accommodate an unusual structure formed by the protein segments after α4 and α6, which protrudes sideways (Hu et al., 2011). The two catalytic aspartate residues (Asp18 and Asp79) are located at the end of helices α1 and α4, respectively. These were found to be structurally uncoupled with a wide space between them, which indicates that the protein switches between at least two conformations (Figure 1.8). In one conformation the two aspartates are at a distance from each other, whilst in the other they are in close proximity for catalysis to occur (Hu et al., 2011). Such an uncoupling mechanism is proposed to be a general mechanism, in all GXGD proteases, to regulate the PilD protease activity.
Figure 1.8 The structure of FlaK.

(A) Two views of FlaK in a ribbon representation. The secondary structural elements (six α-helices, α1-6, and four anti-parallel β-strands, β1-4) and the GXGD motif, which consists of the second aspartate residue of the catalytic site, are labelled. (B) A view from the cytoplasmic site of the membrane of the uncoupling between Asp 18 and Asp 79. Figure from Hu et al. (2011).
The four cysteines initially identified as the catalytic site for both peptidase and methyltransferase activities were shown in a mutagenesis analysis of the *P. aeruginosa* PilD, to be involved in the N-terminal methylation of the mature pilin (Pepe & Lory, 1998). More recently, it was shown in a cell free system that the *P. aeruginosa* PilD binds zinc by these four cysteine residues, which have hallmarks of Zinc-finger-like like motifs, and that zinc is essential for the N-terminal methylation of the mature pilin but not for the cleavage of the prepilin signal peptide (Aly et al., 2013). This finding is highly significant as it helps to differentiate the requirements of the two PilD activities. The zinc-finger like motif was proposed to be involved in the folding and stability of the PilD cytoplasmic domain and/or for the recognition and binding of either the pilin or the methyl donor S-adenosyl methionine. Moreover, this cell free system elegantly demonstrated that PilD and PilE work independently of other Tfp system components, as PilD cleaves and methylates PilE in the absence of any other proteins.

1.2.4.3.2 Tfp assembly

The assembly process requires energy, and this is provided by the cytoplasmic core traffic ATPase, PilF, which is recruited to the cytosolic face of the inner membrane. This was identified in early genetic studies in *P. aeruginosa*, which indicated that extracellular secretion of proteins and assembly of Tfp require traffic ATPases, to provide the energy (Turner et al., 1993). Structural analysis of similar traffic ATPases, most notably PilT, showed that these are dynamic homohexamers that undergo major conformational changes upon ATP binding and hydrolysis, generating mechanical force (Misic et al., 2010, Satyshur et al., 2007). However, the process of how PilF powers assembly of Tfp is unclear. A model has been proposed in which PilF exists in a subcomplex with unknown inner membrane proteins, which are energised by ATP hydrolysis to act like a mechanical piston pushing PilE subunits into a growing pilus (Craig et al., 2006). The presence of such a multi-protein complex has been reported in EPEC where the ATPase, BfpD, is recruited to the inner membrane by binding to the cytoplasmic N-termini of BfpC, a bitopic inner membrane protein, in the presence of BfpE, a conserved polytopic inner membrane protein (Milgotina et
Conversely, it is reported that *T. thermophilus pilF* mutants are piliated, which raises questions as to whether this PilF, which shares sequence similarities with the Tfp biogenesis ATPases, is involved in Tfp assembly (Collins et al., 2013). Also, the *T. thermophilus pilF* mutants displayed reduced competence and the PilF protein was shown to bind to DNA without any sequence specificity, suggesting a role in DNA uptake. Therefore, caution should be taken in extrapolating results between different bacteria, as subtle differences in the way in which assembly systems work may be apparent (Collins et al., 2013).

A study in *N. gonorrhoeae*, reported that a lack of piliation in a *pilC* mutant was suppressed by a second mutation in a gene encoding PilT, the traffic ATPase that powers retraction (Wolfgang et al., 1998b). This demonstrated for the first time that some Pil proteins could be involved in biogenesis, not by participating in the assembly of the pilus *per se* but by counteracting the retraction mediated by PilT. Consequently, lack of piliation in the corresponding mutants would be due to uncontrolled pilus retraction, which could in turn be suppressed upon introduction of a second mutation in *pilT*. This finding inspired a systematic genetic analysis in *N. meningitidis* in which a *pilT* mutation was introduced into each of the identified 15 non-piliated *pil* mutants and piliation of the double mutants was assessed again by IF microscopy using a monoclonal antibody specific for the Tfp fibers (Carbonnelle et al., 2006). This showed that piliation could be restored in the absence of the majority of proteins (PilC1/2, PilG, PilH, PilI, PilJ, PilK, PilQ and PilW) when retraction was abolished by a concurrent mutation in *pilT*, demonstrating that they are not involved in pilus assembly (Carbonnelle et al., 2006) (Table 1.1). Consequently, a surprisingly small number of proteins PilE, PilD, PilF, PilM, PilN, PilO and PilP were predicted to be dedicated in the filament assembly, as piliation was not restored in the corresponding double mutants (Carbonnelle et al., 2006) (Table 1.1). The finding that PilG is not one of the proteins dedicated to the assembly of the Tfp is a paradox, because its status as a core protein, conserved in all Tfp systems, implies that it is a key player in Tfp biology (discussed in section 1.2.4.3.3).
Table 1.1: Components of the machinery involved at various steps of Tfpa biogenesis \textit{(Neisseria} species and \textit{P. aeruginosa}), their homologous proteins in Tfpb biogenesis \textit{(EPEC} and \textit{V. cholerae}) and T2S, and their corresponding main features.

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<td>Class III signal peptide</td>
<td>PilH</td>
<td>FimU</td>
<td></td>
</tr>
<tr>
<td>Class III signal peptide</td>
<td>PilI</td>
<td>PilIV</td>
<td>BfpI</td>
</tr>
<tr>
<td>Class III signal peptide</td>
<td>PilJ</td>
<td>PilIW</td>
<td>BfpJ</td>
</tr>
<tr>
<td>Outer membrane lipoprotein</td>
<td>PilK</td>
<td>PilIK</td>
<td>BfpK</td>
</tr>
<tr>
<td>Secretin</td>
<td>PilQ</td>
<td>PilQ</td>
<td>BfpB</td>
</tr>
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</table>

Since the roles of core proteins PilE, PilD and PilF were already known, the remaining non-core proteins PilM, PilN, PilO and PilP are expected not only to form the inner-membrane multi-protein assembly machinery, but also to convert the chemical energy generated by the hydrolysis of ATP by PilF to mechanical force leading to the extrusion of the pilins out of the inner membrane and to anchor the filaments firmly in the membrane during the powerful cycles of extension/retraction (Pelicic, 2008).

My PhD work mainly focused on these four proteins, so even though other groups and us recently published reports based on their characterization, the following paragraph summarizes the minimal work done up to the year 2009.

PilM, PilN, PilO and PilP are encoded by the \textit{pilMNOPQ} operon, which is conserved among Tfpa-expressing bacteria (Pelicic, 2008). PilM is a cytoplasmic protein, PilN and PilO are bitopic inner membrane proteins and PilP is a lipoprotein (Carbonnelle \textit{et al.}, 2006). PilP was first identified to be involved in Tfp biogenesis in \textit{N. gonorrhoeae} by Drake \textit{et al.} (1997), as gonococcal pilP mutants bearing transposon insertions in the central part of the open reading frame were non-piliated and failed
to express Tfp-mediated phenotypes (Drake et al., 1997). Additionally, this study showed that PilP is a lipoprotein (Drake et al., 1997). Interestingly, it was also observed that in gonococcal pilP mutants the levels of PilQ multimers, for the emergence of Tfp in the surface, were greatly reduced, suggesting that PilP is involved in the stabilization of PilQ multimers in the outer membrane (Drake et al., 1997). However, both M. xanthus and N. meningitidis pilP mutants assembled WT levels of PilQ multimers, contradicting this idea (Nudleman et al., 2006, Carbonnelle et al., 2005). It was later shown in N. meningitidis that the levels of PilQ multimers can be greatly reduced due to a polar effect on pilQ transcription, exhibited by the pilP mutant (Balasingham et al., 2007). Also, PilP has been shown to interact with PilQ, this is discussed in section 1.2.4.3.4 (Balasingham et al., 2007). The solution structure of the N. meningitidis PilP was solved by NMR, minus the first 68 residues within which the cysteine residue residing at position 16 participates in a thioester bond with the fatty acid in the mature lipoprotein (Golovanov et al., 2006) (Figure 1.9). The structure revealed a domain which adopts a simple β-sandwich type fold that consists of a three-stranded antiparallel β-sheet packed against a four-stranded antiparallel β-sheet. Also, at the N-terminus of this domain there is a short 3_10 helix, which makes hydrophobic interactions with residues in both of the β-sheets (Figure 1.9A). In addition, this study identified a potential binding site for a small ligand, as the two β-sheets pack together to form a crevice that is lined with highly conserved hydrophobic residues (Golovanov et al., 2006) (Figure 1.9B). Moreover, PilP has been shown to be located at the inner membrane (Balasingham et al., 2007).
Figure 1.9 The structure of *N. meningitidis* PilP.

(A) Two views of PilP in a ribbon representation with β-strands in yellow and helix in red. The seven β-strands are labelled. (B) The potential binding site of PilP, lined with highly conserved hydrophobic residues. From Golovanov et al. (2006).
1.2.4.3.3 Tfp stabilization and functional maturation

The Tfp stabilization step of biogenesis remains poorly defined. As mentioned above, 7 out of the 15 proteins essential for Tfp biogenesis (PilC1/C2, PilG, PilH, PilI, PilJ, PilK and PilW) act after pilus assembly to counteract PilT-mediated retraction, because piliation was restored in the corresponding *N. meningitidis* mutants, upon introduction of a second mutation into PilT (Carbonnelle *et al.*, 2006) (Table 1.1). This finding highlights the importance of pilus homeostasis in Tfp biogenesis.

Furthermore, this Tfp stabilization step is linked to the functional maturation of the pilus as deduced by Carbonnelle *et al.* (2006). Phenotypic characterization of these double mutants expressing morphologically normal Tfp to WT levels, for all the functions not abolished in the absence of retraction, these are: aggregation and adhesion to human umbilical vein endothelial cells (HUVEC), showed that most of them are non-functional (Carbonnelle *et al.*, 2006). This demonstrated that these proteins are also important in the functional maturation of Tfp, as well. However, the restored fibres in the pilG/T and pilH/T mutants were shown to be partly functional, indicating that PilG and PilH are to some extent dispensable in the functional maturation step of Tfp biogenesis (Carbonnelle *et al.*, 2006). Regardless, of the molecular mechanism of PilT-mediated retraction (section 1.2.5), these seven proteins are hypothesized to interact directly with the Tfp and hinder retraction by acting like a ‘pincer’.

Though the exact functions of PilC1/C2, PilG, PilH, PilI, PilJ, PilK and PilW are not known, recent work improved our general understanding of this step. The two slightly different variants of the PilC protein, PilC1 and PilC2, are independently expressed from separate loci and have been extensively studied in the *Neisseria* species, because apart from being involved in Tfp biogenesis, they are involved in adhesion to human cells (Nassif *et al.*, 1994, Rudel *et al.*, 1995). A series of elegant studies performed using the *P. aeruginosa* PilC homologue, PilY1 have shed light to the function of this protein. Firstly, the crystal structure of the C-terminal domain of PilY1, which is conserved in all PilC proteins (while the N-terminal domains of PilCs are species-specific) revealed a modified β-propeller fold and a distinct EF-hand-like
calcium-binding site (Orans et al., 2010). Strikingly, this calcium binding was shown to be a regulator of twitching motility. In the calcium-bound state PilY1 inhibited PilT-mediated pilus retraction; however, in the calcium-free state, PilY1 was incapable of antagonizing PilT-mediated retraction, leading to loss of cell surface pili (Orans et al., 2010). This characteristic of the conserved calcium-binding site to act as a regulator of twitching motility, has been confirmed to be common to PilC homologues, as it was identified in the two PilC proteins of Kingella kingae (Porsch et al., 2013). However, interestingly it was shown that while this domain is necessary for twitching motility in PilC1, in PilC2 it had a minor influence (Porsch et al., 2013). Moreover, another study reiterated in P. aeruginosa the findings identified originally in N. gonorrhoeae and then in N. meningitidis, whilst the pilY1 mutant is non-piliated, piliation was restored in the pilY1/T mutant (Wolfgang et al., 1998b, Carbonnelle et al., 2006, Heiniger et al., 2010). This confirmed in another different species that although this protein is essential for Tfp biogenesis, it is dispensable for pilus assembly. Lastly, it was shown that the N-terminal domain of P. aeruginosa PilY1 is directly involved in adhesion to host epithelial cell. An integrin-binding arginine-glycine-aspartic acid (RGD) motif was identified in this domain, and the purified PilY1 was shown to bind integrin in vitro, in an RGD-dependent manner (Johnson et al., 2011). Therefore, as this N-terminal domain of PilY1 is involved in adhesion and different species adhere to diverse surfaces, it is logical why this domain is species-specific.

PilG is one of the most peculiar Pil proteins involved in Tfp biogenesis. Its PilC homologue in P. aeruginosa was first identified because the pilC mutant was non-piliated and resistant to infection by bacteriophage PO4, which indicated a role in Tfp biogenesis (Nunn et al., 1990). Similarly, PilG was later identified in N. gonorrhoeae, as the pilG mutant was non-piliated and displayed dramatically reduced competence (Tonjum et al., 1995). Generally in the literature it is hypothesized that PilG is the platform on which the Tfp biogenesis machinery is built, due to its status as a core protein. However, in N. meningitidis, this alleged function is strongly contradicted because PilG is dispensable for the assembly of the filaments, and instead proteins PilM, PilN, PilO and PilP seem to undertake this
function (Carbonnelle et al., 2006). However, though it is not involved in Tfp assembly, studies in Tfpb and T2S indicate that its role in counter-retraction is probably mediated by interacting with the ATPases of the systems. As shown in the two systematic studies in Tfpb system, its homologue BfpE exists in a multiprotein complex with the ATPase BfpD (Ramer et al., 2002, Hwang et al., 2003). Another study showed that BfpD is recruited into the membrane more efficiently when it is expressed in the presence of both BfpC and BfpE (Milgotina et al., 2011). The same study provided evidence for a possible interaction with the putative retraction ATPase, BfpF. Upon overexpression of the cytoplasmic loop of BfpE in WT EPEC, bacteria could aggregate but failed to disaggregate, which is a phenotype exhibited in the bfpF mutant due to an inability to retract pili. This observation was consistent with the concept that cytoplasmic BfpE competes with the WT BfpE for binding to the BfpF, leading to a bfpF mutant phenotype, which implies that BfpF interacts with BfpE (Milgotina et al., 2011). Also, in T2S the homologue of PilG, GspF was shown to co-purify with the complex of GspL-GspE (N-GspL is structurally homologous to N-BfpC and PilM, and GspE is homologous to BfpD and PilF), and in addition its stability was dependent on the simultaneous co-expression of both GspL and GspE (Arts et al., 2007a) (Figure 1.6). Several bioinformatic tools predicted that the tertiary structure of the N. meningitidis PilG consists of four transmembrane helices and it is divided into two unequal halves: an N-terminal cytoplasmic domain of around 170 residues and a C-terminal periplasmic domain of 100 residues which are separated by three transmembrane helices (Collins et al., 2007). Electron microscopy data showed that PilG is a tetramer with cytoplasmic and periplasmic domains protruding from the inner membrane. The top periplasmic domain of the PilG tetramer consists of four periplasmic ‘fins’, which form a shallow, cone-shaped invagination that can potentially form a binding surface (Collins et al., 2007). Consequently, based on the fact that PilG, along with PilH, are the only N. meningitidis proteins involved in counteracting PilT-mediated retraction that are partly dispensable for functional maturation of the Tfp fibers and the evidence from Tfpb and T2S systems that PilG interacts with the ATPases, a possible role of this protein is to couple ATP hydrolysis by PilT to the pilus disassembly.
PilH, PilI, PilJ and PilK are all core pilin-like proteins, which are processed by the pre pilin peptidase PilD. As mentioned before, in the homologous T2S system the minor pseudopilins are involved in the initiation step of the pseudopilus assembly. This was demonstrated in an elegant study of the *Klebsiella oxytoca* T2S, which showed that the two minor pseudopilins Pul and PilJ, form a staggered complex in the inner membrane, to which another minor pseudopilin, PulK, binds. This leads to the stabilization of the complex and also induces an upward movement, causing a partial extraction of PulK from the inner membrane with a displacement of approximately 1 nm with respect to Pul and PilJ, which is identical to the 1 nm axial rise between the major pseudopilins, PulG, in the assembled pseudopilus (Cisneros et al., 2012a). The finding that minor pilins have an intrinsic binding property to form a PilI-PilJ-PilK complex, which acquires a pseudopilus-like structure in the membrane, led to a proposed model in which this complex reduces the kinetic barrier to initiate pseudopilus assembly by coordinating structurally the incoming pseudopilins and bringing the assembly ATPase close to the inner membrane to activate ATP hydrolysis (Cisneros et al., 2012a). Remarkably, a similar role of minor pilins in Tfpb biogenesis is supported by a follow-up study, in which Tfp were assembled heterologously in *E. coli* by the T2S system of *K. oxytoca* (Cisneros et al., 2012b). In the *E. coli* K-12 strain Tfp genes exist but they are silent under standard growth conditions. In this study, it was shown that when Pul T2S system is reconstituted in *E. coli* and the *ppdB* gene, which encodes the *E. coli* K-12 major pilin is expressed, pili are formed (Cisneros et al., 2012b). However, the assembly of pili dependent on the expression of the *K. oxytoca* minor pseudopilin genes pilHIJK, as in the absence of PilH, PilI, PilJ and PilK piliation was abolished (Cisneros et al., 2012b). Moreover, in *E. coli* expressing the T2S *pul* system devoid of all pseudopilin genes, it was shown that assembly of PpdD pilus can be initiated by the minor Tfp pilins, encoded by the *ppdAB-ygdB-ppdC*, when expressed. In accordance with this the minor Tfp pilins PpdA, PpdB, YgdB and PpdC could initiate PulG pilus assembly in *E. coli*, though the pilus was non functional in protein secretion (Cisneros et al., 2012b). However, so far there is no evidence supporting such a role of minor pilins in Tfpb biogenesis. Firstly, as shown in *P. aeruginosa*, minor pilins are incorporated throughout the Tfp filaments (Giltner et al., 2010). Also, it was shown in *N. meningitidis, N. gonorrhoeae*
and *P. aeruginosa* that in *pilH, pill, pilJ* and *pilK* mutants, piliation is restored in a retraction-deficient background (Carbonnelle *et al.*, 2006, Winther-Larsen *et al.*, 2005, Giltner *et al.*, 2010). This indicates that pilus initiation is possible in the absence of these proteins. Nonetheless, it might be possible that this pilus initiation step is dispensable in the absence of pilus retraction, thus it should be further characterized.

PilW is an outer-membrane lipoprotein. This protein is multifunctional, since apart from its role in Tfp stabilization and functional maturation, it is also important in the stability or assembly of PilQ secretin multimers, which form the outer-membrane channel through which Tfp emerge on the bacterial cell surface (section 1.3.3.4) (Carbonnelle *et al.*, 2005). In the absence of PilW only monomeric PilQ was present, which was still localized in the outer membrane, whilst PilQ multimers were detected in the absence of the rest of the Pil proteins (Carbonnelle *et al.*, 2005). The crystal structures of PilW and its *P. aeruginosa* orthologue PilF, revealed that they consist of 13 anti-parallel α-helices that fold into six tetratricopeptide (TPR) motifs (Kim *et al.*, 2006, Trindade *et al.*, 2008, Koo *et al.*, 2008). The TPR motif is a 34 amino acid repeat, with a highly degenerate sequence that is involved in protein-protein interactions, mainly in multiprotein complexes. The overall PilW structure is folded into a right-handed, super-helix of which the two halves form a deep concave area, which is proposed to be a protein-binding region (Trindade *et al.*, 2008). As in *N. meningitidis*, loss of the PilW homologues TgI and PilF in *M. xanthus* and *P. aeruginosa* respectively, abolished piliation and also multimerization of PilQ multimers (Nudleman *et al.*, 2006, Koo *et al.*, 2008). However, in a *pilF* mutant the PilQ monomers were detected only in the inner membrane, which suggested a role of PilF in the localization of PilQ, rather than in its stabilization or multimerization as observed for PilW (Koo *et al.*, 2008). In the absence of the N-terminal lipidation, which mislocalizes the protein to the periplasm, PilF was partly functional as the corresponding strain mediated twitching motility and bacteriophage susceptibility (Koo *et al.*, 2008). Additionally, PilQ multimers were formed but they were detected both in the inner and outer membrane, which suggested that tethering via its lipid anchor promotes the correct localization of PilQ multimers (Koo *et al.*, 2008). A
structure/function analysis of the *N. meningitidis* PilW, similarly showed that PilW is partly functional in the absence of N-terminal lipidation. However, mislocalization of PilW decreased its stability and also reduced the level of PilQ multimers observed (Trindade *et al.*, 2008, Szeto *et al.*, 2011). These results indicated that correct localization of PilW to the outer membrane enhances its stability and functional efficiency to multimerize/stabilize the PilQ multimers. Hence, these findings do not support that PilW is a ‘pilot’ protein, whose role would be to ‘pilot’/localize PilQ to the outer membrane; instead they support a role in the stabilization or formation of PilQ multimers. In agreement with this, the PilW structure is strikingly different from that of the *bona fide* MxiM pilotin of the type III secretion system of *Shigella flexneri*, which strengthens the hypothesis that PilW is not a pilotin (Lario *et al.*, 2005, Trindade *et al.*, 2008, Szeto *et al.*, 2011). The same study showed that conserved residues within a defined deep groove of PilW are important for the Tfp-mediated functions but they are not involved in the multimerization of PilQ (Szeto *et al.*, 2011). This illustrated that the involvement of PilW in the stabilization or assembly of secretin channels can be genetically uncoupled from its role in Tfp functional maturation (Szeto *et al.*, 2011). Significantly, recently an interaction between the *P. aeruginosa* PilF and PilQ proteins was formally demonstrated using pull down assays (Koo *et al.*, 2013). This study also provided evidence that a narrow, defined hydrophobic groove on TPR1 of PilF is important for the multimerization of PilQ, as substitution of residues within this region resulted in loss of PilQ outer membrane localization and/or multimerization, without significantly affecting the stability of PilW (Koo *et al.*, 2013).

1.2.4.3.4 Emergence of Tfp on the bacterial surface

Tfp emerge on the bacterial surface through a pore formed by multimers of a core protein located in the outer membrane, belonging to the secretin family. In *N. meningitidis*, the PilQ secretin is encoded by the last gene of the *pilMNOPQ* operon (Pellicic, 2008). The role of PilQ in Tfp biogenesis was confirmed from studies in *Neisseria* species, which showed that piliation was restored in a double *pilQ/T* mutant, but the fibres remained trapped within the periplasm (Wolfgang *et al.*, ...
2000, Carbonnelle et al., 2006). In *N. gonorrhoeae pilQ/T* mutant, electron microscopy showed that the fibres formed were membrane-bound contained in protrusions, while in *N. meningitidis pilQ/T* mutant, the fibres were released from the periplasm using osmotic shock treatment and they were visualized using IF microscopy (Wolfgang et al., 2000, Carbonnelle et al., 2006).

The PilQ complex, as determined by electron and cryoelectron microscopy, consists of 12 subunits forming a ring-like cylindrical structure with a large funnel-shaped central cavity (Collins et al., 2003, Collins et al., 2004, Berry et al., 2012) (Figure 1.10A). This cavity is sealed at both ends; at the bottom by a ‘plug’ feature and emerging from the top, four arms coil over the cavity forming a dome shaped cap (Collins et al., 2004). As shown *in vitro*, Tfp bind into the PilQ chamber, and electron microscopy data revealed that pilin subunits are able to fill the secretin chamber, inducing structural changes in PilQ (Collins et al., 2005).

PilP has been shown to interact *in vitro* with PilQ (Balasingham et al., 2007). More recently, it was shown using NMR chemical shift perturbations that the C-terminal domain of PilP binds to the first of the two periplasmic α/β domains (namely the N0/N1 domains, located at the N-terminus) of *N. meningitidis* PilQ. This allowed the construction of a structural model of the PilP:PilQ complex, which places the potential PilP binding site exposed on the outer surface (Berry et al., 2012) (Figure 1.10B-D). This model suggested that PilP is needed to stabilize the PilQ oligomer during secretion, by anchoring the PilQ periplasmic domains at the inner membrane (Berry et al., 2012). Moreover, this subcomplex is linked to PilW that also interacts with PilQ (Koo et al., 2013). Additionally, it was suggested by Koo et al. (2008) that the N-terminal α/β domains (N0/N1) of *P. aeruginosa* PilQ interact with PilF (*PilW* orthologue) (Koo et al., 2008). This was proposed as several PilQ mutants were constructed, each consisting of a deletion of a different PilQ domain, which were expressed in *P. aeruginosa* and their expression and ability to form multimers was assessed by Western blotting. Out of all the mutants only two, each consisting of a deletion in one of the two α/β domains, were stably expressed in *P. aeruginosa* but did not form PilQ multimers, which showed that formation of multimers requires these two domains (Koo et al., 2008).
Figure 1.10 Cryoelectron microscopy structure of the *N. meningitidis* PilQ dodecamer and model of the PilP-PilQ assembly.

**A** Left panel: surface-contoured map. Right panel: surface-contoured map, but with half of the volume removed to reveal major domain boundaries. These are: the C-terminal domain, which is embedded in the outer membrane. The N0/N1 domains, which represent constitutively arranged α/β domains. The β domains, which represent structural regions rich in β-structure. **(B)** The B2 domain (left panel) and N0/N1 domain (middle panel) and both (right panel) are docked into the PilQ cryoelectron density map. The B2 domain is shown with a purple to blue gradient from the N- to C-terminus. The N0/N1 domain is shown with an indigo (N0) to green (N1) gradient from the N- to C-terminus. **(C)** Reconstruction of PilQ N0/N1/B2 domain structures (coloured as B) with PilP C-domain bound, which is shown in orange. Left panel: side view with 6 oligomers; right panel: top view with 12 oligomers. **(D)** Detail of two oligomers on opposing sides of the PilQ chamber. From Berry *et al.* (2012).
Secretins sometimes are targeted to the outer membrane with the help of small lipoproteins referred to as ‘pilotins’, which protect them from proteolysis and prevent premature multimerization in the cytoplasm (Guilvout et al., 2006). For instance, in T2S the Gsp pilotin PulS in *K. oxytoca* binds to the secretin PulD and targets it to the outer membrane. This was illustrated in studies showing that when PulS was mislocalised to the periplasm or the inner membrane, it prevented secretin multimerization into the outer membrane (Hardie et al., 1996, Collin et al., 2011).

Even though, PilP and PilW are assigned to be the pilot proteins by several reports, up to now there is no strong evidence supporting such a role. Drake and colleagues reported that PilP was a possible pilotin for PilQ, because in gonococcal *pilP* transposon mutants there was decreased formation of PilQ multimers (Drake et al., 1997). However, PilP protein is precluded to be a pilotin because in subsequent studies PilQ multimers were detected by Western blotting in its absence, in both species *N. meningitidis* and *M. xanthus* (Carbonnelle et al., 2005, Nudleman et al., 2006). Also, it is now understandable that the reduced levels of PilQ multimers observed in the gonococcal *pilP* transposon mutants were possibly due to a polar effect, from the mutation of *pilP* gene on the transcription of *pilQ*, as these two genes are co-transcribed in an operon (Balasingham et al., 2007). The role of PilW as pilotin is rather contentious. Even though studies in *N. meningitidis*, *M. xanthus* and *P. aeruginosa* agree that PilW is involved in the multimerization of PilQ multimers, as in all species PilQ multimers were not formed in the absence of PilW, studies in *N. meningitidis* and *P. aeruginosa* disagree regarding the role of PilW as a PilQ pilotin, due to key differences observed when PilW was mis-targeted to the periplasm (these are discussed in 1.2.4.3.3) (Carbonnelle et al., 2005, Nudleman et al., 2006, Koo et al., 2008, Szeto et al., 2011). However, concluding on the fact that unlike bona fide pilotins, when PilW was mislocalized to the periplasm in both *N. meningitidis* and *P. aeruginosa*, PilQ multimers were able to form, piliation was not abolished and Tfp were partly functional, PilW is not a pilotin (Koo et al., 2008, Szeto et al., 2011).

Moreover, the tertiary structure of PilW differs markedly from the well-characterized MxiM pilotin in the type III secretion system of *S. flexneri*, which further supports that PilW is not a pilotin (Lario et al., 2005, Trindade et al., 2008).
1.2.5 Proteins dispensable for Tfp biogenesis but playing significant roles in Tfp biology

Seven proteins (PilT, PilT2, PilU, PilV, PilX, ComP and PilZ), dispensable for piliation in *N. meningitidis*, were identified by a systematic study to be key players in Tfp biology, since their corresponding mutants displayed dramatically affected Tfp-mediated functions. Also, three of these proteins (PilT, PilU and PilZ) are conserved in Tfpa-expressing species (Brown *et al.*, 2010).

The most well known protein of all is PilT, which is the traffic ATPase that powers pilus retraction, a process that involves removal of pilin subunits from the base of the pilus, hence PilT antagonizes the core ATPase PilF. In its absence, most Tfp functions are affected as it leads to loss of twitching motility and DNA competence and increased aggregation and adhesion (Brown *et al.*, 2010). PilT is found in all Tfpa systems, but so far it has been identified only in Bfp pili of Tfpb system (Bieber *et al.*, 1998). PilT, like PilF, is a dynamic homohexameric assembly, formed mainly by extensive ionic interactions. Moreover, PilT belongs to the same family of secretion ATPases, conserved in T2S and Tfp systems, which share a relatively common structure consisting of an N-terminal domain, followed by the conserved C-terminal domain which encompasses four conserved sequence motifs: the Walker A box, the atypical Walker B box, the His box, and the Asp box (Misic *et al.*, 2010).

The molecular mechanism used by PilT to mediate retraction has not yet been determined. Structural studies in *P. aeruginosa* revealed that it is impossible for the pilin subunits to disassemble by passing through PilT as the base opening is not wide enough to accommodate the pilus (Misic *et al.*, 2010). This led to two possible mechanisms being proposed. The first one is referred as the ‘direct interaction’ model, in which PilT interacts with the bottommost pilin subunit through its N-terminal α-helix, and pulls and transfers it to the inner membrane. However, such an interaction between PilT and the N-terminal α-helix of PilE has not been identified, and also the N-terminal α-helix of PilE is unlikely to extend that far across the inner membrane to interact extensively with PilT (Misic *et al.*, 2010). The second model involved interaction of PilT with a conserved inner-membrane protein, which
upon ATP hydrolysis would change orientation to remove the last pilin subunit within the filament.

However, the molecular mechanism of PilT-mediated retraction is probably more complex than those described above because of the presence of PilT paralogs. In *N. meningitidis*, there are two PilT paralogs, PilU and PilT2 (*Neisseria*-specific), which are also dispensable for Tfp biogenesis but are involved in fine-tuning of the Tfp-linked functions (Brown *et al.*, 2010). These belong to the family of Type II/IV secretion system ATPases and they show a high degree (60%) of amino acid conservation with PilT, however they differ from PilT by the presence of terminal extensions (Chiang *et al.*, 2008, Brown *et al.*, 2010). PilT2 is an antagonist of Tfp assembly/stability and probably works synergistically with PilT, since a pilT2 mutant showed increased piliation (Brown *et al.*, 2010). Moreover, in another report PilT2 was shown to modulate the retraction speed by enhancing the speed of twitching motility, as in the pilT2 mutant the speed of twitching motility was significantly reduced (Kurre *et al.*, 2012). The role of PilU remains unclear mainly because pilU mutants of different organisms display different phenotypes. In *P. aeruginosa*, the pilU mutant like the pilT mutant displayed defective twitching motility as it lacked the spreading-colony morphology, which is characteristic of twitching motility, and it was also hyperpiliated (Whitchurch & Mattick, 1994). However, in contrast to the pilT mutant, the pilU mutant was not resistant to infection by bacteriophage POA (Whitchurch & Mattick, 1994). In *N. gonorrhoeae*, the pilU mutant displayed twitching motility, lacked aggregation but surprisingly displayed increased bacterial adherence for human epithelial cells (ME-180 human cervical carcinoma cell line), which was eight-fold higher compared to the WT (Park *et al.*, 2002). This is very unusual as aggregation and adhesion are closely linked Tfp-mediated properties and typically loss of aggregation is associated with a decrease in adhesion to human cells. In *N. meningitidis*, the pilU mutant showed only faster adhesion to HUVEC (Brown *et al.*, 2010). However, a more recent study in *N. meningitidis* showed that the pilU mutant delayed microcolony formation, and proposed that the faster adhesion observed in the original study is because both the pilU mutant and WT strain bind epithelial cells as single diplococci, and the inherent increase in cellular adhesion
displayed by pilU mutant is not coupled to the ability to form microcolonies (Eriksson et al., 2012). Therefore, overall these phenotypes of the N. meningitidis pilU mutant somewhat resemble those of the N. gonorrhoeae pilU mutant.

ComP, PilV and PilX are all minor pilins, harbouring the class III signal peptide and also the C-terminal D-region. Also, they are all processed by prepilin peptidase and co-purify with Tfp (Wolfgang et al., 1999, Brown et al., 2010, Winther-Larsen et al., 2001). A structure/function analysis of PilX revealed that its surface exposed D-region is involved in aggregation and adhesion, since deletion of the majority of this region abolished aggregation and adherence was reduced by 1000-fold when compared with the WT strain (Helaine et al., 2007). This led to a model being proposed. In WT cells, the PilX subunits within Tfp of interacting bacteria, are proposed to counteract PilT-mediated retraction, by bracing against each other through the D-region, resulting in formation of bacterial aggregates (Helaine et al., 2007).

In a N. gonorrhoeae study examining the presence of type IV prepilin-like protein, a protein was identified whose corresponding mutant was piliated, displayed unaltered twitching motility, aggregation and adhesion to human epithelial cells but was not competent (Wolfgang et al., 1999). This protein was designated ComP for competence associated prepilin (Wolfgang et al., 1999). A subsequent study in N. gonorrhoeae, showed that ComP is required for DNA binding. Upon ComP overexpression there was a 20-fold increase in DNA binding and in a pilT mutant background, where DNA uptake is abolished, ComP overexpression resulted in a 5-fold increase in DNA binding (Aas et al., 2002). The result of the original study was reproduced in N. meningitidis in a systematic functional analysis of the seven Pil proteins dispensable for piliation. This analysis showed that ComP was the only protein, apart from PilT, whose absence resulted in loss of competence. A follow-up study identified ComP as the DUS-specific DNA receptor using a multidisciplinary approach (Cehovin et al., 2013). Firstly, ComP was shown to be required by Tfp for efficient DNA binding, by testing the ability of filaments from a WT stain and a comP mutant to bind DNA using ELISA. Subsequently, ComP’s exquisite preference for DUS was demonstrated using EMSAs by comparing its affinity for DUS and two other
scrambled sequences. Lastly, NMR analysis of ComP in the presence of DUS showed that it binds DUS through an electropositive stripe predicted to be exposed on the filaments surface (Cehovic et al., 2013).

A similar study in *N. gonorrhoeae* as the one that identified ComP identified another type IV prepilin-like protein, PilV (Winther-Larsen et al., 2001). The *pilV* mutant showed unaltered twitching motility, DNA competence, and although it could form aggregates, adherence to human corneal epithelial cells was 100-fold reduced compared to the WT strain. Also, the *pilV/T* mutant did not display adhesion, whereas *pilT* mutant did, which indicated that dynamics of Tfp retraction are not responsible for the lack of adhesion in the *pilV* mutant. Interestingly, purified pili from *pilV* mutants contained PilC, which is involved in adhesion, at reduced levels (Winther-Larsen et al., 2001). Consequently, it was concluded that PilV is involved in adhesion, probably by promoting the functional display of PilC within the Tfp (Winther-Larsen et al., 2001). In contrast, in a study investigating host cell surface reorganization, the *N. meningitidis pilV* mutant adhered normally to epithelial cells compared to the WT strain, however it was unable to induce host cell membrane remodelling to form cell membrane projections that are found inside and around microcolonies adhering to the cell surface (Mikaty et al., 2009). Microcolonies formed by this mutant were found to be highly sensitive to shear stress, thus the PilV dependent cellular projections were confirmed to be required to generate strains resistant to high external forces (Mikaty et al., 2009). The more recent systematic functional analysis performed in *N. meningitidis* demonstrated that PilV modulates weakly multiple functions, which explain the low resistance of *pilV* microcolonies to shear stress observed by Mikaty et al. (2009). The *pilV* mutant showed increased competence, faster formation of aggregates, and an increase in twitching motility of the adhering aggregates that are more loosely associated (Brown et al., 2010). Thus, the adhesion of looser and ‘hypertwitching’ *pilV* aggregates seems less efficient and aggregates are probably more fragile with low resistance to shear stress. Overall, these phenotypes of *N. meningitidis pilV* mutants suggest that PilV is an intrinsic antagonist of competence and aggregation (Mikaty et al., 2009, Brown et al., 2010).
Similarly to other pilus types, it has also been attempted to create vaccines against Tfp. However, development of a Tfp-based vaccine was abandoned due to the extreme antigenic variation of the main Tfp pilin PilE, which resulted in incapable anti-PilE antibodies to provide protection against strains expressing different pili (Helm & Seifert, 2010, Boslego et al., 1991). In contrast, the minor pilins PilV, PilX and ComP are not only exposed in the Tfp filaments but they are also conserved between different serogroups (Cehovin et al., 2010). A study using sera of patients convalescent from meningococcal disease, showed that these proteins are immunogenic during meningococcal infection as antibodies were raised against them (Cehovin et al., 2011). Significantly, these antibodies were able to interfere selectively with the Tfp-linked functions, which concluded that PilV, PilX and ComP are promising candidates to be included in a multicomponent vaccine against N. meningitidis (Cehovin et al., 2011).

Lastly, PilZ was first identified in P. aeruginosa as an essential protein for Tfp biogenesis because the pilZ mutant is not piliated (Alm et al., 1996). However, in N. meningitidis, the corresponding pilZ mutant was found unexpectedly to be piliated with a defect in bacterial aggregation, hence it was categorized in the group of dispensable proteins for Tfp biogenesis that play roles in Tfp functionality. PilZ is predicted to be a cytoplasmic protein. Interestingly, this protein could not be detected in WT N. meningitidis strain or in pilZind strain, which contains a second chromosomal copy of pilZ under the transcriptional control of an IPTG-inducible promoter (Brown et al., 2010). This suggested that either PilZ is expressed at very low levels or it has an extremely short half-life (Brown et al., 2010). Nonetheless, the systematic functional analysis of N. meningitidis PilZ revealed that it has many functions, since the pilZ mutant showed defects in both aggregation and adhesion. Surprisingly, both defects were restored in a pilZ/T mutant, which suggested that PilZ is involved in counterbalancing PilT-mediated retraction. Also, PilZ in Xanthomonas axonopodis is reported to associate with the traffic ATPase PilF, therefore it is possible that PilZ and PilF work synergistically in antagonizing PilT (Guzzo et al., 2009). Moreover, PilZ has limited homology to a domain called ‘PilZ domain’ which binds the secondary messenger bis-(3’-5’)-cyclic dimeric guanosine monophosphate
(c-di-GMP) (Amikam & Galperin, 2006, Brown et al., 2010). However, it is unlikely that PilZ has a regulatory function by binding c-di-GMP. This is because there are no known proteins in N. meningitidis involved in synthesis or degradation of c-di-GMP (Brown et al., 2010). Also, the crystal structure of X. campestris PilZ showed that it lacks an essential N-terminal motif for binding of c-di-GMP and it has been shown that the P. aeruginosa PilZ does not bind c-di-GMP (Li et al., 2009, Merighi et al., 2007, Brown et al., 2010).

1.3 Aim of this study

Even though probably all of the Pil proteins involved in Tfp biology have been identified in N. meningitidis and other bacterial species, the exact function played by each protein is still to be determined. As mentioned earlier, it is generally accepted that these proteins function within a large multiprotein complex, like the T2S secreton. Although systematic studies to identify protein-protein interactions between Tfp proteins have been performed in Tfpb-expressing organism EPEC, unfortunately the results could not be extrapolated to Tfpa–expressing bacteria due to the significant differences between the two systems. Consequently, further advances in improving our understanding of this machinery require the characterization of the interactions between the Pil proteins.

The overall aim of my study was to improve the general understanding of the Tfpa machinery in N. meningitidis, by identifying fundamental protein-protein interactions. Hence, in this study I performed for the first time a systematic large-scale analysis to identify the interactions between 11 Pil proteins of Tfpa machinery, using a bacterial two-hybrid system. The results provided me with a strong foundation to proceed to the central aim of my study, which was to perform a functional analysis on the poorly characterized proteins (PilM, PilN, PilO and PilP) predicted to be involved in pilus assembly. This analysis involved a combination of approaches including membrane topology determination of PilN and PilO, mapping of interaction domains between PilE, PilM, PilN and PilO, stability assays and co-immunoprecipitation studies. The final aim was to reconstitute a minimal Tfpa assembly system in a heterologous non-piliated organism, similarly to what was
done for the Tfpb system by expressing the *bfp* operon into the non-EPEC strain BL21 (Sohel *et al.*, 1996). Such a system could improve our understanding of TfpA not only by confirming which proteins are sufficient for Tfp assembly, but by providing a simplified model to further study the biology of Tfp.
Chapter 2: Methods and Materials
2.1 Bacterial strains and growth conditions

The *E. coli* strains used in this study and their descriptions are listed on Table 2.1. *E. coli* DH5α was used for cloning and topology determination experiments, *E. coli* BTH101 was used for BACTH assays, and *E. coli* BL21(DE3) was used for protein expression and purification experiments. Strains were typically grown at 37°C on Luria-Bertani (LB) agar plates (Difco), or in liquid LB medium (Difco) with shaking at 180 revolutions per minute (rpm). The optical density (OD) of liquid cultures was determined using a Biochrom Libra S11 spectrophotometer at 600 nm. When required media were supplemented with 100 µg/ml spectinomycin (spec), 100 µg/ml ampicillin (amp), 50 µg/ml kanamycin (kan), 100 µg/ml streptomycin (strep) and 30 µg/ml nalidixic acid (nal) (all from Sigma) for antibiotic selection. Ultracompetent cells were prepared as described in section 2.3.1.

The WT strain of *N. meningitidis* used in this study is a recently sequenced and systematically mutagenized variant of the serogroup C clinical isolate 8013. It is encapsulated, expressing a highly adhesive pilin variant and no opacity proteins (Geoffroy *et al.*, 2003). *N. meningitidis* was grown on Gonococcal medium base (GCB) agar plates (Difco) containing the following three supplements. Supplement 1 contains 400 g glucose and 20 mg thiamine dissolved in 1 L of Milli Q water, and was added to a final concentration of 1% in the media. Supplement 2 contains 5 g Fe(NO₃)₄ dissolved in 1 L of Milli Q water, and was added to a final concentration of 0.1% in the media. Supplement 3 contains 10 g L-glutamine dissolved in 1 L of Milli Q water, and was added to a final concentration of 1% in the media. Plates were incubated in a moist atmosphere containing 5% CO₂. Also when required 100 µg/ml kan and 3 µg/ml erythromycin (ery) (Sigma) were used for antibiotic selection. The mutant *N. meningitidis* stains derived from stain 8013 were constructed as described in section 2.8 and are listed in Table 2.2.
Table 2.1 *E. coli* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Antibiotic resistance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F−, φ80lacZΔM15, Δ(lacZYA-argF), U169, recA1, endA1, hsdR17 (r−, m−), phoA, supE44 λ−, thi-1, gyrA96, relA1</td>
<td>N/A</td>
<td>Invitrogen</td>
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<tr>
<td>BL21 (DE3)</td>
<td>F−, ompT, hsdS8 (r8-m8−), gal, dcm, (DE3)</td>
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<td>Invitrogen</td>
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<tr>
<td>BTH101</td>
<td>F−, cya-99, araD139, galE15, galK16, rpsL1, hsdR2, mcrA1, mcrB1</td>
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<td>Euromedex</td>
</tr>
<tr>
<td>DHM1</td>
<td>F−, cya-854, recA1, endA1, gyrA96, thi1, hsdR17, spoT1, rfbD1, glnV44 (AS)</td>
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<td>Euromedex</td>
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</table>

Table 2.2 *N. meningitidis* strains used in this study.

<table>
<thead>
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<th>Description</th>
<th>Antibiotic resistance</th>
<th>Reference</th>
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</thead>
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<td>Kan</td>
<td>This study</td>
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<td>pilN mutant</td>
<td>Kan</td>
<td>This study</td>
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<td>pilO mutant</td>
<td>Kan</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpilP</td>
<td>pilP mutant</td>
<td>Kan</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpilM/pilM&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>Complemented pilM mutant</td>
<td>Kan, Ery</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpilN/pilN&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>Complemented pilN mutant</td>
<td>Kan, Ery</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpilO/pilO&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>Complemented pilO mutant</td>
<td>Kan, Ery</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpilP/pilP&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>Complemented pilP mutant</td>
<td>Kan, Ery</td>
<td>This study</td>
</tr>
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<td>This study</td>
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Table 2.3 Plasmids used in this study.

<table>
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<th>Description/Purpose</th>
<th>Antibiotic resistance</th>
<th>Source/Reference</th>
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<td>PCR8/GW/TOPO</td>
<td>TA cloning vector for direct ligation of PCR products</td>
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<td>Invitrogen</td>
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<td>Vector designed to determine the topology of a protein by fusing it at its C-terminus with T25; p15 ori; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Kan</td>
<td>Karimova et al., 2009</td>
</tr>
<tr>
<td>pKTop pilID</td>
<td>BACTH vector expressing PilD-T25</td>
<td>Kan</td>
<td>This study</td>
</tr>
<tr>
<td>pKTop pilIE</td>
<td>BACTH vector expressing PilE-T25</td>
<td>Kan</td>
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</tr>
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<td>Kan</td>
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<td>Mehr et al., 2000</td>
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<td>Ery</td>
<td>This study</td>
</tr>
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<tr>
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<td>This study</td>
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<td>Co-expression vector</td>
<td>Amp</td>
<td>Novagen</td>
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<td>This study</td>
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<td>This study</td>
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<td>Name</td>
<td>Description/Purpose</td>
<td>Antibiotic resistance</td>
<td>Source/Reference</td>
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<td>---------------------------------------------------</td>
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<td>Strep</td>
<td>Novagen</td>
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<td>pCDFDuet-1 derivative for expressing PilE</td>
<td>Strep</td>
<td>This study</td>
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<td>pCDFDuet pilD-pilE</td>
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<td>Strep</td>
<td>This study</td>
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<td>Streo</td>
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<td>Novagen</td>
</tr>
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<td>Amp</td>
<td>This study</td>
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<td>This study</td>
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<tr>
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<td>pET-21b derivative for expressing PilD, PilE, PilF, PilM, PilN, PilO and PilP</td>
<td>Amp</td>
<td>This study</td>
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</table>
2.2 Recombinant DNA methods

2.2.1 Polymerase Chain reaction (PCR)

Amplifying target genes by PCR for cloning was performed using the high fidelity DNA polymerases, PfuUltra II (Agilent) and Herculase II Fusion (Agilent). For analytical PCRs, such as colony PCRs and to confirm the insertion of genes into plasmids, Taq DNA polymerase (New England Biolabs) was used. It was also rarely used to amplify genes when the high fidelity enzymes failed to work. All of the reaction mixtures (50 μl) were set up according to manufacturer’s instructions (Table 2.4). PCRs were completed in a thermocycler with the PCR parameters described in Table 2.5. Control reactions were performed that did not contain DNA template. Primers used are listed in Table 2.6.

For colony PCR, to screen colonies for the presence of a desired insert, each PCR reaction was inoculated with a colony directly from the plates using a pipette tip. Also, the initial step of denaturation at 95°C was performed for 5 minutes, instead for the standard 2 minutes.
Table 2.4 PCR mixes for each DNA polymerase.

<table>
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<tr>
<th></th>
<th>Pfu</th>
<th>Herculase</th>
<th>Taq</th>
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<tr>
<td>Buffer</td>
<td>5 µl</td>
<td>10 µl</td>
<td>5 µl</td>
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<tr>
<td>dNTPs (25mM)</td>
<td>0.4 µl</td>
<td>0.5 µl</td>
<td>0.4 µl</td>
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<tr>
<td>Forward Primer (25µM)</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
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<tr>
<td>Reverse Primer (25µM)</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>DNA</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Polymerase</td>
<td>1 µl</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
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<tr>
<td>H₂O</td>
<td>41.6 µl</td>
<td>37 µl</td>
<td>42.1 µl</td>
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Table 2.5 PCR cycling parameters for each PCR polymerase.

<table>
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<tr>
<th>Temperature</th>
<th>Pfu Time</th>
<th>Herculase Time</th>
<th>Taq Time</th>
<th>Cycles</th>
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<tr>
<td>95°C</td>
<td>2 minutes</td>
<td>2 minutes</td>
<td>2 minutes</td>
<td>Initial denaturation</td>
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<tr>
<td>95°C</td>
<td>20 seconds</td>
<td>20 seconds</td>
<td>30 seconds</td>
<td>Denaturation</td>
</tr>
<tr>
<td>55°C</td>
<td>20 seconds</td>
<td>20 seconds</td>
<td>30 seconds</td>
<td>Primer annealing</td>
</tr>
<tr>
<td>72°C</td>
<td>15 seconds/kb</td>
<td>30 seconds/kb</td>
<td>1 minute/kb</td>
<td>Extension</td>
</tr>
<tr>
<td>72°C</td>
<td>10 minutes</td>
<td>10 minutes</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td>10°C</td>
<td>∞</td>
<td>∞</td>
<td>∞</td>
<td>Hold</td>
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Table 2.6 Primers used in this study.

<table>
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<th>Used for</th>
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<td>PCR8/GW/TOPO specific for sequencing</td>
</tr>
<tr>
<td>GW2</td>
<td>GTTCGACAAATTATGTAGGACATCAG</td>
<td>PCR8/GW/TOPO specific for sequencing</td>
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<td>dir PilD</td>
<td>cccggatccATGTCTGGTATTGCTGTATTGCG</td>
<td>Cloning pilD in BACTH vectors</td>
</tr>
<tr>
<td>rev PilD</td>
<td>cccggatccCAACCGCAGGATGGTACGACCCAC</td>
<td>Cloning pilD in BACTH vectors</td>
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<td>Cloning pilN in BACTH vectors</td>
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<td>Cloning pilN in BACTH vectors</td>
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<td>Used for</td>
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<td>pilMNOPOperon-IR3</td>
<td>CTGGATAGCGTTATCGCGCC</td>
<td>Sequencing internal region of optimized pilMNOP</td>
</tr>
</tbody>
</table>

* Lower-case is used for overhangs. Restriction sites are underlined. Mismatched bases generating mutations are in bold.
2.2.2 Cloning in TOPO vectors

All PCR products were cloned directly into pCR8/GW/TOPO (Invitrogen). The ligation between the PCR product and the linearized TOPO vector requires a single deoxyadenosine (A) to the 3’ end of the PCR product as the TOPO vector has overhanging 3’ deoxythymidine (T) residues. The Taq polymerase has a nontemplate dependent terminal activity and adds 3’ As at the ends to the PCR products, so the PCR product could be directly cloned in the vector. However, on amplifying the genes using PfuUltra II, the PCR product was purified and eluted in 40 µl (section 2.2.9) and the 3’ As were inserted by adding 0.5 µl Taq polymerase, 5 µl of 0.2 µM dATP and 5 µl of Taq buffer and next incubating the reaction in the thermocycler at 72°C for 10 minutes. The cloning reaction was performed following the manufacturer’s instructions. Subsequently, 2 µl of the reaction was used to transform 200 µl of competent DH5α cells.

2.2.3 Plasmid preparation

For small-scale plasmid purification, a single colony was used to inoculate 5 ml of liquid LB medium with appropriate antibiotics, which was incubated overnight at 37°C, shaking at 180 rpm. The plasmid DNA was purified using either the QIAprep spin mini-prep kit (Qiagen) or the Nucleospin plasmid kit (Macherey-Nagel) both used as per the manufacturer’s instructions.

When larger quantities of plasmids were required, 50 ml overnight cultures were set up and the plasmid DNA was purified using the QIAGEN plasmid plus midi kit (Qiagen) according to the manufacturer’s handbook. All plasmids were stored at -20°C.

2.2.4 DNA Sequencing

DNA Sequencing reactions were prepared using: 150 ng of plasmid DNA for plasmids up to 3 kb or 500-600 ng for plasmids over 3kb, 3.2 pmoles of primer made up in 10 µl total volume. Next, they were sent to be sequenced to the MRC Clinical Sciences
Centre Genomics Core Laboratory, Imperial College. All sequences were then analyzed using DNA Strider and 4Peaks.

2.2.5 Quantification of DNA concentration

DNA concentrations were determined using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific). 1.5 µl of DNA was required to measure the concentration (ng/µl) spectrophotometrically at 260nm and purity using the 260/280 nm ratio. The blank used for each sample was the buffer in which the DNA was resuspended.

2.2.6 Restriction endonuclease digestion of DNA

Digestion reactions were performed using enzymes and buffers purchased from New England Biolabs (NEB). A typical digestion reaction, prior to ligation of target DNA into suitable vectors included: 1 µl of restriction endonuclease, 1x restriction endonuclease buffer, 1x bovine serum albumin (BSA) if required, 2 µg of DNA completed to a volume of 100 µl with Milli Q water. Double digestions were performed using a compatible buffer for both enzymes if possible or the first digestions was performed followed by a DNA purification step (section 2.2.9) and subsequently the second digestion was performed in a different buffer.

Analytical digestions to verify successful ligations were performed using 4 µl of plasmid DNA obtained by mini-prep, 1 µl of restriction endonuclease, 1x restriction endonuclease buffer, 1x bovine serum albumin (BSA) if required, completed to a volume of 20 µl with Milli Q water.

Digestions were performed in water baths (usually at 37°C) for 1-4 hours.

2.2.7 Alkaline phosphatase treatment

After restriction endonuclease digestion, linearized plasmid DNA was dephosphorylated using FastAP Thermosensitive Alkaline Phosphatase (FastAP) (Thermo Fisher Scientific) in order to prevent re-ligation. For this reaction, 1 µl of FastAP and 1x FastAP buffer were added to 2 µg of digested vector and the reaction was completed to 30 µl with Milli Q water. Reaction was then incubated at 37°C for
10 minutes. Subsequently, the enzyme was heat inactivated by incubating at 75°C for 5 minutes. The FastAP treated plasmids were stored at -20°C.

2.2.8 Agarose gel electrophoresis

DNA was analyzed using agarose gel electrophoresis. The gels were prepared by adding agarose (Fisher) into 1x Tris-Acetate-EDTA buffer (TAE) (Fisher) and heating in a microwave to dissolve. SYBR Safe DNA gel stain (invitrogen) was added (1/20,000) to the molten agarose before pouring into a casting tray with a comb and allowing to set. The percentage of agarose (0.6%-1%) used depended on the size of the DNA analyzed. DNA samples were prepared by mixing them with a 6x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water). 1 kb Plus DNA ladder (Invitrogen) was run alongside the samples as a size marker. Electrophoresis was performed at 100 V for approximately 40-60 minutes, depending on the separation required, using the Mini-Sub Cell GT electrophoretic system (Bio-rad). The DNA bound SYBR safe dye was visualized using a Safe Image Blue Light Transilluminator (Invitrogen). Gel images were captured using a Gel Doc™ EZ Imager (BioRad).

2.2.9 DNA purification using PCR purification and gel extraction kits

Digested plasmids and PCR products were purified for downstream applications using the QIAquick PCR purification kit (Qiagen) from a solution and the QIAquick gel extraction kit (Qiagen) from agarose gels. Both procedures were performed as described in the manufacturer’s handbook. Purified DNA was stored at -20°C.

2.2.10 Ligation reactions

All ligations were carried out on linearized vector/insert DNA with cohesive ends, previously produced by enzymatic cleavage (section 2.2.6), using Ready-To-Go DNA Ligase (Amersham Biosciences). Briefly, 30 femtomoles (fmoles) of both DNA insert and vector, completed to 20 µl with Milli Q water, was added to one tube of Ready-To-Go DNA Ligase. The ligation reactions were incubated at 16°C for 45 minutes. 4 µl
of ligation reaction was used to transform 200 µl of competent *E. coli* DH5α cells (section 2.3.2).

### 2.2.11 Site directed mutagenesis (SDM)

*pilN* point mutant alleles were generated using the Quickchange site-directed mutagenesis kit (Stratagene) as described in the instruction manual. A series of long complementary primers were synthesized (25-45 bases) that contain the desired mutation approximately in the centre of each primer (Table 2.6) and pYU61 was used as DNA template. PCRs were performed using PfuUltra II and were completed in a thermocycler consisting of the PCR parameters displayed in Table 2.7.

**Table 2.7 PCR cycling parameters for SDM.**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Pfu Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>2 minutes</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>95°C</td>
<td>30 seconds</td>
<td>Denaturation</td>
</tr>
<tr>
<td>55°C</td>
<td>1 minute</td>
<td>Primer annealing</td>
</tr>
<tr>
<td>68°C</td>
<td>15 seconds/kb</td>
<td>Extension</td>
</tr>
<tr>
<td>68°C</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td>10°C</td>
<td>∞</td>
<td>Hold</td>
</tr>
</tbody>
</table>

Following PCRs, the products were treated with *DpnI* that is specific for digesting methylated and hemimethylated DNA, i.e. template DNA, enabling the selection of the mutation-containing newly synthesized DNA. Thus, to each PCR product 1 µl *DpnI* (NEB) and 5.6 µl buffer 4 (NEB) were added and digestions were performed at 37°C for 1 hour. Then, 2 µl of the PCR product was used to transform 200 µl of DH5α cells and plates were incubated overnight at 37°C (section 2.3.2). Next, the plasmids were purified using the QIAprep spin mini-prep kit (Qiagen) and the presence of desired mutations in *pilN* was verified by sequencing.
2.3 Transformation

2.3.1 Preparation of *E. coli* ultracompetent cells

*E. coli* ultracompetent cells were prepared using the Inoue method (Inoue *et al.*, 1990). Bacteria from frozen glycerol stocks were streaked onto LB agar plates and incubated overnight at 37°C. Next, 10-12 large colonies were picked from the plate to inoculate 250 ml of SOB (Difco) and the culture was grown at 16°C with shaking at 180 rpm. When the optical density at 600 nm (OD₆₀₀) reached approximately 0.6, the culture was incubated on ice for 10 minutes to chill and the cells were harvested by centrifuging at 2,500 xg for 10 minutes at 4°C. The pellet was gently resuspended in 80 ml of ice-cold Inoue transformation buffer (ITB) (55mM MnCl₂·4H₂O, 15mM CaCl₂·2H₂O, 250 mM KCl, and 0.5 M PIPES pH 6.7), incubated on ice for 10 minutes and centrifuged at 2,500 xg for 10 minutes at 4°C. The pellet was gently resuspended in 20 ml of ice-cold ITB to which 1.5 ml of dimethyl sulfoxide was added. The cell suspension was incubated on ice for 10 minutes and it was divided in 0.2 ml aliquots in pre-chilled 1.5 ml sterile tubes, which were flash-frozen in a dry ice/ethanol bath. Competent cells were stored at -80°C.

2.3.2 *E. coli* transformation

For transformation, 200 µl of *E. coli* ultracompetent cells were slowly thawed on ice, DNA was added (<5 µl), mixed by quickly flicking the tube and incubated at 4°C for 30 minutes. Next, they were subjected to a 30 second ‘heat shock’ at 42°C and were returned immediately on ice for 5 minutes. 800 µl of prewarmed SOB containing 20 mM glucose was added and the transformed cells were left to recover for 1 hour at 37°C, shaking at 180 rpm, before they were plated on LB agar plates containing an appropriate antibiotic. Plates were then incubated overnight at 37°C. For the bacterial adenylate cyclase two-hybrid (BACTH) transformations, refer to section 2.4.
2.3.3 *N. meningitidis* transformation

*N. meningitidis* was grown overnight at 37°C on GCB agar. A loop of bacteria was resuspended in 1 ml of GCG liquid medium (15 g Proteose peptone, 4 g K₂HPO₄, 1 g KH₂PO₄ and 5 g NaCl dissolved in 1 L of ddH₂O) containing 5 mM MgCl₂, known as GCB ‘transfo’, and 200 µl of the bacterial suspension was added to the wells of a 24-well tissue culture plate. DNA containing the DNA uptake sequence, which is essential for transformation of *N. meningitidis*, was added to the well, at a concentration of 1 µg/ml, which is saturating. Plates were incubated for 30 minutes at 37°C with gentle rocking, approximately 140 rpm, in a moist atmosphere containing 5% CO₂. Then, 0.8 ml of GCB transfo was added to each well and the plate was further incubated for 3 hours at 37°C without shaking. Transformed cells were plated on GCB agar plates containing appropriate antibiotics and plates were incubated for 16-24 hours in a moist atmosphere containing 5% CO₂. Transformants were re-streaked on the same plates for further analysis.

2.4 Bacterial adenylate cyclase two-hybrid system

The recombinant plasmids with the full-length *pil* genes used in the BACTH assays were constructed previously in the lab by Vladimir Pelicic, as follows. The full-length *pilD, pilE, pilF, pilG, pilM, pilN, pilO, pilT, pilT2, pilU* and *pilZ* genes were amplified from WT strain 8013 genomic DNA (section 2.7) using PfuUltra II and suitable primers (Table 2.6). PCR products were cloned into pCR8/GW/TOPO and all the inserts were sequenced to ensure they contain no errors. Next, each *pil gene* was digested by *Bam*HI and *Kpn*I, gel-extracted and subcloned into each BACTH vector (pUT18, pUT18C, pKT25 and pKNT25) cut with the same enzymes (Table 2.3).

The same cloning strategy was used to produce BACTH plasmids in which truncated versions of *pilE, pilN* and *pilO* were amplified using suitable primers (Table 2.6), cloned into pCR8/GW/TOPO, checked by sequencing, gel-extracted and subcloned into the BACTH vectors (Table 2.3). The mutant *pilN* alleles prepared by side-directed mutagenesis were also directly subcloned from the corresponding pCR8/GW/TOPO derivatives into the BACTH vectors as described above.
To perform the assay, one hundred µl of competent BTH101 cells were co-transformed with 20 ng of two recombinant plasmids encoding fusions to T18 and T25 proteins respectively. Two hundred µl of the transformed cells was plated on MacConkey agar base medium supplemented with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Merck chemicals), 1% maltose solution (Sigma), 100 µg/ml ampicilllin and 50 µg/ml kanamycin. Plates were incubated at 30°C and the colour of the colonies was scored after 40-48 hours. In every assay, positive and negative controls were included. As a positive control, the pKT25-zip and pUT18C-zip plasmids were co-transformed into BTH101 cells, and generated deep purple colonies. As a negative control, pKT25 and pUT18C plasmids with no inserts were co-transformed into BTH101 cells, and generated white colonies (Karimova et al., 1998). All the positive plasmid combinations, i.e. generating coloured colonies, were transformed again for confirmation of the phenotypes.

2.5 β-galactosidase assays

The efficiency of functional complementation in BACTH, between T18 and T25, for the positive plasmid combinations was quantified by measuring β-galactosidase activities in liquid culture (Karimova et al., 1998). Single colonies of BTH101 transformants to be assayed were picked to inoculate 5 ml of liquid LB medium supplemented with 0.5 mM IPTG, 100 µg/ml ampicilllin and 50 µg/ml kanamycin and cultures were grown at 30°C for 14-16 hours, with shaking at 180 rpm. At least three independent cultures were performed for each transformant to be tested. Next, these were diluted 1/5 in M63 broth (15 mM (NH₄)₂SO₄, 100 mM KH₂PO₄, 1.8 µM FeSO₄.7H₂O, 3 µM vitamin B1, pH 7.0) and the OD₆₀₀ was recorded. Cells were then permeabilized by adding 20 µl of chloroform and 20 µl of 0.1% SDS to 1.5 ml bacterial suspension, followed by vortexing for 10 seconds and incubating at 37°C for 40 minutes with shaking.

For the enzymatic reaction 10 µl of the cells was added to 990 µl of PM2 (70 mM Na₂HPO₄.12H₂O, 30 mM NaH₂PO₄.H₂O, 1 mM MgSO₄, 0.2 mM MnSO₄, pH 7.0) containing 100 mM β-mercaptoethanol and incubated in a heat block at 28°C for 5 minutes. The reaction was started by adding 0.25 ml of 0.4% O-nitrophenol-β-
galactoside (ONPG) (Sigma) in PM2 buffer (without β-mercaptoethanol) pre-equilibrated at 30°C. The reaction was incubated at 28°C before being stopped by the addition of 0.5 ml of 1M Na₂CO₃. This occurred after 20 minutes for positive samples and after 60 minutes for negative samples. These time points were standardized for all experiments after several time points had been tested for sufficient yellow colour development. The OD₄₂₀ and OD₆₀₀ were then recorded and the enzymatic activity (A) (in units per millilitre) was calculated using the following formula:

\[ A = 200 \times (\text{OD}_{420}/\text{minutes of incubation}) \times \text{dilution factor} \]

However, the results are expressed as units of enzymatic activity per milligram of bacterial dry weight (U/mg), where 1 unit corresponds to 1 nmol of ONPG hydrolyzed per minute at 28°C (Karimova et al., 1998). To calculate this, it was considered that 1 ml of culture at an OD₆₀₀ of 1 corresponds to 300 µg dry weight bacteria. Thus, the following formula was used:

\[ \text{U/mg} = \frac{A}{[(\text{OD}_{600} \times 300)/1000]} \]

### 2.6 Membrane topology analysis

To determine the topology of the bitopic proteins PilN and PilO, the vector pKTop was used which contains a dual pho-lac reporter (Karimova et al., 2009). Full length and truncated versions of the pilN and pilO genes were digested from the corresponding PCR8/GW/TOPO derivatives with BamHI and KpnI, gel-extracted and subcloned into pKTop digested with the same enzymes (Table 2.3). Hence, the pil genes were fused in frame with the dual reporter. The resulting recombinant pKTop plasmids were transformed into competent E. coli DH5α cells which were plated on LB agar plates supplemented with 80 µg/ml X-phos (5-bromo-4-chloro-3-indolyl phosphate disodium salt), 100 µg/ml Red-Gal (6-chloro-4-indolyl-β-D-galactoside) (both from Sigma), 1mM IPTG, 50 mM phosphate buffer (pH 7.0) and 50 µg/ml kanamycin. As controls, we used E. coli DH5α cells transformed with pKTop, containing no insert. The plates were incubated overnight at 37°C before coloration was scored. The experiment was later modified by using plates supplemented with
80 µg/ml X-phos, 1mM IPTG, 50 mM phosphate buffer (pH 7.0) and 50 µg/ml kanamycin, thus excluding Red-Gal. As controls, we additionally used two previously published fusions with the *E. coli* YmgF polytopic protein, YmgF$_{1-32}$-PhoLac and YmgF$_{1-72}$-PhoLac, directing the reporter to the periplasm and the cytoplasm respectively (Karimova *et al.*, 2009).

### 2.7 *N. meningitidis* genomic DNA extraction

Genomic DNA extraction from *N. meningitidis* was performed using the Wizard Genomic DNA purification kit (Promega) according to manufacturer’s instructions. Concisely, a loopful of bacteria, grown overnight on GCB agar plates at 37°C, was resuspended in 600 µl of Nuclei lysis solution and incubated at 80°C for 5 minutes. The sample was then cooled to room temperature and 200 µl of Protein precipitation solution was added and mixed by vortexing, before incubating for 5 minutes at 4°C. Following, the sample was centrifuged at 15,000 xg for 3 minutes, the supernatant was carefully transferred to a clean tube containing 600 µl of isopropanol and centrifuged at 15,000 xg for 5 minutes. The supernatant was discarded and the DNA pellet was washed with 600 µl of 70% ethanol. Subsequently, the sample was centrifuged at 15,000 xg for 5 minutes, ethanol was removed and the DNA pellet was air-dried. Finally, the pellet was resuspended in 100 µl of Milli Q water and 1 µl of RNAse solution was added.
2.8 Construction of *N. meningitidis* mutants

### 2.8.1 Construction of non-polar *N. meningitidis* mutant strains by splicing PCR

*N. meningitidis* non-polar ΔpilM, ΔpilN, ΔpilO and ΔpilP mutants were constructed by splicing PCR as described by de Berardinis et al. (2008). Two sets of primers (F1/R1 and F2/R2) (Table 2.6) were used to amplify approximately 500 bp fragments upstream and downstream from each target gene, respectively. The R1 and F2 primers were designed to delete the coding region of the mutagenized genes from the start codon to approximately 30 bp before the stop codon, in order to preserve the ribosomal binding sites used by downstream genes. Also, these two primers contained 23-mer overhangs that are complementary to the aphF and aphR primers used to amplify the promoterless *aphA*-3 gene that encodes kanamycin resistance from start to stop codons, respectively. Primers F1 and/or R2 contained 12-mer overhangs corresponding to the DNA uptake sequence that is essential for DNA to be taken up by the meningococcus during natural transformation. In the first step, three PCR fragments were amplified using F1/R1, F2/R2 and aphF/aphR, and the high-fidelity Herculase II Fusion DNA polymerase to obtain the two flanking regions of the target genes and the kanamycin resistance integration cassette, respectively. Next, the three PCR fragments were combined and spliced together using the same enzyme and the F1 and R2 primers. The spliced PCR fragments were then directly transformed into *N. meningitidis* and mutants were selected on GCB agar plates supplemented with 100 µg/ml kanamycin. For each mutant, at least two colonies were isolated and further verified by PCR using the F1 and R2 primers.

### 2.8.2 Construction of *N. meningitidis* complemented strains

To create the *N. meningitidis* ΔpilM/pilM<sub>WT</sub>, ΔpilN/pilN<sub>WT</sub>, ΔpilO/pilO<sub>WT</sub> and ΔpilP/pilP<sub>WT</sub> complemented strains, the chromosomal DNAs were extracted from the ΔpilM, ΔpilN, ΔpilO and ΔpilP mutants (section 2.7). Subsequently, they were used to transform strains in which the WT alleles were previously integrated in the genome, by allelic exchange, under the transcriptional control of an IPTG-inducible promoter.
(Carbonnelle et al., 2006). Complemented mutants were selected on GCB agar plates supplemented with 100 µg/ml kanamycin, 3 µg/ml erythromycin and 0.25 mM IPTG.

2.8.3 Construction of *N. meningitidis* pilN point mutant strains: ΔpilN/pilN<sub>N8</sub>A, ΔpilN/pilN<sub>L9</sub>A and ΔpilN/pilN<sub>P11</sub>A strains

The pilN point mutant alleles in PCR8/GW/TOPO were amplified using suitable primers (pilN-Ind, Table 2.6) flanked by PacI sites and cloned back into PCR8/GW/TOPO. This was performed to allow the genes to be cloned into the PacI restriction site of pGGC4 (Mehr et al., 2000). Subsequently, the pilN alleles were sequenced to ensure that no errors were introduced during PCR. The genes were then excised from PCR8/GW/TOPO by PacI digestion (NEB) and sub-cloned into pGCC4 restricted with the same enzyme. This placed the pilN point mutant alleles under the transcriptional control of an IPTG-inducible promoter, within a region of the gonococcal chromosome conserved in *N. meningitidis*, allowing homologous recombination of the genes into the chromosome. The resulting pGCC4 pilN<sub>N8</sub>A, pGCC4 pilN<sub>L9</sub>A and pGCC4 pilN<sub>P11</sub>A plasmids were linealized by NotI digestion and used to transform the WT strain 8013 in which genome they integrated by allelic exchange. The endogenous pilN copy was then interrupted, by transforming these strains with genomic DNA extracted from a ΔpilN non-polar mutant. The resulting strains were grown on GCB agar plates supplemented with 100 µg/ml kanamycin, 3 µg/ml erythromycin and 0.25 mM IPTG before they were analyzed for piliation (section 2.15).
2.9 Preparation of protein extracts

2.9.1 Preparation of whole protein extracts from *E. coli*

For *E. coli*, total protein extracts were prepared by centrifuging 1 ml of culture at 11,000 xg for 4 minutes. The pellet was resuspended in 10 µl of Laemmli sample buffer (Bio-rad) per 0.1 OD_{600} of the culture. The samples were next heated at 100°C for 5-7 minutes and stored at -20°C.

2.9.2 Preparation of whole protein extracts from *N. meningitidis*

A loop of *N. meningitidis* grown on GCB plates was resuspended in 1 ml of PBS and the OD_{600} was recorded. The cells were centrifuged at 11,000 xg for 5 minutes and the pellet was resuspended in 10 µl of Laemmli sample buffer per 0.1 OD_{600} of the cell suspension and subsequently heated at 100°C for 10 minutes.

For immunoprecipitations, protein extracts were prepared using the B-PER bacterial protein extraction reagent (Pierce), which is a mild nonionic detergent that enables mild extraction of proteins without mechanical disruption. In brief, bacteria grown overnight on GCB agar plates at 37°C were resuspended in 1ml of PBS to an OD_{600} of 1.5-3, and centrifuged at 11,000 xg for 5 minutes. Subsequently, the pellet was resuspended in 500 µl of B-PER, containing 0.1 mg/ml lysozyme, until homogeneous by vortexing for 1 minute. Following resuspension, the sample was incubated at room temperature for at least 15 minutes and then centrifuged at 13,000 xg for 15 minutes at 4°C to pellet the cell debris. The supernatant containing the soluble protein fraction was recovered and quantified (section 2.10.1). Samples were then stored at -20°C.
2.10 Analysis of protein samples

2.10.1 Protein quantification

Protein concentration was determined using the Bio-Rad protein assay according to the manufacturer’s instructions. Briefly, several dilutions of the protein sample were made, by completing to 800 µl with Milli Q water. To the 800 µl diluted samples, 200 µl of the Bio-Rad protein assay solution was added, mixed and incubated for 5 minutes at room temperature. The absorbance of the bound form of Coomassie Brilliant Blue G-250 dye, present in the Bio-Rad protein assay, is proportional to the sample protein concentration, so the absorbance of the protein samples at 595 nm was analyzed. The absorbance values were compared to a standardized curve using known concentrations of BSA, in order to determine the protein sample concentration.

2.10.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out to analyze protein samples. SDS-PAGE gels were prepared at an appropriate percentage depending on the molecular weight of the target protein, and they were assembled and run in the Mini-PROTEAN Tetra Cell system (Bio-rad) with 1x Tris glycine sodium dodecyl sulphate buffer (TGS) (10x TGS, 0.25 M Tris, 1.92 M glycine, 1% SDS pH 8.3.) as described in the manufacturer’s handbook. The compositions of the gels commonly used are shown in Table 2.8. The samples were electrophoresed for 1h at 200 V.

Table 2.8 Composition of SDS-PAGE gels.

<table>
<thead>
<tr>
<th></th>
<th>Resolving gels</th>
<th></th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10%</td>
<td>12%</td>
<td>15%</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.0 ml</td>
<td>3.3 ml</td>
<td>2.3 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>3.3 ml</td>
<td>4.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>1.5M Tris pH 8.8</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>1.0M Tris pH 6.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µl</td>
<td>4 µl</td>
<td>4 µl</td>
</tr>
</tbody>
</table>
2.10.3 Coomassie staining

The gels were stained using Bio-Safe Coomassie (Bio-rad) as directed by the manufacturer. Gel images were captured using a Gel Doc™ EZ Imager (BioRad).

2.10.4 Western Blotting

Proteins were first separated by SDS-PAGE and subsequently they were blotted to Amersham Hybond ECL nitrocellulose membrane (GE Healthcare) using the Mini Trans-Blot electrophoretic transfer cell system (Bio-rad) as described in manufacturer’s handbook. The transfer was carried out in ice-cold transfer buffer (39 mM glycine, 48 mM Tris base, 0.037 % SDS and 20% isopropanol) for 1 hour at 100 V. The membrane was incubated overnight at 4°C in blocking solution (PBS pH 7.4 with 0.1% Tween-20 (PBS-T), with 5% skimmed milk powder). Then the membrane was washed twice in PBS-T for 5 minutes and it was incubated for 1 hour at room temperature with the primary antibody, amount and type as specified in Table 2.9, diluted in 10 ml blocking solution. After incubation the membrane was washed at least three times for 5 minutes with PBS-T and then incubated with the secondary antibody, either ECL anti-rabbit IgG Horseradish peroxidase linked whole antibody or ECL anti-mouse IgG Horseradish peroxidase linked whole antibody (GE Healthcare), depending on where the primary antibody was raised, for 1 hour at room temperature. The secondary antibodies were used at a 1/10,000 dilution in 10 ml blocking solution. The membrane was washed at least three times for 5 minutes with PBS-T and quickly rinsed twice with PBS. The detection step was carried out using ECL Prime western blot detection reagent (GE Healthcare) as described by the manufacturer. The image was captured using Hyperfilm ECL (GE Healthcare) in an X-ray film cassette and developed using an automated developer (Agfa Curix) (Rusniok et al., 2009).
Table 2.9 Primary antibodies used for Western blotting in this study.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Proteins used for production</th>
<th>Immunized animal</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PilD</td>
<td>Two peptides: PilD_{48-63} and PilD_{145-156}</td>
<td>Rabbit</td>
<td>1/2,000</td>
</tr>
<tr>
<td>Anti-PilE</td>
<td>Recombinant polyhistidine PilE_{36-133}</td>
<td>Rabbit</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Anti-PilF</td>
<td>Two peptides: PilF_{158-173} and PilF_{451-466}</td>
<td>Rabbit</td>
<td>1/5,000</td>
</tr>
<tr>
<td>Anti-PilM</td>
<td>Recombinant polyhistidine-PilM</td>
<td>Rabbit</td>
<td>1/5,000</td>
</tr>
<tr>
<td>Anti-PilN</td>
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<td>Rabbit</td>
<td>1/3,000</td>
</tr>
<tr>
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<td>Two peptides: PilO_{45-59} and PilO_{169-183}</td>
<td>Rabbit</td>
<td>1/50,000</td>
</tr>
<tr>
<td>Anti-PilP</td>
<td>Recombinant polyhistidine-PilP_{17-145}</td>
<td>Rabbit</td>
<td>1/50,000</td>
</tr>
<tr>
<td>Anti-T18 (CyA)</td>
<td>Bordetella pertussis CyaA_{370-400}</td>
<td>Mouse</td>
<td>1/1,000</td>
</tr>
<tr>
<td>20D9</td>
<td>Purified pili from 8013 strain</td>
<td>Mouse</td>
<td>1/10,000</td>
</tr>
</tbody>
</table>

2.11 Immunoprecipitations (IPs)

To optimize immunoprecipitations of PilM, PilN, PilO and PilP from *N. meningitidis* protein extracts prepared using B-PER bacterial protein extraction reagent, three different immunoprecipitation kits were tried.

2.11.1 TrueBlot Immunoprecipitation kit

Immunoprecipitations were performed using the TrueBlot anti-rabbit Ig IP beads and rabbit IgG TrueBlot HRP as described in manufacturer’s protocol. In summary, 50 µl of TrueBlot anti-rabbit Ig IP bead slurry was washed by centrifuging at 2,500 xg for 3 minutes at 4°C and resuspending in 50 µl of B-PER bacterial protein extraction reagent. 100 µg of *N. meningitidis* protein extract was then precleared by incubating it with the 50 µl of pre-washed TrueBlot anti-rabbit Ig IP bead slurry for 60 minutes at 4°C. Next, the protein extract/TrueBlot anti-rabbit Ig IP beads mixture was centrifuged at 2,500 xg for 3 minutes at 4°C. The supernatant was retrieved and incubated with 5 µg of purified anti-PilN antibody for 60 minutes at 4°C on a rotating wheel. Following this, 50 µl of bead slurry was added to capture the immune complexes and incubated for further 60 minutes at 4°C, before washing the beads five times with 500 µl of B-PER reagent by centrifuging at 2,500 xg for 3 minutes at 4°C. After the last wash, the supernatant was aspirated completely and 50 µl of Laemmli sample buffer with 25 mM DTT as a reducing agent was added to bead
pellet to elute the antigen. The beads were vortexed and heated at 100°C for 10 minutes and subsequently centrifuged at 10,000 xg for 5 minutes. The supernatant was collected carefully, loaded onto SDS-PAGE gels and analyzed by Western blotting. The secondary antibody Rabbit IgG TrueBlot HRP was used, at a 1/1000 dilution in blocking solution, which preferentially detects the native form of rabbit IgG over the SDS-denatured form of IgG, to eliminate signal interference by the heavy and light chains of the immunoprecipitating antibody.

2.11.2 Dynabeads Protein A

Magnetic Dynabeads Protein A (Invitrogen) were used as directed by the manufacturer. Firstly, *N. meningitidis* protein extract was precleared. This was performed by washing twice 50 µl of Dynabeads Protein A with 200 µl of B-PER reagent, using the magnet (Invitrogen). Next, 100 µg of *N. meningitidis* protein extract was incubated with prewashed beads for 20 minutes at 4°C and the supernatant was harvested. Following, 50 µl of Dynabeads Protein A was washed twice with 200 µl of B-PER reagent, before incubating with 5 µg of purified anti-PilN antibody for 10 minutes at room temperature on a rotating wheel. The Dynabeads-antibody complex was then washed twice with 200 µl of B-PER reagent and incubated with the precleared protein extract for 25 minutes at 4°C. The Dynabeads-antibody-antigen complex was washed three times with 200 µl B-PER reagent and incubated with 20 µl of Laemmli sample buffer containing 50 mM DTT at 100°C for 10 minutes to elute the antigen. The supernatant was removed using the magnet and the antigen was analyzed by Western blotting.

2.11.3 Crosslink Immunoprecipitation kit

The Crosslink Immunoprecipitation kit (Pierce) involved covalent crosslinking of antibodies onto Protein A/G Plus Agarose and it was performed according to manufacturer’s instructions as follows. 20 µl Protein A/G Plus Agarose was washed twice with 200 µl of Coupling buffer (Pierce), before binding to the antibody by incubating for 60 minutes at room temperature on a rotating wheel. Washing steps were performed by centrifuging at 1,000 xg for 1 minute and antibodies were used
at the following amounts: 5 µg of purified anti-PilM, anti-PilN and anti-PilO antibodies and 8 µl of anti-PilP serum. Next, the antigen-resin complex was washed three times with 300 µl of Coupling buffer and covalently cross-linked by incubating with 50 µl of 450 µM disuccinimidyl suberate (DSS) (Perce) in DMSO for 60 minutes at room temperature on a rotating wheel. The antibody-crosslinked resin was then washed three times with 100 µl of Elution buffer (Pierce) to remove any non-crosslinked antibody and the flow-throughs were analyzed by Western blotting to verify antibody crosslinking. Next, 500 µg of N. meningitidis protein extract was immunoprecipitated by incubating with the antibody-crosslinked resin overnight at 4°C on a rotating wheel. Following two washing steps with 200 µl of Lysis/Wash buffer (Pierce), the precipitated protein was eluted in 50 µl of Elution buffer and analyzed by Western blotting.

2.12 Construction of pilD, pilE, pilF, pilM, pilN, pilO and pilP co-expression systems

The pilE, pilF, pilMNOP and pilGD genes were synthesized and optimized for E. coli expression by GeneArt (Invitrogen).

2.12.1 pilD, pilE, pilF, pilM, pilN, pilO and pilP co-expression using the pETDuet vectors

Two pET-duet vectors were used, pETDuet-1 and pCDFDuet-1, each having two multiple cloning sites (MCS), each of which is preceded by a T7lac promoter and a ribosomal binding site (rbs). The optimized pilMNOP operon was synthesized with ribosomal binding sites added in front of the pilNOP genes, and with flanking Ndel and Xhol sites. The optimized pilE was synthesized on its own with flaking Ndel and Xhol sites. The optimized pilF was synthesized on its own with flanking BspHI and NotI sites while the pilGD was synthesized with ribosomal binding site added in front of pilD gene and with flanking Ncol and NotI sites.

First, pilE and pilMNOP were digested from their corresponding pMAT-T and pMK-RQ derivatives respectively, with Ndel and Xhol, gel extracted and subcloned into the
MCS2 of both pETDuet-1 and pCDFDuet-1 digested with the same enzymes. This generated four different vectors: pETDuet pilE, pCDFDuet pilE, pETDuet pilMNOP and pCDFDuet pilMNOP. pilF was subsequently digested from its pMK-RQ derivative using BspHI and NotI and subcloned into the MCS1 of pETDuet pilMNOP and pCDFDuet pilMNOP cut with Ncol and NotI. pilD was amplified from pMK-RQ pilGD using suitable primers (Table 2.6) to introduce a flanking BspHI site on its 5’end. Next, it was cloned into pCR8/GW/TOPO, checked by sequencing, digested with BspHI and NotI and subcloned into the MCS1 of pETDuet pilE and pCDFDuet pilE cut with Ncol and NotI. The Ncol and BspHI restriction endonucleases used to digest the vector and the insert respectively generate compatible cohesive ends that can be ligated. Four different constructs were thus produced: pETDuet pilD-pilE, pCDFDuet pilD-pilE, pETDuet pilF-pilMNOP and pCDFDuet pilF-pilMNOP. At each cloning step expression of proteins was assessed as described in section 2.13 by Western blotting.

2.12.2 pilD, pilE, pilF, pilM, pilN, pilO and pilP co-expression by constructing a unique synthetic operon

The optimized pilD, pilE, pilF, pilMNOP were amplified using appropriate primers (Table 2.6) to introduce an NdeI restriction site at their 5′ ends and Nhel and Xhol restrictions sites at their 3’ends, and they were cloned individually into PCR8/GW/TOPO. Subsequently, pil genes were verified by sequencing to ensure no errors were introduce in PCRs. The genes were then digested from PCR8/GW/TOPO by NdeI and Xhol, gel extracted and subcloned into pET-21b restricted with the same enzymes. Each pil genes was then sequentially added in alphabetical order to form a unique synthetic operon as follows. In the first step, pilE was excised from pET-21b by XbaI and Xhol, gel extracted and then subcloned into pET-21b pilD, cut with Nhel and Xhol, which is the primary vector used for consecutive ligations. The Nhel and XbaI restriction endonucleases used to digest the vector and the insert respectively, produce compatible cohesive ends that can be ligated. Subsequently, pilF was introduced in the same way into pET-21b pilDE and finally pilMNOP into pET-21b
pilDEF. At each cloning step expression of proteins was assessed as described in section 2.13 by Western blotting.

2.13 Testing protein expression

pET plasmids containing genes under the T7 promoter were transformed into E. coli BL21 (DE3) strain and transformants were selected on LB agar plates containing appropriate antibiotics and incubated overnight at 37°C. A small-scale culture was set up by inoculating 10 ml of liquid LB medium containing suitable antibiotics with a single colony, and incubated overnight at 37°C, shaking at 180 rpm. Then, 0.5-1 ml of this overnight culture was used to inoculate 50 ml of liquid LB medium with suitable antibiotics and culture was incubated at 37°C, with shaking at 180 rpm, until OD$_{600}$ reached 0.5-0.6. At this instant, 1 ml of the culture was removed and prepared for SDS-PAGE analysis to provide the non-induced control and immediately after protein expression was induced by the addition of 1mM IPTG to the culture. The culture was grown for further 3 hours at 37°C and 1 ml of the induced culture was removed and prepared for SDS-analysis. Non-induced and induced samples were analyzed by Coomassie staining and Western blotting.

2.14 E. coli sphaeroplast formation

E. coli sphaeroplasts were prepared from bacteria grown on plates and in cultures, by submitting them to a cold osmotic shock treatment as described by Cisneros et al. (2012a). When grown on plates, a loopful of bacteria grown overnight at 37°C on LB agar plates, containing appropriate antibiotics and 1mM IPTG, was resuspended in 0.25 ml of osmotic shock solution (0.1 M Tris pH 7.5, 0.5 M sucrose and 5 mM EDTA). 0.1 mg/ml lysozyme was then added followed by immediate addition of 0.25 ml of ice-cold Milli Q water. After 5 minute incubation at 4°C, 18mM MgSO$_4$ was added to stabilize the sphaeroplasts. Next, bacteria were fixed on coverslips and piliation was assessed by IF microscopy.

To prepare sphaeroplasts from bacteria grown in cultures, 1ml of an overnight culture was used to inoculate 50 ml liquid LB medium with appropriate antibiotics.
The cultures were grown at 37°C, with shaking at 180 rpm, until an OD<sub>600</sub> of approximately 0.5-0.6 was reached. Protein expression was induced with 1 mM IPTG and cultures were grown for further 3 hours at 37°C. Next, 10 ml of the cultures was centrifuged at 1,200 x g for 10 minutes at 4°C. The pellets were washed by gently resuspending in 10 ml of PBS, before centrifuging at 1,200 x g for 10 minutes at 4°C. Finally, the pellets were gently resuspended in 0.25 ml of Osmotic shock solution and sphaeroplasts were prepared as above.

2.15 Immuno-fluorescence (IF) microscopy

IF microscopy was performed to assess piliation of <i>N. meningitidis</i> and <i>E. coli</i> sphaeroplasts. IF microscopy on <i>N. meningitidis</i> samples was performed by Vladimir Pelicic, as previously describe by Helaine et al. (2005), with minor differences . A loop of <i>N. meningitidis</i> grown overnight was resuspended in 1 ml of PBS and heated to 56°C for 30 minutes to kill the bacteria. 20 µl of each bacterial suspension was added in a well of a 10-well slide and air-dried. Samples were then fixed using 2.5% paraformaldehyde (PFA) in PBS for 20 minutes, and the reaction was stopped by incubating for 5 minutes with 0.1 M glycine in PBS, which serves as a quenching agent. Next, samples were blocked with 0.2% gelatine in PBS (PBS-Gelatine) for 20 minutes. Tfp were specifically labelled with anti-Tfp 20D9 monoclonal antibody used at a 1/100 dilution in PBS-Gelatine for 30 minutes at room temperature. Wells were then washed three times in PBS, before incubating for 30 minutes with the secondary goat anti-mouse antibody conjugated with Alex Fluor 488 (Molecular Probes), also used at 1/100 dilution in PBS-Gelatine. This solution also contained 100 ng/ml of 4’, 6-diamidino-2-phenylindol (DAPI) (Invitrogen) for staining the bacteria. Finally, the wells were washed 3-4 times with PBS, mounted with Aqua-Poly/Mount (Polysciences, Inc.) to enhance and retain fluorescent intensity and sealed with a coverslip. Samples were viewed using a Nikon Eclipse E600 microscope, with a 40x objective lens.

To examine pilus production on <i>E. coli</i> sphaeroplasts, 20 µl of sphaeroplasts was diluted 1/10 in osmotic shock solution (containing also 18mM MgSO₄), and added in the well of a 10-well slide. IF microscopy was performed as described above with
four main differences. Firstly, for blocking and antibody incubation steps, PBS with 5% skimmed milk powder was used instead of PBS-Gelatine. Secondly, 10 µg/ml of propidium iodide (Invitrogen) was used to stain bacteria rather than DAPI. The secondary goat anti-mouse antibody conjugated with Alex Fluor 488 (Molecular Probes), was used at 1/1,000 dilution and lastly samples were viewed using a Zeiss Axio Imager D1 microscope, with a 63x objective lens.
Chapter 3: Using the bacterial adenylate cyclase two-hybrid (BACTH) system to identify interactions between 11 *N. meningitidis* Pil proteins
3.1 Introduction

Deciphering individual protein-protein interactions is an essential step to improve understanding of the composition and organization of a multiprotein machinery. At the beginning of my PhD project, only a handful of protein-protein interactions between the Pil proteins of Tfpa machinery were known, so it was critical and intriguing to perform for the first time a systematic analysis. To accomplish that, I used a bacterial two-hybrid system and identified multiple binary interactions between 11 *N. meningitidis* Pil proteins.

The work described in this chapter has been published in: Large-scale study of the interactions between proteins involved in type IV pilus biology in *Neisseria meningitidis*: characterization of a subcomplex involved in pilus assembly. *Mol Microbiol.* (2012) 84: 857-873 (found in Appendix).

3.2 Bacterial adenylate cyclase two-hybrid (BACTH) system

3.2.1 Principle of BACTH

The BACTH system is a sensitive, simple and very reliable system, based on the interaction-mediated reconstitution of a cyclic AMP (cAMP) signalling cascade in *E. coli*. It employs the catalytic domain of *Bordetella pertussis* calmodulin (CaM) dependent adenylate cyclase (CyaA), which exhibits high catalytic activity, i.e. cAMP synthesis, in the presence of CaM and low but detectable activity in its absence (Ladant, 1988). This catalytic domain resides in the first 400 amino acids of the 1706 amino acid long CyaA protein and it can be proteolytically cleaved into two complementary fragments, T25 and T18 corresponding to amino acids 1-224 and 225-399 respectively (Ladant *et al.*, 1989). These fragments when co-expressed as independent polypeptides remain connected in the presence of CaM in a fully active ternary complex, but in the absence of CaM they are physically separated and they are unable to interact to reconstitute a functional enzyme and yield the basal CaM-independent activity (Ladant *et al.*, 1989) (Figure 3.1). Therefore, when interacting proteins are genetically fused to T25 and T18 and co-expressed in an *E. coli cya*
mutant strain (E. coli lacking CaM), interaction between the two-hybrid proteins brings T18 and T25 in close proximity and results in a functional complementation between T18 and T25 and hence cAMP synthesis (Karimova et al., 1998). cAMP in turn binds to the catabolite gene activator protein (CAP). The cAMP/CAP complex is a pleiotropic regulator of gene transcription, and turns on the expression of several genes including those involved in the catabolism of carbohydrates such as the maltose and lactose operons, which can easily be scored on indicator agar plates (Figure 3.1) (Karimova et al., 1998).

Figure 3.1 Principle of BACTH.

(A) The catalytic domain of B. pertussis adenylate cyclase, when expressed in E. coli, exhibits basal CaM-independent activity, which leads to cAMP synthesis. (B) The T18 and T25 fragments, when co-expressed as separate entities in E. coli, are unable to interact and thus no cAMP synthesis occurs. (C) The T18 and T25 fragments when fused to interacting proteins and co-expressed in E. coli, are brought close together, which results in functional complementation between the T18 and T25 fragments, leading to cAMP synthesis. (D) In an E. coli cya mutant strain, the cAMP synthesized by the complementing T18 and T25 binds to the catabolite gene activator protein, CAP. The cAMP/CAP complex turns on the expression of several genes, including the lac and mal operons, which can be easily visualized on indicator plates. This figure was inspired from Karimova et al. (1998).
The main advantage of this system that made it appealing for studying the Tfpa machinery, is that many of the Pil proteins are in the inner membrane and BACTH allows the study of interactions between membrane proteins. This was demonstrated in a systematic analysis used to identify interactions between the *E. coli* cell division proteins (Karimova *et al.*, 2005). In this study, 9 proteins of the divisome: the cytoplasmic tubulin-like protein FtsZ forming the Z-ring, the actin-like peripheral membrane protein FtsA, together with 7 Fts integral membrane proteins (FtsB, FtsI, FtsL, FtsN, FtsQ, FtsW and FtsX) and an integral membrane protein of unknown function YmgF, were fused to the C-terminus of T18 and T25 and strikingly multiple interactions were identified between them (Karimova *et al.*, 2005). Unlike in other systems, including the popular yeast two-hybrid system or the activator-dependent transcriptional bacterial two-hybrid system, the interaction between the two hybrid proteins does not need to occur close to the transcription machinery (Fields & Song, 1989, Dove & Hochschild, 2004). It can occur either in the cytosol or as explained below at the inner membrane provided that the T18 and T25 fragments are facing the cytoplasm. This is because upon interaction between the two hybrid T18 and T25 proteins, cAMP is synthesized which is freely diffusible in the cytoplasm and can therefore diffuse from the site of synthesis and bind to CAP leading to the transcription activation events and selectable phenotypes (Karimova *et al.*, 1998). Another important advantage is that analysis is carried out in *E. coli*, which is the basic ‘tool’ for molecular biology, making the system easy to handle.

The main limitation of BACTH is that cAMP needs to be produced in the cytoplasm because it cannot cross the lipid bilayer, prohibiting the analysis of proteins that have no cytoplasmic domain i.e. proteins localized in the outer membrane or periplasm. The second minor limitation, which is common to all two-hybrid systems, is potential false negative results. This assay requires the spatial proximity between T25 and T18 fragments, thus if there is a steric constraint and the CyaA fragments are held too far apart, they cannot interact leading to the absence of functional complementation between T18 and T25 fragments. However, interacting functional domains are in the range of 100-200 amino acids, and probably interactions mediated by domains of this size can be detected using BACTH. To minimize the
occurrence of false negatives we fused all target proteins to both the N- and C-termini of T18 and T25 to test all possible configurations.

### 3.2.2 Selection of proteins for BACTH analysis

There are 23 Pil proteins involved in Tfp biology in *N. meningitidis* separated into two classes: the proteins essential for the biogenesis of Tfp and a set of accessory proteins, dispensable for Tfp biogenesis but that fine-tune multiple functions mediated by Tfp. To decide which proteins could be analyzed using BACTH, a bioinformatic analysis was performed on all 23 proteins, to predict their sub-cellular localization using Psort (http://www.psort.org/psortb/index.html) (Yu et al., 2010). This analysis revealed that only five proteins could not be analyzed by BACTH due to the topological limitations of the system; these are PilC1/PilC2, PilQ and PilW, which are localized in the outer membrane and PilP, which is an inner membrane-anchored lipoprotein. Of all the proteins that are pilins (PilE, PilH, PilI, PilJ, PilK, ComP, PilV and PilX), I decided to focus only on PilE to facilitate the analysis, otherwise it would have been too labour intensive. The relevant characteristics of the 23 proteins including their cellular localization, molecular weight and isoelectric point (pI) are displayed on Table 3.1.
Table 3.1 Defining features of the *N. meningitidis* Pil proteins.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Gene label</th>
<th>Protein length (aa)</th>
<th>Molecular weight (Da)</th>
<th>Isoelectric point (pI)</th>
<th>Predicted localization</th>
<th>Analyzed in BACTH</th>
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</thead>
<tbody>
<tr>
<td><strong>Pilus assembly</strong></td>
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<td></td>
<td></td>
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<tr>
<td>PilE</td>
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<td>31345</td>
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<td>61979</td>
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<td>41367</td>
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<td>PilC1</td>
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<td><strong>Accessory proteins playing important roles in Tfp biology</strong></td>
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<td>ComP</td>
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<td>√</td>
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Of 18 cytoplasmic/inner membrane proteins, we selected 11 for a large-scale identification of their binary interactions (Table 3.1). These include six out of the seven proteins thought to be essential for Tfp assembly: the major pilin PilE, the prepilin peptidase PilD, the traffic ATPase that powers pilus assembly PilF, and PilM, PilN and PilO that together with PilP are likely to constitute the essence of the Tfp assembly machinery (Carbonnelle et al., 2006). Also, PilG was included that is a universally conserved inner membrane protein whose exact role is unclear. It is important to mention that on deciding whether to include the four inner membrane proteins PilD, PilG, PilN, PilO we further extended their bioinformatic analysis using the topology prediction server TMHMM (Krogh et al., 2001). This analysis indicated that all of the proteins apart from PilD have at least one cytoplasmic terminal end, which is absolutely essential for BACTH (Figure 3.2). Nevertheless, PilD was included in the study because bioinformatic analyses are not 100% accurate and also due to its key role in Tfp biogenesis as processing of PilE by PilD is a pre-requisite for filament assembly. The interaction between PilD-PilE has been demonstrated many times. The original study by Nunn and Lory (1991) showed that *P. aeruginosa* PilD purified by anion-exchange and immunoaffinity chromatography efficiently cleaves purified prepilins (PilA), without requiring any additional protein cofactors. PilD activity was assessed by a size shift between the prepilin and its processed mature form using SDS-PAGE (Nunn & Lory, 1991). Also, recently it was elegantly demonstrated by Aly et al. (2013) in a cell-free translation system. In this study *P. aeruginosa* PilD was co-synthesized in vitro with its substrate, the full-length PilA, in the presence of unilamellar liposomes. PilD was biologically active and led to a complete cleavage of the PilA signal peptide, shown by a size shift between the full-length and processed PilA using Western blotting (Aly et al., 2013).

Moreover, four other proteins were included from the accessory-protein class. These are PilZ, which is a cytoplasmic protein of unclear function, and the other three Pil traffic ATPases: PilT that mediates pilus retraction, PilT2 that probably works synergistically with PilT, and PilU whose role remains unclear (Brown et al., 2010).

The main reason that all pilin-like proteins (PilH, PilL, PilJ, PilK, ComP, PilV and PilX), were left out from the analysis, apart from the fact that the analysis would have
been huge, is that they were all reported to co-purify with Tfp, indicating that the pilus is their localization site and most likely their main interaction partner is PilE (Brown et al., 2010, Winther-Larsen et al., 2005).
The probability for transmembrane helix, inside or outside displayed for the prepilin peptidase PilD, the core polytopic PilG protein and the assembly proteins PilN and PilO. The plot is obtained by calculating the total posterior probability that a residue is in a transmembrane helix, on the cytoplasmic side or on the periplasmic side, over all possible paths through the model (Krogh et al., 2001). **(A)** TMHMM plot reveals that PilD has 6 transmembrane helices, 3 cytoplasmic domains, 2 periplasmic domains and N- and C-terminal periplasmic tails. **(B)** TMHMM plot reveals that PilG has a large cytoplasmic N-terminal domain, 3 transmembrane helices, another large cytoplasmic domain, a small periplasmic domain and a C-terminal periplasmic tail. **(C)** TMHMM plot predicts that PilN has a small N-terminal cytoplasmic segment, a single transmembrane helic and a large C-terminal periplasmic domain. **(D)** TMHMM plot predicts that PilO has a small N-terminal periplasmic domain, a single transmembrane helix, and a large C-terminal cytoplasmic domain.
3.2.3 Construction of recombinant plasmids used in BACTH

The full-length pil genes were first amplified from genomic DNA extracted from the N. meningitidis WT strain 8013. The PCR products were cloned directly into pCR8/GW/TOPO and sequenced. Next, genes containing no errors were selected and subcloned into the BamHI and KpnI restriction sites of the four BACTH vectors to create in-frame fusions at the N- and the C- termini of both T18 and T25 (Figure 3.3). The pUT18 and pUT18C vectors encode the T18 fragment, whereas the pKNT25 and pKT25 vectors encode the T25 fragment. The only difference between the two vectors expressing the same CyaA fragment is the position of the MCS. In pUT18 and pKNT25 the MCS is at the 5’ end of the T18 and T25 fragments respectively, to construct in-frame fusions at the N-terminal end of the CyaA polypeptides. In pUT18C and pKT25 it is at the 3’ end of the T18 and T25 fragments respectively, to create in-frame fusions at the C-terminal end of the CyaA polypeptides (see Chapter 2, section 2.4) (Karimova et al., 2001). Thus, for each protein four different plasmids were generated: X-pUT18, pUT18C-X, X-pKNT25 and pKT25-X. However, the following nomenclature is used for clarity which directly illustrates the nature of the engineered fusions: for instance, T18-PilD and PilD-T18 indicate that the T18 domain has been fused to the N- or C-terminus of PilD respectively.

Also, in order for co-expression of the two hybrids T18 and T25 proteins in E. coli to be possible, the plasmids expressing T18 (pUT18C and pUT18) and T25 (pKT25 and pKNT25) have compatible origins of replication and different antibiotic resistance genes for selection (Figure 3.3). The pUT18 and pUT18C vectors carry the ColE1 origin of replication and ampicillin resistance selection marker, while pKT25 and pKNT25 carry the p15A origin of replication and kanamycin resistance selection marker (Karimova et al., 2001).

When analyzing a potential interaction between any two proteins, all possible combinations between the four plasmids encoding each protein were tested resulting in eight different plasmid combinations in total (X-pUT18/Y-pKNT25, X-pUT18/pKT25-Y, pUT18C-X/ Y-pKNT25, pUT18C-X/pKT25-Y and Y-pUT18/X-pKNT25, Y-pUT18/pKT25-X, pUT18C-Y/ X-pKNT25, pUT18C-Y/pKT25-X) (Figure 3.3). So on
analysing the interactions between the 11 Pil proteins, the number of assays performed grew exponentially to 484 combinations.

In these vectors the hybrid protein to be expressed is under the transcriptional control of a lac promoter. Therefore, during the experiment, full expression of hybrid proteins requires, in addition to IPTG, the presence of the cAMP/CAP complex. This means that when proteins interact and the cAMP level rises, a positive feedback loop is driven and allows full expression of the hybrid proteins. This threshold effect contributes to the reliability of the system as it minimizes chances of false positive interactions caused by protein overexpression.
Figure 3.3 Schematic representation of the BACTH plasmid maps and the hybrid T18 and T25 proteins.

(A) Each gene was subcloned into the four BACTH vectors to create in-frame fusions at the N- and the C- termini of T18 and T25. Four BACTH plasmids were used. pUT18 and pUT18C encode the T18 fragment and they both carry the ColE1 origin of replication and ampicillin resistance selection marker. pKNT25 and pKT25 encode the T25 fragment and they both carry the p15A origin of replication and a kanamycin resistance selection marker. On pUT18 and pKNT25 the MCS is located at the 5’ end of T18 and T25 fragments respectively, to create in-frame fusions at the N-terminal end of the CyaA polypeptides. Whereas on pUT18C and pKT25 the MCS is located at the 3’end of the T18 and T18 fragments respectively to create in-frame fusions at the C-terminal end of the CyaA polypeptides (Karimova et al., 2001). (B) Upon analyzing a potential interaction between any two proteins (X and Y), eight different plasmid combinations were tested.
3.2.3 Identification of protein-protein interactions between Pil proteins

Before beginning the systematic analysis, all conditions were optimized. Functional complementation between T18 and T25 could be detected by plating *E. coli cya* transformants on two types indicator plates, either LB agar containing X-gal or MacConkey agar supplemented with maltose and observing the coloration within 40-48 hours at 30°C. The expression of the lacZ gene encoding β-galactosidase is positively controlled by cAMP/CAP. In the absence of functional complementation between T18 and T25 fragments, bacteria on LB agar containing the blue chromogenic β-galactosidase substrate X-gal, form white colonies; whilst when functional complementation occurs, bacteria become able to express β-galactosidase which hydrolyzes X-gal, and thus form blue colonies. In the absence of functional complementation between T18 and T25 fragments, bacteria on MacConkey agar supplemented with maltose form white colonies, whereas when functional complementation occurs, bacteria become able to ferment maltose leading to acidification of the medium and hence colour change of the dye phenol red, forming pink colonies (Karimova et al., 1998).

Therefore we tested two *E. coli cya* mutant strains DHM1 and BTH101 on the two types of indicator plates using many different T18/T25 plasmid combinations including the positive and negative control plasmids, described below, to select the best combination of *E. coli cya* mutant strain and indicator agar for the analysis. We found that positive clones in BTH101 cells showed a stronger interaction signal than in DHM1 cells using both types of indicator agar, which denoted that functional complementation between the hybrid T18 and T25 proteins is more efficient within BTH101. Also, upon incubating negative BTH101 colonies longer than 48 hours at 30°C, we found that they remained white on MacConkey agar supplemented with maltose, whereas on LB-agar supplemented with X-gal they always showed a blue spot in the centre of the colony which casted doubts on positive results. Consequently, for the subsequent large-scale analysis we selected BTH101 cells and MacConkey agar supplemented with maltose.
Also, it is important to mention that all variables per transformation i.e. the volume of cells used and plated and the amount of plasmids used were standardized, to obtain on plates approximately the same number of colonies of the same size (~200 colonies per plate) (see Chapter 2, section 2.4). This is because overcrowding the indicator plates resulted in smaller colonies which made detection of positive coloured colonies difficult, and also because too few colonies on indicator plates (<50 colonies per plate) made scoring statistically unreliable. Moreover, incubation time of the transformation plates was also standardised; interactions were first scored after 40 hours and then after 48 hours. This was performed as a general good experimental practice since sometimes incubation shorter than 48 hours was not sufficient for weak interactions to develop pink colonies, and prolonged incubation (>3 days) resulted in weak pink spot in the centre of negative colonies which could have been scored as positive leading to false positive results. Pictures of the plates were always taken after 48 hours of incubation for reference.

To identify putative Pil-Pil interactions, the *E. coli cya* mutant strain BTH101 was co-transformed with all possible pairs of T18 and T25 plasmids i.e. a total of 484 combinations. As a negative control, BTH101 cells were co-transformed with pUT18C/pKT25 plasmids containing no inserts, which always yielded white colonies. As a positive control, cells were co-transformed with pUT18C-zip/pKT25-zip plasmids in which the 35-amino acid-long leucine zipper derived from yeast protein GCN4 is fused to T18 and T25 and yielded deep purple colonies (Karimova *et al.*, 1998). In the analysis, interactions rarely produced as deep purple colonies as the positive control. Figure 3.4 displays the colony colour yielded by the positive and negative controls and also the different colony colours yielded by the positive clones, which varied from purple to light pink. The BACTH assays were repeated for all of the plasmid combinations that produced coloured colonies for confirmation of the phenotypes.
Figure 3.4 Screening of interacting proteins with the BACTH system on MacConkey agar supplemented with maltose.

Variation of colony colour observed using BACTH. (A) As a positive control, BTH101 cells were co-transformed with pUT18C-zip and pKT25-zip, in which the T18-zip and T25-zip hybrid proteins interact through a leucine zipper motif, and generated deep purple colonies. (B) As a negative control, BTH101 cells were co-transformed with pUT18C and pKT25 plasmids containing no inserts, and generated white colonies. (C) Purple colonies formed by the interaction between two hybrid Pil proteins. (D) Light pink colonies formed by the interaction between two hybrid Pil proteins.
This systematic analysis was very successful as it revealed protein-protein interactions many of which are novel. Remarkably, out of the 484 T25/T18 plasmids combinations, 483 could be scored (Figure 3.5). Only the PilT2-T18/PilT2-T25 combination could not be scored as it generated microscopic colonies even after prolonged incubation at 30°C, suggesting that for an unknown reason this combination is toxic. Forty-five combinations (9.3%) yielded coloured colonies with coloration varying between light pink and purple (Figure 3.5). In 11 out of the 45 positive combinations (24.4%) only a fraction of the colonies, approximately 5%, were coloured (Figure 3.5). These are believed to be real interactions but rather weak and transient occurring in part of the cells, and this is supported by the fact that six of these interactions are confirmed to be positive using different plasmid combinations.

Notably only one protein, the prepilin peptidase PilD, yielded no interactions with any other protein, which is surprising considering its role in processing the leader peptide of prepilins; it was expected to interact at least with PilE (Figure 3.5) (Strom et al., 1993b). However, these results agree with the predicted topology of PilD using the membrane protein topology server TMHMM (Figure 3.3). This showed that most likely both the N- and C- terminus of PilD, which are fused to the T18 and T25 fragments, are on the periplasmic side of the inner membrane (Krogh et al., 2001). Hence, the lack of identified interactions with PilD is probably attributed to the topology of PilD being incompatible with the BACTH system.

The major pilin PilE was found to interact with itself identified in one plasmid combination, T18-PilE/T25-PilE. This interaction was expected, as PilE must interact extensively with itself within the Tfp. Also, we found that PilE interacts with: PilG (T18-PilE/T25-PilG), PilN (T18-PilN/T25-PilE) and PilO (T18-PilO/T25-PilE, T18-PilE/T25-PilO). The assembly ATPase PilF was found to interact with itself (PilF-T18/T25-PilF and T18-PilF/PilF-T25) and with two other proteins: PilT2 (PilT2-T18/PilF-T25 and T18-PilF/ PilT2-T25) and PilZ identified in six plasmid combinations (PilZ-T18/T25-PilF, T18-PilZ/T25-PilF, PilZ-T18/PilF-T25, T18-PilZ/PilF-T25, T18-PilF/T25-PilZ and T18-PilF/PilZ-T25). The analysis also identified four interactions with the conserved inner membrane protein PilG: with PilE stated above, itself (T18-
PilG/T25-PilG), PilO (T18-PilO/T25-PilG and T18-PilG/T25-PilO) and PilT2 (PilT2-T18/T25-PilG and T18-PilT2/T25-PilG). Three interactions were identified with PilM: with itself (PilM-T18/T25-PilM and T18-PilM/PilM-T25), PilN (T18-PilN/T25-PilM and T18-PilM/T25-PilN) and PilT (PilM-T18/T25-PilT). PilN was found to interact with three other proteins: PilM stated above, PilO (T18-PilO/T25-PilN and T18-PilN/T25-PilO) and PilT (T18-PilN/T25-PilT). PilO was identified to interact with itself (T18-PilO/T25-PilO) and with the three proteins PilE, PilG and PilN, stated above. Moreover, the analysis found five interactions with the ATPase PilT; with PilM and PilN stated above, with itself (PilT-T18/T25-PilT, PilT-T18/PilT-T25 and T18-PilT/PilT-T25), PilT2 (PilT2-T18/PilT-T25, T18-PilT2/PilT-T25, T18-PilT/PilT-T25 and PilU (T18-PilU/PilT-T25). PilT2 was identified to interact with itself (PilT2-T18/T25-PilT2, T18-PilT2/T25-PilT2, and T18-PilT2/PilT2-T25), with four other protein: PilG, PilF and PilT stated above, and PilU (PilU-T18-T25-PilT2, T18-PilU/T25-PilT2, PilU-T18/PilT2-T25 and T18-PilU/PilT2-T25). PilU as mentioned above, was identified to interact with two proteins PilT and PilT2. Lastly PilZ was found to interact with PilF, stated earlier, and with itself (PilZ-T18/T25-PilZ and T18-PilZ/PilZ-T25).

Interestingly, these results showed that 8 out of the 11 proteins (PilE, PilF, PilG, PilM, PilO, PilT, PilT2, PilZ) analyzed were able to dimerize or multimerize, indicating the existence of an even more complex interaction network than originally thought.
Figure 3.5 Binary protein-protein interactions between 11 *N. meningitidis* Pil protein using the BACTH system.

The proteins, labelled by their corresponding letter, were fused to the both N- and C-termini of the *B. pertussis* adenylate cyclase fragments T18 and T25. All the possible plasmid combinations, 484 in total, were co-transformed in the *E. coli* cya strain BTH101 and plated on MacConkey agar plates supplemented with maltose. Functional complementation between the T18 and T25 fragments, which occurs upon interaction of the hybrid proteins triggers the expression of *mal* genes and yields pink to purple colonies (Karimova et al., 1998). +, pairs that yielded coloured colonies. +/-, only a fraction of colonies were pink. NT, this combination could not be tested because the colonies were microscopic even after prolonged incubation.
As described above, when complementation between the two-hybrids proteins occurred, it could not be detected in all configurations, i.e. using the eight different plasmid combinations. This is likely to be because the end of the proteins fused to T18 or T25 is located in the periplasm or because of steric hindrance. Table 3.2 summarizes all of the protein-protein interactions identified using BACTH and the number of times each interaction was identified using different plasmid combinations. This reveals that overall 22 different binary protein-protein interactions were identified using BACTH.

Table 3.2 The protein-protein interactions identified using BACTH and the number of times they were identified.

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Number of times identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 PilE-PilE</td>
<td>1</td>
</tr>
<tr>
<td>2 PilE-PilG</td>
<td>1</td>
</tr>
<tr>
<td>3 PilE-PilN</td>
<td>1</td>
</tr>
<tr>
<td>4 PilE-PilO</td>
<td>2</td>
</tr>
<tr>
<td>5 PilF-PilF</td>
<td>2</td>
</tr>
<tr>
<td>6 PilF-PilT2</td>
<td>2</td>
</tr>
<tr>
<td>7 PilF-PilZ</td>
<td>6</td>
</tr>
<tr>
<td>8 PilG-PilG</td>
<td>1</td>
</tr>
<tr>
<td>9 PilG-PilO</td>
<td>2</td>
</tr>
<tr>
<td>10 PilG-PilT2</td>
<td>2</td>
</tr>
<tr>
<td>11 PilM-PilM</td>
<td>2</td>
</tr>
<tr>
<td>12 PilM-PilN</td>
<td>2</td>
</tr>
<tr>
<td>13 PilM-PilT</td>
<td>1</td>
</tr>
<tr>
<td>14 PilN-PilO</td>
<td>2</td>
</tr>
<tr>
<td>15 PilN-PilT</td>
<td>1</td>
</tr>
<tr>
<td>16 PilO-PilO</td>
<td>1</td>
</tr>
<tr>
<td>17 PilT-PilT</td>
<td>3</td>
</tr>
<tr>
<td>18 PilT-PilT2</td>
<td>3</td>
</tr>
<tr>
<td>19 PilT-PilU</td>
<td>1</td>
</tr>
<tr>
<td>20 PilT2-PilT2</td>
<td>3</td>
</tr>
<tr>
<td>21 PilT2-PilU</td>
<td>4</td>
</tr>
<tr>
<td>22 PilZ-PilZ</td>
<td>2</td>
</tr>
</tbody>
</table>
3.3 Quantification of the strength of identified protein-protein interactions

The different range of colony colours generated upon functional complementation between T18 and T25 fragments is probably indicative of the strength of the interactions between the hybrid proteins (Figure 3.4). The interaction between the leucine zipper hybrid proteins of the positive control is exceptionally strong and in the analysis only three positive plasmid combinations (T18-PiT2/T25-PiT2, T18-PiT2/PiT2-T25 and T18-PiE/T25-PiE) formed as deep purple colonies as the positive control, while the rest of the positive combinations varied from purple to pink colour.

Another advantage of BACTH is that the efficiency of functional complementation between the T18 and T25 fragments can be quantified by measuring β-galactosidase activities in liquid culture to accurately determine the strength of the interactions (Karimova et al., 1998, Karimova et al., 2005). This is because as mentioned before, in E. coli the lacZ gene encoding β-galactosidase is positively controlled by cAMP/CAP, thus bacteria expressing interacting hybrid proteins subsequently express β-galactosidase. This assay involves permeabilization of the cells and their subsequent incubation with a colourless β-galactosidase substrate, ONPG, which upon hydrolysis turns into the yellow chromophore O-nitrophenol (ONP). The intensity of the yellow colour is directly proportional to the amount of β-galactosidase produced and hence the strength of the interaction between the interacting hybrid proteins. ONP formation was followed spectrophotometrically at OD<sub>420</sub> and used to calculate the β-galactosidase activities using the following formula: \( A = 200 \times \frac{\text{OD}_{420}}{\text{minutes of incubation}} \times \text{dilution factor} \). The results were expressed as units of enzymatic activity per milligram of bacterial dry weight (U/mg), where 1 unit corresponds to 1 nmol of ONPG hydrolysed per minute at 28°C (Karimova et al., 1998). For each plasmid combination yielding coloured colonies in the BACTH, the β-galactosidase activity of three independent cultures was measured, thus for the 45 positive plasmid combinations identified using BACTH, a total of 135 assays were performed.
An increasing number of studies have optimized measuring β-galactosidase activity in a 96-well plate format for more efficient processing of large number of samples (Griffith & Wolf, 2002). However, on comparing the 96-well plate format and the traditional single tube format described by Karimova et al. (2001) using duplicate samples, we found that when samples were prepared in a 96-well plate the OD_{420} readings of the samples fluctuated widely, whereas when prepared in tubes the OD_{420} readings were always consistent. Hence, we performed the β-galactosidase assays in the traditional single tube method, even though it was more tedious and time consuming.

Also, the original protocol stated that the reaction should be stopped after sufficient yellow colour has developed, however defining sufficient yellow colour was very judgemental (Karimova et al., 2001). On optimizing the assay we found that strong interactions, such as the positive control, developed sufficient yellow colour within a few seconds. Whereas weak interactions developed sufficient yellow colour within 10-20 minutes that was never as intense as the colour of strong interactions, and the negative control developed faint yellow colour within an hour. Thus, on performing 135 assays, the intensity of yellow colour could not be used as a constant factor in determining the incubation time with ONPG of each assay. Instead, we decided to standardise the incubation time, which is the second variable in the equation used to calculate the enzymatic activity. To do that we tested numerous samples to determine the best time to stop the reactions and, based on our results, we established an optimized protocol in which reactions were stopped after 20 minutes for positive samples and 60 minutes for negative samples.

As shown in Figure 3.6, a background β-galactosidase activity of 205 ± 47 U/mg was measured in the negative control, while the positive control generated activity of 5,247 ± 1,339 U/mg. The activities generated using the 45 positive plasmid combinations, ranged from 7,910 ± 262 U/mg for T18-PilT2/T25-PilT2, which is even higher than the activity of the positive control, to 160 ± 9 U/mg for PilM-T18/T25-PilT, which is below the background level measured in the negative control. However, only two combinations, the already mentioned PilM-T18/T25-PilT and T18-PilN/T25-PilT generated β-galactosidase activities (160 ± 9 U/mg and 173 ± 10 U/mg
respectively), below the background level (Figure 3.6). It should be stressed that in these two combinations only a fraction of the colonies were coloured (Figure 3.5). The other 43 combinations identified using BACTH could be confirmed, as they all generated β-galactosidase activities higher than the negative control activity. Of the 43 combinations, the lowest activity generated was 483 ± 28 U/mg for T18-PilM/T25-PilN, which is more than 2-fold higher than the activity of the negative control. Twenty-nine interactions generated β-galactosidase activities greater than 1,000 U/mg and were classified as strong, while 14 interactions were weaker and generated activities lower than 1,000 U/mg (Figure 3.5).

Interestingly, the three T18/T25 combinations that yielded deep purple colonies in the BACTH screening (T18-PilT2/T25-PilT2, T18-PilT2/PilT2-T25 and T18-PilE/T25-PilE) are the ones that generated as high or even higher β-galactosidase activities than the positive control (Figure 3.6).
Figure 3.6 Quantification of Pil-Pil interactions identified by BACTH.

The efficiency of functional complementation between the indicated hybrid proteins was quantified by measuring the β-galactosidase activities. The results are expressed as units of β-galactosidase activity per milligram of bacteria (dry weight) and are the mean ± standard deviation of at least three different independent experiments. The red line indicates the background β-galactosidase activity measured in the negative control. The blue line indicates the threshold used to define strong and weak interactions.
3.4. Summary

In summary, using the BACTH system and the β-galactosidase assays, we identified 43 interactions between 10 Pil proteins. Some interactions were identified multiple times using different plasmid combinations, for instance the PilF-PilZ interaction was identified with six different plasmid combinations. Thus, overall this analysis identified 20 different binary protein-protein interactions and represents the most complex interaction network between Pil proteins to date (Figure 3.6). Significantly, until then many of these interactions had never been reported elsewhere and these are: PilE-PilN, PilE-PilO, PilG-PilE, PilG-PilO, PilG-PilT2, PilM-PilM, PilT2-PilT, PilT2-PilU, PilT2-PilF and PilT-PilU. Also, the PilM-PilN was novel at the time we identified it, but immediately after completing the analysis it was reported in T. thermophilus using other approaches before our results were published.

Schematic representation of the topology of this network reveals the existence of two subcomplexes linked though an interaction between PilT2-PilG (Figure 3.7). The first subcomplex consists of the four traffic ATPases (PilF, PilT, PilT2 and PilU) and PilZ that interacts with PilF (Figure 3.7). The interactions between the different ATPase monomers (PilF-PilT2, PilT-PilT2, PilU-PilT and PilU-PilT2) provide evidence that different ATPases (homohexamers) interact with each other, but also suggest the existence of possible heteromultimers. Remarkably, we discovered that PilT2 interacts with all of the other ATPases acting as the ‘nucleus’ of the interaction network. The second subcomplex consists of the five proteins predicted to be essential for Tfp assembly, which interact in a highly ordered fashion: PilM-PilN-PilO-PilE, and PilG that acts after the assembly step and interacts with PilE bridging the two subcomplexes together (Figure 3.7). The discovery of this inner membrane subcomplex using this study is highly significant, as previously there was only limited evidence for its existence in P. aeruginosa by Ayers et al. (2009), and for the first time we had a more clear view of the assembly machinery providing the framework for further characterization.
Figure 3.7 Schematic presentation of the protein network that was identified by BACTH.

The thickness of the edges between the nodes is proportional to the number of times that link has been identified (between one and six times).
Chapter 4: Functional characterization of the PilM-PilN-PilO-PilP subcomplex involved in pilus assembly
4.1. Introduction

PilM, PilN, PilO and PilP are encoded by the *pilMNOPQ* operon, which is highly conserved among Tfpa-producing bacteria (Pelicic, 2008). These proteins were identified to be essential for Tfp assembly by Carbonnelle *et al.* (2006) as described in Introduction (section 1.2.4.3.2) and they possibly constitute the essence of the pilus assembly machinery. However, at the beginning of my PhD very little was known about them.

The BACTH systematic analysis I performed was the first direct evidence we had for the existence of the PilM-PilN-PilO-PilE subcomplex. Thus, this analysis prompted us to further investigate these proteins, in order to delineate architecture of this subcomplex and the mechanism of pilus assembly. However, it is important to mention that key studies were later published during my PhD, using the *P. aeruginosa* and *Thermus thermophilus* homologues that together shaped a more complete picture of the subcomplex. The main features of these four proteins are displayed on Table 3.1.

The work described in this chapter has been published in: Large-scale study of the interactions between proteins involved in type IV pilus biology in *Neisseria meningitidis*: characterization of a subcomplex involved in pilus assembly. *Mol Microbiol.* (2012) 84: 857-873 (found in Appendix).

4.2 Experimental determination of the membrane topology of PilN and PilO

The first step of the functional analysis of the Tfpa assembly machinery was to determine the topology of some of its components. As discussed in the introduction the topology of PilE is well established, it consists of a long α-helix that is inserted in the inner membrane when not in filaments, and a C-terminal globular domain in the periplasm. Also, previous studies have determined that PilM is a cytoplasmic protein and PilP is a lipoprotein. Thus, the only proteins of the complex whose topology was unclear were PilN and PilO.
Bioinformatic analyses of PilN and PilO proteins performed using the tools TMPred, TopPred and TMHMM revealed that both PilN and PilO are bitopic inner membrane proteins with a short N-terminal segment, followed by a single transmembrane domain and a large C-terminal domain (data only shown for TMHMM predictions, Figure 3.2C-D). However, although the servers agree that PilN is localized with its N-terminus in the cytoplasm and C-terminus in the periplasm, they predict different topologies for PilO. Also, based on the BACTH analysis, we observed that the bitopic inner membrane proteins PilE, PilN and PilO only interacted when their N-termi

were fused to the T18 and T25 fragments. This suggested that the proteins might be localized with their N-termi

n in the cytoplasm and their globular C-terminal domains in the periplasm, which agrees with the already established PilE topology. Consequently, we experimentally determined the membrane topology of PilN and PilO using the plasmid pKTop that encodes a dual pho-lac reporter system (Karimova et al., 2009). This vector contains the E. coli alkaline phosphatase fragment PhoA_{42-472} fused with the α-peptide of E. coli β-galactosidase, LacZ_4-60. The E. coli alkaline phosphatase is a periplasmic protein that is enzymatically inactive in the cytoplasm because its cysteines become fully reduced and hence fail to acquire disulphide bonds, which are important for its activity (Derman & Beckwith, 1991). The E. coli β-galactosidase is a cytoplasmic protein that is enzymatically inactive in the periplasm because its cysteines become oxidized and form improper intermolecular disulphide bonds (Snyder & Silhavy, 1995). Thus, a periplasmic location of the reporter leads to a high alkaline phosphatase activity and a low β-galactosidase, whilst for a cytoplasmic location it is the opposite (Karimova et al., 2009).

Using this approach, pil genes were inserted at the 5’ end of the dual pho-lac reporter to create in-frame fusions at the N-terminal end of the reporter. The full-length pilN and pilO genes and their corresponding truncated variants pilN_{1-50} and pilO_{1-50}, consisting of the first 50 residues of the proteins, were amplified from genomic DNA extracted from WT 8013 strain. Then, they were cloned into pCR8/GW/TOPO and sequenced. Genes with correct sequences were selected and cloned into the BamHI and KpnI sites of pKTop. Subsequently, the recombinant plasmids were transformed into E. coli DH5α cells, and transformants were streaked
on LB agar plates containing both a blue chromogenic substrate (X-phos) of alkaline phosphatase and a red chromogenic substrate (Red-gal) of β-galactosidase (Karimova et al., 2009). As a control, we used E. coli DH5α cells transformed with pKTop, containing no insert, in which the PhoLac reporter remains in the cytosol and yields red colonies. As can be seen on Figure 4.1A, while the PilN-PhoLac and PilO1.50-PhoLac exhibited blue phenotype, the PilO-PhoLac and PilN1.50-PhoLac exhibited a purple phenotype, which is the result of a mixture of red and blue colonies. This means that in the latter two fusions there was a combination of reporter activities, similarly to what was previously reported in another study using the same dual reporter (Karimova et al., 2009). This is explained by the fact that when a fusion protein that directs the dual reporter to the periplasm is overexpressed, the cellular secretion machinery can presumably get jammed. Hence, it is not unusual for a fraction of the fusion protein to remain trapped in the cytoplasm, leading to both alkaline phosphatase and β-galactosidase activities. Importantly, this purple coloration was observed only when the fusion proteins were actively exported to the periplasm, and it never occurred in the negative control, the pKTop plasmid with no insert, which further supports our explanation.

We next attempted to lower the level of expression of the fusion proteins to examine if it would eliminate the residual β-galactosidase activity. However, this was not successful as purple colonies could be observed even with 10 µM IPTG, which is 100-fold lower than the recommended protocol concentration.

Consequently, we modified the experiment by using plates containing only the blue chromogenic substrate (X-phos) of alkaline phosphate and we also included two controls, which are previously published fusions with the E. coli YmgF polytopic protein, YmgF1.32-PhoLac and YmgF1.72-PhoLac that direct the reporter to the periplasm and the cytoplasm respectively (Karimova et al., 2009). This time, all of the cells expressing PilN-PhoLac, PilO-PhoLac, PilN1.50-PhoLac and PilO1.50-PhoLac exhibited a blue phenotype, which indicates a periplasmic location of the reporter and thus of the C-terminus of PilN and PilO proteins (Figure 4.1B).
Figure 4.1 Topology analysis of PilN and PilO.

*pilN* and *pilO* genes encoding both full length and truncated proteins, corresponding to the first 50 residues, were fused in-frame to a dual *pho-lac* reporter in the pKTop (Karimova et al., 2009). (A) *E. coli* DH5α cells expressing the PilN-PhoLac, PilO-PhoLac, PilN<sub>1-50</sub>-PhoLac and PilO<sub>1-50</sub>-PhoLac fusions were plated on LB agar containing two chromogenic substrates, Red-Gal for β-galactosidase activity and X-phos for alkaline phosphate activity. As a control, we used *E. coli* DH5α cells transformed with pKTOP, in which the PhoLac reporter remains in the cytosol. Blue colony coloration generated by high phosphatase activity, indicates a periplasmic location of the reporter. Red colony coloration generated by high β-galactosidase activity, indicates a cytoplasmic location of the reporter. (B) *E. coli* DH5α cells expressing the PilN-PhoLac, PilO-PhoLac, PilN<sub>1-50</sub>-PhoLac and PilO<sub>1-50</sub>-PhoLac fusions were plated on LB agar containing only X-phos, for alkaline phosphate activity. As controls, we used two previously published fusions with the *E. coli* polytopic protein YmgF, directing the reporter to the periplasm (YmgF<sub>1-32</sub>-PhoLac) and the cytoplasm (YmgF<sub>1-72</sub>-PhoLac) (Karimova et al., 2009). Blue colony coloration generated by high phosphatase activity, indicates a periplasmic location of the reporter. No colony coloration indicates a cytoplasmic location of the reporter.
Therefore, based on our results and TMHMM predictions, PilN and PilO have a short N-terminal segment of 20-26 amino acids in the cytoplasm, one transmembrane helix and a large C-terminal periplasmic domain consisting of 153/199 amino acids for PilN and 173/214 amino acids for PilO (Figure 4.2).

Figure 4.2 Schematic representation of the PilN and PilO topology.

PilN and PilO have a similar topology consisting of a short N-terminal cytoplasmic segment followed by one transmembrane helix and a large C-terminal periplasmic domain. The transmembrane helices start and end point residues were predicted using TMHMM. IM, inner membrane.
4.3 Mapping of the interaction domains between PilE, PilM, PilN and PilO

Following the determination of PilN and PilO membrane topology, we decided to further investigate the identified interactions between PilE, PilM, PilN and PilO, by mapping the critical domains for protein-protein interaction using BACTH. To achieve this, we generated truncated variants of PilE, PilN and PilO corresponding to approximately the first 39-50 residues of the proteins, which consist of the short N-terminal cytoplasmic domain and the transmembrane helix. The underlying principle was that removing the C-terminal periplasmic domains would help determine their contribution to the interactions identified. Hence, a decrease in the strength of the interaction with a truncated variant compared to the strength of the interaction with the full-length protein, means that the C-terminal periplasmic domain contributes to the interaction.

Subsequently, PilE\textsubscript{1-39}, PilN\textsubscript{1-50} and PilO\textsubscript{1-50} were generated by amplifying the genomic DNA of N. meningitidis WT strain 8013 using suitable primers. These shorter versions were cloned into pCR8/GW/TOPO, verified by sequencing and subcloned into the BamHI and KpnI restriction sites of only two BACTH vectors (pUT18C and pKT25) to create in-frame fusions at the C-terminus of T18 and T25. This is because as mentioned before, interactions with the full-length PilE, PilN and PilO proteins were identified using BACTH only when in pUT18C and pKT25 plasmids. Putative interactions were identified by co-transforming the corresponding plasmids into E. coli BTH101 cells and screening for functional complementation between the T18 and T25 fragments on MacConkey agar supplemented with maltose (Karimova et al., 1998). Figure 4.2 displays the interactions studied and which ones yielded coloured colonies (Figure 4.3).
Truncated variants of PilE, PilN and PilO (PilE_{1-39}, PilN_{1-50} and PilO_{1-50}) were fused to the C-terminus of T18 and T25 fragments. The plasmid combinations indicated were co-transformed in the strain *E. coli* cya BTH101 and plated on MacConkey agar supplemented with maltose. Functional complementation between the T18 and T25 fragments, which occurs upon interaction of the hybrid proteins triggers the expression of *mal* genes and yields pink to purple colonies (Karimova *et al*., 1998). +, pairs that yielded coloured colonies. -., pairs that yielded white colonies.

The functional complementation between the T18 and T25 fragments was quantified by measuring β-galactosidase activities in liquid culture and compared to the activities generated by the full-length proteins. This was performed for all plasmid combinations tested, shown in Figure 4.3, including the ones that yielded white colonies.

Based on the topology analysis (Figure 4.2) we could conclude that PilN interacts with the cytoplasmic PilM protein through its short N-terminal cytoplasmic domain. To demonstrate this, we first examined the PilM-PilN interaction, which was identified in two combinations, T18-PilM/T25-PilN and T18-PilN/T25-PilM. The T18-PilN_{1,50}/T25-PilM interaction (447 ± 41 U/mg) showed no significant difference in strength compared to the original T18-PilN/T25-PilM interaction (479 ± 7 U/mg) (Figure 4.4). Interestingly, the T18-PilM/T25-PilN_{1,50} interaction (787 ± 112 U/mg) generated 1.7-fold higher β-galactosidase activity than the original T18-PilM/T25-PilN interaction (450 ± 13 U/mg) (Figure 4.4). A possible explanation for this
observation is that the shorter PilN fusion is more stable, however we could not investigate this further because the anti-PilN antibody we have recognizes the deleted part of the protein. Thus, as the interaction between PilN_{1-50} and PilM was as strong as the original interaction with the full-length PilN protein, this analysis showed that the C-terminal periplasmic domain is not involved at all in the PilM-PilN interaction and it ascertained that the N-terminus of PilN is the only domain of PilN critical of the PilM-PilN interaction.

The next interaction we examined was PilN-PilO, which was as well identified in two combinations, T18-PilO/T25-PilN and T18-PilN/T25-PilO. In the first combination, T18-PilO/T25-PilN, the functional complementation between T18 and T25 was abolished with T18-PilO_{1-50}/T25-PilN, as the β-galactosidase activity generated (120 ± 2 U/mg) was below the activity measured of the negative control (127 ± 25 U/mg) (Figure 4.4). The T18-PilO/T25-PilN_{1-50} plasmids generated considerable β-galactosidase activity (609 ± 36 U/mg) that was approximately 5-fold higher than the negative control (127 ± 25 U/mg) (Figure 4.4). However, this activity generated with PilN_{1-50} was lower than that measured with the original T18-PilO/T25-PilN interaction (1049 ± 129 U/mg). In the second combination, T18-PilN/T25-PilO, no functional complementation between T18 and T25 was detected with either T18-PilN_{1-50}/T25-PilO (127 ± 3 U/mg) or T18-PilN/T25-PilO_{1-50} (123 ± 11 U/mg) plasmids. In summary, these results reveal that the PilN-PilO interaction is mainly mediated by the C-terminal periplasmic domains of PilN and PilO, but also partly by the N-terminus and transmembrane helix of PilN, since T25-PilN_{1-50} was capable of interacting with T18-PilO.

The last interaction we examined was the PilO-PilE interaction, which was also identified in two combinations, T18-PilO/T25-PilE and T18-PilE/T25-PilO. In the first combination, T18-PilO/T25-PilE, there was no functional complementation between T18 and T25 with the shorter versions of the proteins in T18-PilO_{1-50}/T25-PilE (137 ± 7 U/mg) and T18-PilO/T25-PilE_{1-39} (127 ± 5 U/mg) (Figure 4.4). In the second combination, T18-PilE/T25-PilO, while there was no functional complementation between T18 and T25 with T18-PilE/T25-PilO_{1-50} (140 ± 8 U/mg), the T18-PilE_{1-39}/T25-PilO plasmids generated substantial β-galactosidase activity (493 ± 89 U/mg) (Figure
4.4). However, compared to the β-galactosidase activity generated with the full-length proteins T18-PiLE/T25-PiLO (1448 ± 350 U/mg), there was a 3-fold decrease. Taken together, these results indicate that the C-terminal periplasmic domains of PiLE and PiLO mainly contribute to the PiLE-PiLO interaction, but also the N-terminus of PiLE participates in this interaction, as T18-PiLE_{1-39} was capable of interacting with T25-PiLO.

In conclusion, PiLN interacts with PiLM through its N-terminal cytoplasmic segment, but with PiLO along the whole length of both proteins. Also, PiLO-PiLE interaction occurs along the whole length of the two proteins. However, it is important to highlight that though PiLO interacts along its whole length with PiLE and PiLN, these results indicate that interactions occur predominantly through its C-terminal periplasmic domain.
Figure 4.4 Quantification of protein interactions between PilE<sub>1-39</sub>, PilN<sub>1-50</sub> and PilO<sub>1-50</sub> and full-length proteins.

Truncated variants of PilE, PilN and PilO (PilE<sub>1-39</sub>, PilN<sub>1-50</sub> and PilO<sub>1-50</sub>) were fused to the C-terminus of T18 and T25 fragments. The strength of each interaction was quantified by measuring the β-galactosidase activities and compared to the strength of the interaction between the full-length hybrid proteins. The results are expressed as units of β-galactosidase activity per milligram of bacteria (dry weight) and are the mean ± standard deviation of at least three independent experiments. The red line indicates the background β-galactosidase activity measured in the negative control.
The decrease in β-galactosidase activity or lack of activity shown with the shorter variants of PilE, PilN and PilO, in the PilN-PilO and PilO-PilE interactions, could also be interpreted as a general instability of the proteins upon removing the C-terminal periplasmic domain, rather than this missing protein domain contributing to the interactions. Therefore, to investigate these possibilities, we tested by Western blotting using an anti-T18 antibody the stability of all shorter variants fused to T18 in the different plasmid combinations studied using BACTH. Also, we tried two different anti-T25 antibodies, to test the stability of all shorter variant fused to T25, however none of them worked (data not shown). The whole-cell protein samples were prepared from *E. coli* BTH101 transformants grown overnight in liquid LB medium supplemented with appropriate antibiotics and 0.5 mM IPTG, and equal amounts of the samples were loaded in each well of the SDS-PAGE gels. The anti-T18 Western blots (Figure 4.5) were of very bad quality, primarily because of non-specific binding of the anti-T18 antibody. However, this is possibly because on performing these Western blots, I did not carry out the secondary antibody incubation steps in blocking solution (see section 2.10.4) as I always did later on, which might have contributed to the high background signal. Nonetheless, they were not repeated, because as explained below, they proved that the shorter PilE, PilN and PilO variants fused to T18 are stable.

Interestingly, as observed in Figure 4.5 expression of the T18 hybrid proteins could be definitely confirmed only when the proteins were in the presence of an interacting protein partner. When the T18 hybrid proteins were present alone or with a non-interacting protein, the protein expression level was really low. This is evident from the detection of massive signal only by the full-length proteins in the original T18/T25 combinations and the T18-PilO/T25-PilN$_{1-50}$ and T18-PilE$_{1-39}$/T25-PilO combinations that generated significant β-galactosidase activities (Figure 4.5).

Though this observation briefly troubled us, soon we realized it is logical considering that the expression of the proteins is under the transcriptional control of a *lac* promoter. As explained in Chapter 3 (section 3.2.3), full expression of the hybrid proteins in a *cya* mutant strain requires apart from IPTG the presence of the cAMP/CAP complex i.e. interaction between the two hybrid proteins is leading to
amplification of protein expression. Also, it is possible that the interaction between the two proteins contributes to the stabilization of the proteins and thus to a better detection signal. Therefore, when there is no interaction between the two proteins tested or when only one protein is present, the level of induction of gene expression is low because of lack of cAMP and possibly expressed proteins are unstable because of lack of interacting partners.

Consequently, these Western blots show that the proteins are stable upon removing the C-terminal periplasmic domain and confirm that our conclusions based on the BACTH results are accurate.
Figure 4.5 Detection of T18-hybrid proteins to confirm stability of PilE_{1-39}, PilN_{1-50} and PilO_{1-50}.

Western blotting using anti-T18 antibody on whole-cell protein extracts from BTH101 cells transformed with the different T18/T25 plasmid combinations. For each blot equal volumes of whole-cell protein extracts were loaded in each lane. (A) T18-PilO and T18-PilO_{1-50} detected in the T18-PilO/T25-PilN combination. (B) T18-PilN and T18-PilN_{1-50} detected in the T18-PilN/T25-PilO combination. (C) T18-PilO and T18-PilO_{1-50} detected in the T18-PilO/T25-PilE combination. (D) T18-PilE and T18-PilE_{1-39} detected in the T18-PilE/T25-PilO combination.
4.4 Assessment of the functional importance of a conserved N-terminal motif in PilN

4.4.1 Role of the conserved N-terminal motif of PilN in PilM-PilN interaction

A previous study in *P. aeruginosa* showed using amino acid sequence alignment of PilN orthologues from a variety of Gram-negative bacteria, that the cytoplasmic segment of the protein has a highly conserved motif, INLLP, between residues 7 to 10 (Figure 4.6) (Sampaleanu et al., 2009).

![Figure 4.6 Amino acid sequence alignment of PilN proteins from different bacterial species.](image)

PilN sequences from *N. meningitidis*, *N. gonorrhoeae*, *X. campestris*, *P. aeruginosa*, *L. pneumophila* and *T. thermophilus* were aligned. For shading to occur, all residues of PilN sequences from different species had to be similar or identical. Conserved amino acids are shown in black boxes with white writing, while similar amino acids are shown in gray boxes and black writing. The red-boxed region is the highly conserved cytoplasmic N-terminal motif ‘INLLP’. The blue-boxed region is the transmembrane domain, as predicted by TMHMM.

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Since we found that PilN interacts with the cytoplasmic PilM protein through its N-terminal cytoplasmic domain, we next investigated if this PilN conserved cytoplasmic motif plays a role in the PilM-PilN interaction. This was achieved by constructing three different PilN variants, in which different residues within the INLLP motif were substituted with alanine using site-directed mutagenesis (PilN_{N8A}, PilN_{L9A} and PilN_{P11A}). This approach is quite common in determining the contribution of a specific residue to the function of the protein because alanine possesses one of the most chemically inert, simple side chains and it does not change main-chain conformations or impose extreme steric or electrostatic effects (Cunningham & Wells, 1989). The mutant pilN alleles were subcloned from the pCR8/GW/TOPO derivatives into the BamHI and KpnI sites of pUT18C and pKT25 to create in-frame fusions with the C-terminus of T18 and T25. Subsequently, interactions between the corresponding PilN hybrid proteins and PilM were identified by co-transforming the plasmids into E. coli BTH101 cells and plating on MacConkey agar supplemented with maltose (Figure 4.7). The strength of the interactions were quantified by measuring their β-galactosidase activities in liquid culture, and compared to the strength of the original interactions T18-PilM/T25-PilN and T18-PilN/T25-PilM.

We found that functional complementation between T18 and T25 was abolished with PilN_{N8A} and PilN_{L9A} variants in both PilM-PilN combinations, as they generated β-galactosidase activities lower than the background activity yielded by the negative control (172 ± 62 U/mg) (Figure 4.8). In contrast, PilN_{P11A} interacts with PilM as well as PilN_{WT}, since it generated 545 ± 29 U/mg and 492 ± 123 U/mg in the T18-PilM/T25-PilN_{P11A} and T18-PilN_{P11A}/T25-PilM combinations respectively, compared to 653 ± 169 U/mg and 711 ± 210 U/mg generated by the corresponding interactions with PilN_{WT} (Figure 4.8).

As a control experiment, to verify that that the elimination of the interaction between PilN and PilM with the PilN_{N8A} and PilN_{L9A} variants was not due to a major protein instability or a lack of production of the PilN variants, we assessed the interactions between the three different PilN variants and PilO using BACTH. The rationale of this experiment is based on our finding that PilN interacts with PilO not only through its N-terminal cytoplasmic segment but also along its whole length.
Thus, absence of functional complementation between T18 and T25 with the PilN variants and PilO should be interpreted as problem with protein stability or production. The results showed that all PilN variants were able to interact with PilO in the T18-PilO/T25-PilN and T18-PilN/T25-PilO combinations, which rules out that the absence of interaction of PilN_{N8A} and PilN_{L9A} with PilM might be because of lack of production or major instability. However, the β-galactosidase activities generated by the PilN_{N8A} variant were reduced when compared to those of PilN_{WT}, generating 456 ± 29 U/mg versus 1212 ± 468 U/mg in the T18-PilO/T25-PilN and 439 ± 36 U/mg versus 1049 ± 114 U/mg in the T18-PilN/T25-PilO combinations (Figure 4.8).

**Figure 4.7 Binary protein-protein interactions between the SDM-PilN mutants, and PilM and PilO.**

Three PilN variants PilN_{N8A}, PilN_{L9A} and PilN_{P11A} were fused to the C-terminus of T18 and T25 fragments. The plasmid combinations indicated were co-transformed in the strain *E. coli* cya BTH101 and plated on MacConkey agar supplemented with maltose. Functional complementation between the T18 and T25 fragments, which occurs upon interaction of the hybrid proteins triggers the expression of *mal* genes and yields pink to purple colonies (Karimova *et al.*, 1998).+ , pairs that yielded coloured colonies. -, pairs that yielded white colonies.
Figure 4.8 Quantification of interactions between the SDM-PilN mutants, and PilM and PilO.

Three PilN variants PilN_{N8A}, PilN_{L9A} and PilN_{P11A} were fused to the C-terminus of T18 and T25 fragments. The efficiency of functional complementation between these hybrid proteins and PilM and PilO was quantified by measuring the β-galactosidase activities and compared to the strength of the interaction with the WT proteins. The results are expressed as units of β-galactosidase activity per milligram of bacteria (dry weight) and are the mean ± standard deviation of at least three different independent experiments. The red line indicates the background β-galactosidase activity measured in the negative control.
This decreased activity of PilN_{N8A} with PilO could be interpreted as a general instability of the PilN protein or as a role of these residues in the interaction with PilO. Therefore, to further investigate this, we tested by Western blotting using the anti-T18 antibody the stability of all variants in the T18-PilN/T25-PilM and T18-PilN/T25-PilO combinations. Whole-cell protein samples were prepared from *E. coli* BTH101 transformants grown overnight in liquid LB medium supplemented with appropriate antibiotics and 0.5 mM IPTG, and equal amounts of the samples were loaded in each well of the SDS-PAGE gels. As can be seen in Figure 4.9, in the T18-PilN/T25-PilM combination, while the level of PilN_{P11A} was the same as the PilN_{WT}, the levels of PilN_{N8A} and PilN_{L9A} were reduced which was expected because of lack of positive feedback loop driven by cAMP upon protein-protein interaction to allow full hybrid protein expression (see section 4.3). In the T18-PilN/T25-PilO combination the same amount of PilN was detected in every variant (Figure 4.9) and confirms that the weaker interaction of PilN_{N8A} with PilO is not due to a general instability of the PilN protein, but possibly the residue could be involved to some extent in the PilN-PilO interaction.

![Western blot showing PilN variants](image)

**Figure 4.9 Detection of T18-hybrid proteins to confirm stability of PilN_{N8A}, PilN_{L9A} and PilN_{P11A}.

Western blotting using anti-T18 antibody on whole cell protein extracts from BTH101 cells transformed with the different T18/T25 plasmid combinations. Equal volumes of whole-cell protein extracts were loaded in each lane.
Taken together, these results show that the N8 and L9 residues within the INLLP motif are important for the interaction with PilM, while N8 has a minor role in the PilN-PilO interaction.

### 4.4.2 Assessing piliation of *N. meningitidis pilN* mutant strains

Based on our finding that the PilN conserved cytoplasmic motif INLLP is involved in the PilM-PilN interaction, it was hypothesized that mutations within it in *N. meningitidis* will interfere with piliation. Therefore, to investigate the functional significance of this sequence and confirm our assumption, we assessed whether these PilN variants were able to restore piliation in a *N. meningitidis pilN* mutant.

To do this, we cloned the three different *pilN* alleles constructed by site-directed mutagenesis, into pGCC4 plasmid under the control of an IPTG-inducible *lacP* promoter within a gonococcal region conserved in *N. meningitidis*. Subsequently, expression and hence stability of each PilN variant from the pGCC4-based derivatives was confirmed in *E. coli*. Small volume liquid cultures of *E. coli* DH5α transformants were induced with 0.5 mM IPTG for 3 hours and whole-cell protein samples produced prior to and after IPTG induction were separated by SDS-PAGE and gels were then stained with Bio-Safe Coomassie (Figure 4.10A). Upon confirmation that the proteins are expressed, the induced whole cell protein samples were subjected to Western blotting using the anti-PilN antibody to ensure that all PilN variants are stable by detecting them at similar levels (Figure 4.10B).
A. Coomassie

![Coomassie stained gel showing protein bands](image)

B. Anti-PiIN Western blot

![Western blot showing PiIN bands](image)

**Figure 4.10 Detection of pGCC4-based PiIN\textsubscript{NBA}, PiIN\textsubscript{L9A} and PiIN\textsubscript{P11A} proteins in *E.coli*.**

Whole cell protein extracts from *E. coli* DH5\(\alpha\) cells transformed with pGCC4 pilIN\textsubscript{NBA}, pGCC4 pilIN\textsubscript{L9A} and pGCC4 pilIN\textsubscript{P11A} plasmids and induced with 0.5 mM IPTG, were separated by SDS-PAGE. *E. coli* transformed with pGCC4 pilIN\textsubscript{WT} (pYU26) was included as control. (A) Coomassie staining. (B) Western blotting using anti-PiIN antibody.
Next, the resulting pGCC4 pilN\textsubscript{N8A}, pGCC4 pilN\textsubscript{L9A} and pGCC4 pilN\textsubscript{P11A} plasmids were used to transform \textit{N. meningitidis} WT strain 8013, in which the \textit{pilN} variants integrated ectopically in the genome by homologous recombination. The endogenous \textit{pilN} gene was then interrupted with genomic DNA extracted from a \textit{ΔpilN} non-polar mutant, constructed by splicing PCR as described in Figure 4.13 (de Berardinis \textit{et al.}, 2008). The stability of each PilN variants expressed in \textit{N. meningitidis} strains \textit{ΔpilN/pilN\textsubscript{N8A}}, \textit{ΔpilN/pilN\textsubscript{L9A}} and \textit{ΔpilN/pilN\textsubscript{P11A}} was tested by Western blotting using the anti-PilN antibody (Figure 4.11). This showed similar levels of PilN variant detected in all strains, compared to the PilN levels of the WT and the \textit{ΔpilN/pilN\textsubscript{WT}} complemented strains, confirming that they are all stable in \textit{N. meningitidis}.

![Western blotting](image)

\textbf{Figure 4.11 Detection of PilN\textsubscript{N8A}, PilN\textsubscript{L9A} and PilN\textsubscript{P11A} proteins in \textit{N. meningitidis}.}

In \textit{N. meningitidis} \textit{ΔpilN/pilN\textsubscript{N8A}}, \textit{ΔpilN/pilN\textsubscript{L9A}} and \textit{ΔpilN/pilN\textsubscript{P11A}} strains, the mutant \textit{pilN} alleles generated by site-directed mutagenesis were placed under the control of an IPTG-inducible promoter and were integrated ectopically in the genome of a \textit{ΔpilN} non-polar mutant. Whole cell protein extracts from \textit{N. meningitidis} \textit{ΔpilN/pilN\textsubscript{N8A}}, \textit{ΔpilN/pilN\textsubscript{L9A}} and \textit{ΔpilN/pilN\textsubscript{P11A}} strains were subjected to Western blotting using anti-PilN antibody. The WT strain, \textit{ΔpilN} mutant and \textit{ΔpilN/pilN\textsubscript{WT}} complemented mutant were included as controls. For each gel, equal volumes of whole-cell protein extracts were loaded in each lane.
Piliation of the *N. meningitidis* ΔpilN/pilN_{N8A}, ΔpilN/pilN_{L9A} and ΔpilN/pilN_{P11A} strains was assessed in the presence of IPTG by IF microscopy using the anti-Tfp 20D9 antibody that is specific for the 8013 strain’s Tfp (Pujol *et al.*, 1997). As controls, we included the WT strain, ΔpilN mutant and ΔpilN/pilN_{WT} complemented mutant. As shown in Figure 4.12, no Tfp could be detected in the ΔpilN/pilN_{N8A} and ΔpilN/pilN_{L9A} strains, indicating that the PilN_{N8A} and PilN_{L9A} are unable to promote Tfp biogenesis. However, in the ΔpilN/pilN_{P11A} strain piliation was restored, as equal amounts of Tfp could be detected as in the ΔpilN/pilN_{WT} complemented mutant, which indicates that PilN_{P11A} is functional with respect to Tfp biogenesis. Therefore, these results further reinforce the BACTH results, that the N8 and L9 residues within the INLLPY motif are essential for the PilM-PilN interaction.

To conclude, taken together these data confirm that the highly conserved N-terminal motif is crucial for the protein’s function in the assembly process of Tfp by mediating the interaction with PilM.
Figure 4.12 Piliation as assessed by immunofluorescence microscopy in *N. meningitidis* ΔpilN/pilN₈₄₈, ΔpilN/pilN₉₉₉ and ΔpilN/pilN₁₁₁ strains.

Tfp (green) were detected with a monoclonal mouse antibody 20D9, which is specific for *N. meningitidis* 8013 filaments (Pujol *et al.*, 1997). Bacterial (red) were stained with DAPI. The WT strain, ΔpilN mutant and ΔpilN/pilNWT complemented mutant were included as controls. Scale bars represent 10 µm.
4.5 Identification of a complex between PilP, PilM, PilN and PilO by stability assays

Up to this point our study of Tfp assembly proteins was mainly focused on the three proteins PilM, PilN and PilO, because as mentioned previously the BATCH system did not allow testing of PilP which is a lipoprotein that does not possess a cytoplasmic domain. Therefore, to further improve our understanding of the assembly machinery, it was necessary to employ alternative methods to confirm the existence of the PilM-PilN-PilO subcomplex but also to investigate PilP’s involvement in the complex.

Previously studies in EPEC and P. aeruginosa identified interactions between proteins involved in the biogenesis of Tfpb and PilM-PilN-PilO-PilP proteins respectively, by measuring changes in the stability of one protein in the absence of another one (Ramer et al., 2002, Ayers et al., 2009). The logic of this assay lies in the fact that often protein-protein interactions contribute to the stability of each partner within a protein complex, thus when a specific member of the complex is missing, the other proteins with which it physically interacts are often less stable. Therefore, we decided to use a battery of specific antibodies available to determine if deletion of one of the four Pil proteins (PilM, PilN, PilO and PilP) has a negative impact on the stability of the remaining three.

To achieve this, we first constructed N. meningitidis non-polar deletion mutants by splicing PCR method adapted from a large mutagenesis study in Acinetobacter baylyi ADP1, described in Figure 4.13 (de Berardinis et al., 2008). This was performed in several PCR steps in which the coding region of the mutagenized genes was replaced from the start codon to approximately 30 bp before the stop codon, in order to maintain the ribosomal binding sites used by downstream genes, with a kanamycin resistance cassette. The spliced PCR fragment was then used to transform N. meningitidis and transformants, in which the WT gene was replaced by allelic exchange, were selected on plates containing kanamycin. As controls, complemented strains were constructed as well, in which a WT copy of the
corresponding genes was expressed ectopically under the transcriptional control of an IPTG-inducible promoter.

Figure 4.13 Construction of *N. meningitidis* non-polar Δ*pilM*, Δ*pilN*, Δ*pilO* and Δ*pilP* mutant strains by splicing PCR.

Two sets of primers (F1/R1 and F2/R2) were used to amplify approximately 500 bp fragments, upstream and downstream from each target *pil* gene, respectively. The R1 and F2 primers were designed to delete the coding region of the mutagenized genes from the start codon to approximately 30 bp before the stop codon, to preserve the ribosomal binding sites of downstream genes. Two other primers, *aphF* and *aphR*, were used to amplify a kanamycin resistance cassette. Primers, F1 and/or R2 contained the DNA uptake sequence (DUS), which is essential for DNA to be taken up by meningococcus during natural transformation. Three PCR fragments were amplified using F1/R1, F2/R2 and *aphF*/*aphR* and they were combined and spliced together using the F1 and R2 primers. The resulting spliced PCR fragment was then directly transformed into *N. meningitidis*, in which the WT genes was replaced by allelic exchange and mutants were selected on kanamycin plates.
To test PilM, PilN, PilO and PilP protein levels, Western blots were performed on equal amounts of whole-cell protein extracts of non-polar ΔpilM, ΔpilN, ΔpilO and ΔpilP mutants and also of ΔpilM/pilMWT, ΔpilN/pilNWT, ΔpilO/pilOWT and ΔpilP/pilPWT complemented mutants using rabbit anti-PilM, anti-PilN, anti-PilN and anti-PilP antibodies. As shown in Figure 4.14, PilM levels remained unaffected by the absence of PilN, PilO and PilP proteins, and also loss of PilM had no effect on their levels. In contrast, the other three proteins showed mutually stabilizing effects. PilN and PilO are strongly dependent on each other for stability as loss of either protein eliminated/dramatically reduced the level of the other. Moreover, the absence of either PilN or PilO resulted in slightly reduced levels of PilP and importantly, in the absence of PilP there was a dramatic decrease in the levels of PilN and PilO. These results were confirmed to be due to the specific absence of the protein partners and not due to polar effects because protein levels were restored to WT levels in the complemented mutants.

Overall, these results show that PilN, PilO and PilP interact and are highly significant as they confirm the existence of an assembly complex in vivo.
Figure 4.14 Determination of the stability of PilM, PilN, PilO and PilP proteins in non-polar ΔpilM, ΔpilN, ΔpilO and ΔpilP mutants.

PilM, PilN, PilO and PilP were detected by Western blotting in whole-cell protein extracts of non-polar ΔpilM, ΔpilN, ΔpilO and ΔpilP mutants and ΔpilM/pilM<sub>WT</sub>, ΔpilN/pilN<sub>WT</sub>, ΔpilO/pilO<sub>WT</sub> and ΔpilP/pilP<sub>WT</sub> complemented strains. The WT strain was included as a positive control. For each blot, equal volumes of whole-cell protein extracts were loaded in each lane.
4.6 Identification of interactions between PilP and PilM, PilN and PilO by co-immunoprecipitation studies

All our data supporting the existence of the PilM-PilN-PilO-PilP complex was based on genetic studies, so to further characterize the complex between PilM, PilN, PilO and PilP we used a biochemical approach by performing co-immunoprecipitation (co-IPs) studies. This is one of the classical, most widely used methods and it is considered the gold standard for identifying protein-protein interactions. It generally involves incubation of whole protein extracts with an antibody specific for one protein of a protein complex and subsequently the antibody-protein complex is precipitated using several types of beads, washed and the proteins bound to the antibody are eluted and analyzed by Western blotting.

To prepare the *N. meningitidis* whole protein extracts we used the B-PER bacterial extraction reagent, which is a mild nonionic detergent and allows extraction of proteins without mechanical disruption. Thus, this method was relatively easy and particularly suitable for extracting soluble proteins (PilM), but most importantly membrane proteins (PilN, PilO and PilP). It is important to mention that prior to performing any immunoprecipitations, the presence of PilM, PilN, PilO and PilO in the whole protein extracts was always confirmed by Western blots.

Though the principle of immunoprecipitating proteins from whole protein extracts is simple, we found that the experimental procedure required considerable optimization. One of the main problems was that on performing Western blots on immunoprecipitated proteins, the heavy and light chains, of size 55 kDa and 23 kDa respectively, of the immunoprecipitating antibody could also be detected together with the primary Western blot antibody. This posed a significant problem since the light chain could hinder detection signal of PilO protein, which is 23.32 kDa. Therefore, to successfully overcome this problem we tested three different kits as described below by immunoprecipitating PilN which is slightly smaller than the light chain, and thus if precipitated it should be detected.
4.6.1 TrueBlot immunoprecipitation kit

The TrueBlot immunoprecipitation kit involved incubating 100 µg of whole protein WT *N. meningitidis* extract with 5 µg of anti-PilN antibody, and subsequently the antibody-antigen complex was captured by TrueBlot anti-rabbit Ig IP bead slurry, which are agarose beads coupled with the goat anti-rabbit IgG. The antigen was eluted by incubating the beads-antibody-antigen complex at 100°C with Laemmli buffer containing DTT, which is a reducing agent (see section 2.11.1). The supernatant was then analyzed by Western blotting using rabbit anti-PilN as a primary antibody, and the rabbit IgG TrueBlot as a secondary antibody instead of the conventional secondary ECL anti-rabbit IgG HRP linked whole antibody. The reason for that is that this anti-rabbit IgG preferentially detects the non-reduced form of rabbit IgG over the reduced, SDS denatured form, and therefore interference by the heavy and light chains of the immunoprecipitating antibody should be in theory minimized. As a control, the experiment was repeated without adding the immunoprecipitating anti-PilN antibody.

As can be seen in Figure 4.15, PilN could be precipitated successfully, but the heavy and light chains were clearly visible as well, indicating that the secondary rabbit IgG TrueBlot antibody was not efficient in eliminating the signal. Also, the control reveals that the high signal corresponding to the light and heavy chains is not due to the anti-PilN antibody, as it is absent in the control, but due to the goat anti-rabbit IgG bound on the TrueBlot Ig IP beads (Figure 4.15). This means that the secondary anti-rabbit IgG TrueBlot cross-reacts with the goat anti-rabbit IgG of the beads. Thus, performing immunoprecipitations with this kit was not an option as we would have been unable to detect PilO.
Figure 4.15 Immunoprecipitation of PilN using TrueBlot kit.

B-PER protein extract (100 µg) from *N. meningitidis* WT strain was immunoprecipitated using anti-PilN antibody. As a control, immunoprecipitation was performed without using the anti-PilN antibody.

4.6.2 Dynabead Protein A

Our second attempt was using Dynabead Protein A, which are magnetic beads (Dynabead) with recombinant Protein A coupled to their surface. Protein A has the capacity to bind immunoglobulins via their Fc domains and it has a high affinity for rabbit IgG. The main advantage of this kit is that Dynabeads are handled easily using a magnet, which is used to remove the beads following the washing steps, and incubations with the immunoprecipitating antibody and the antigen. This magnetic separation technology saves time and minimizes loss of material from the centrifugation steps, thus it allows simple and efficient washing. Also, another critical advantage of this kit is that the beads are not covered by antibodies, which could contribute to the unwanted detection signal of heavy and light chains on Western blots.

This kit involved incubating first Dynabeads Protein A with 5 µg of anti-PilN antibody and subsequently the Dynabeads-antibody complex was incubated with 100 µg of *N. meningitidis* whole protein extract. The antigen was eluted from the beads by incubating at 100°C the beads with Laemmli buffer containing DTT. The supernatant was analyzed by Western blotting using the anti-PilN antibody. As a control, the
experiment was performed without adding the immunoprecipitating anti-PiLN antibody.

As observed in Figure 4.16, though PiLN did precipitate, there is a massive background above PiLN on the blot even two hours after the detection step, using the ECL Prime Western blot detection reagent. Also, the background is present in the control, which indicates that the strong band close to PiLN is not the immunoprecipitating antibody but probably contaminants from the beads. This means that using this kit it would have been impossible to detect PiLM and PiLO, which are larger proteins than PiLN.

![Image of gel with labeled bands]

**Figure 4.16 Immunoprecipitation of PiLN using Dynabead Protein A.**

B-PER protein extract (100 µg) from *N. meningitidis* WT strain was immunoprecipitated using anti-PiLN antibody. As a control, immunoprecipitation was performed without using the anti-PiLN antibody. This blot is developed two hours after the detection step because of the high background.
4.6.3 Crosslink Immunoprecipitation kit

Our last attempt was using the Crosslink immunoprecipitation kit, which unlike the other kits described above, enables covalent cross-linking of the antibodies onto the beads. This kit employed Protein A/G Plus agarose resin, which are agarose beads coupled to recombinant Protein A/G. While Protein A binds exclusively to the Fc region of the antibody heavy chains, Protein G binds to the Fc region but also in some cases it binds to the Fab region. Additionally, both proteins have different binding strengths to different species of immunoglobulins and their subclasses, however both proteins have a strong affinity for rabbit IgGs which is highly significant as all our antibodies, anti-PilM, anti-PilN, anti-PilO and anti-PilP, were generated in rabbits. The engineered recombinant Protein A/G combines four Protein A and two Protein G antibody-binding sites.

The first step in using this kit was to covalently link the Protein A/G agarose to 5 µg of the anti-PilN antibody using the water-insoluble crosslinker DSS in order to prevent elution of the immunoprecipitating antibody along with the antigen. Subsequently, 500 µg of \textit{N. meningitidis} protein extract was incubated with the antibody-crosslinked resin. The precipitated protein was finally eluted using the kit elution buffer, which is an amine-containing buffer of low pH, pH 2.5-3, that effectively dissociates most protein-protein interactions. As a control, I repeated the immunoprecipitation using whole protein extracts from \textit{N. meningitidis} non-polar \emph{ΔpilN} mutant. As shown in Figure 4.16B, PilN was successfully immunoprecipitated from the WT strain with minimal interference by the light chain of the immunoprecipitating antibody but it was not immunoprecipitated from the \emph{ΔpilN} mutant strain.

Since this immunoprecipitation kit proved to be the most successful out of all those tested, we consequently used it to perform immunoprecipitation of all four proteins PilM, PilN, PilO and PilP from WT \textit{N. meningitidis} whole protein extracts and their corresponding non-polar deletion mutants as described above. Each antibody immunoprecipitated the corresponding protein from the WT strain but not from the non-polar deletion mutant strains (Figure 4.17A-D). The quality of these blots is not
good, however they were not repeated as they showed that all proteins were immunoprecipitated successfully.

Figure 4.17 Immunoprecipitations of PilM, PilN, PilO and PilP using Crosslink immunoprecipitation kit.

Identical amounts of B-PER protein extracts (500 μg) from N. meningitidis WT strain were immunoprecipitated using anti-PilM, anti-PilN, anti-PilO and anti-PilP antibodies. As controls, immunoprecipitations were performed on ΔpilM, ΔpilN, ΔpilO and ΔpilP mutants. Also, WT and the corresponding non-polar mutant N. meningitidis extract were included in each western blot, as controls. (A) PilM immunoprecipitation. (B) PilN immunoprecipitation. (C) PilO immunoprecipitation. (D) PilP immunoprecipitation.
Subsequently, precipitated samples were subjected to Western blotting using the anti-PilP antibody to probe for the presence of PilP. As shown in Figure 4.18, PilP co-immunoprecipitates with PilM, PilN and PilO, while in the control experiments with protein extracts prepared from the non-polar deletion ΔpilM, ΔpilN, ΔpilO and ΔpilP mutants, PilP was not precipitated. This shows that PilM, PilN, PilO and PilP proteins dedicated to pilus assembly form an inner membrane assembly complex in *N. meningitidis*.

![Figure 4.18 Co-immunoprecipitations of PilP using Crosslink immunoprecipitation kit.](image)

Identical amounts of B-PER extracts (500 µg) were immunoprecipitated from *N. meningitidis* WT strain using anti-PilM, anti-PilN, anti-PilO and anti-PilP antibodies. As controls, immunoprecipitations were performed on ΔpilM, ΔpilN, ΔpilO and ΔpilP mutants. Ten microliters of all precipitates were probed for the presence of PilP by Western blotting using an anti-PilP serum. It should be noted that since the signal was much stronger in the precipitates of the WT strain obtained using anti-PilP and anti-PilN antibodies, these have been diluted prior to SDS-PAGE 100-fold and 50-fold respectively.

It is important to point out that initially our aim was to confirm the interactions between the PilM-PilN-PilO proteins identified by BACTH, using co-immunoprecipitations as performed with PilP. However, unfortunately this was not possible because unlike the anti-PilP antibody, the other antibodies are not sufficiently sensitive to detect the minute amount of proteins precipitated in the experiments. This was evident as on using the anti-PilM, anti-PilN and anti-PilO antibodies to test by Western blotting whether PilM, PilN and PilO were present in the different precipitates, they failed to produce a signal (data not shown).
Nonetheless, co-immunoprecipitation of PilP with the other proteins is the most important of all possible co-immunoprecipitations, because this is the only protein involved in the pilus assembly that we could not test by BACTH.
Chapter 5: Attempts of reconstituting a TfpA minimal system in *E. coli*
5.1 Introduction

Proteins PilD, PilE, PilF, PilM, PilO, and PilP are the only proteins of Tfp biogenesis in *N. meningitidis* predicted to be dedicated in the assembly of Tfp, based on the systematic genetic analysis performed by Carbonnelle *et al.* (2006). The functional analysis of the PilM, PilN, PilO and PilP proteins in Chapter 3 and Chapter 4 showed that these proteins exist in a complex in *N. meningitidis* and significantly the BACTH identified a strong interaction between PilO and the major pilin PilE, providing a snapshot of the assembly machinery with the main pilus component. Notably, in our published paper of the work described so far, it was also shown by Marta Castagnini that this PilM-PilN-PilO-PilP subcomplex could self-assemble in the absence of other Pil proteins in *E. coli* (Georgiadou *et al.*, 2012). This was performed by co-expressing PilM, PilN, PilO and PilP in *E. coli* and subsequently co-immunoprecipitating PilP with PilM, PilN and PilO, as I performed in section 4.6.3. Collectively, these results paved the way to the final part of the functional analysis of the assembly machinery, which was to attempt to reconstitute a minimal Tfp assembly system in a heterologous non-piliated organism, similarly to what was previously done for the Tfpb system by expressing the *bfp* operon into the non-EPEC strain BL21 (Sohel *et al.*, 1996). The existence of such a system could be highly powerful, as it would confirm that the seven proteins are sufficient for Tfp assembly and also it would provide a constructive model to further characterize the fundamental molecular mechanisms of pilus assembly.

To create a minimal Tfp assembly system we expressed the *pilE, pilD* and *pilF* genes along with the *pilMNOP* operon in the *E. coli* BL21 (DE3) strain, which is non-piliated. On over-expressing any heterologous gene in *E. coli*, one of the main causes preventing protein production is biased codon usage (Wu *et al.*, 2004). To explain, each organism has its own codon usage predisposition, thus the tRNA population reflects the codon bias of the mRNA population. As a result, when a heterologous gene is overexpressed, the tRNAs corresponding to the rare codons are depleted. This can lead to translational stalling, premature translation termination, amino acid misincorporation and translation frameshifting, which all stop heterologous protein production (Wu *et al.*, 2004). Consequently, since the task of co-expressing seven
genes in *E. coli* was already challenging, to minimize the possibility of having protein production problems attributed to the codon bias of *E. coli*, I did not use the *N. meningitidis* WT genes but instead I used genes synthesized and optimized for *E. coli* expression by GeneArt. Moreover, to co-express the optimized genes I employed two different strategies described in the following two sections. The first strategy was to clone the genes into two compatible pETDuet vectors, while the second was to create a unique synthetic pilDEFMNOP operon.

To test whether a minimal Tfpa system was successfully reconstituted in *E. coli*, piliation of the cells was assessed by IF microscopy using the anti-Tfp 20D9 antibody that is specific for *N. meningitidis* 8013 filaments (Pujol *et al.*, 1997). However, any Tfp assembled were expected to be periplasmic and hence undetectable using the conventional IF protocol. This is because the *pilQ* gene, which encodes the PilQ secretin that allows the emergence of Tfp on the bacterial surface, was not included in the experiment. To assess for the presence of intra-periplasmic Tfp, *E. coli* sphaeroplasts were prepared by subjecting *E. coli* cells to a cold osmotic shock treatment to remove the outer membrane and release the periplasmic proteins.

### 5.2 *pilD, pilE, pilF, pilM, pilN, pilO* and *pilP* co-expression using the pETDuet vectors

The *pilD, pilE, pilF, pilM, pilN, pilO* and *pilP* genes were co-expressed in *E. coli* using two different pETDuet vectors, pETDuet-1 and pCDFDuet-1. Each vector has two multiple cloning sites (MCS1 and MCS2), each of which is preceded by a T7lac promoter and a ribosomal binding site. Also, the two vectors are compatible to allow their co-existence in the bacterial cells; with different origins of replication (ColE1 in pETDuet-1 and CloDF13 in pCDFDuet-1) and antibiotic resistance markers (amp in pETDuet-1 and strep in pCDFDuet-1).

Firstly, the optimized *pilE* and *pilMNOP* genes were digested from the corresponding pMAT-T and pMK-RQ plasmids constructed by GeneArt, with *NdeI* and *XhoI* and subsequently subcloned into the MCS2 of both pETDuet-1 and pCDFDuet-1 cut with the same enzymes. It is worth mentioning that cloning of *pilMNOP* into pCDFDuet-1
was particularly difficult, as I repeated that ligation numerous times over a period of one month to manage and get a single clone.

Consequently, gene expression and hence production of proteins from all constructs was assessed in *E. coli*. The resulting plasmids were transformed into *E. coli* BL21 (DE3) strain and grown in small cultures (50 ml) to an OD$_{600}$ of 0.5-0.6, at which point a sample of each culture was taken to provide the non-induced control. Immediately after, 1 mM IPTG was added and the cultures were further grown for 3 hours before removing the induced whole-cell protein samples. Non-induced and induced samples were analyzed following SDS-PAGE separation by both Coomassie staining (not shown) and Western blotting. As shown in Figure 5.1A, following IPTG induction PilE was expressed successfully from the pETDuet *pilE* and pCDFDuet *pilE*, though it was much better from the latter. Also, there was good expression of PilM, PilN, PilO and PilP proteins from pETDuet *pilMNOP* and pCDFDuet pilMNOP (Figure 5.1B). It is worth noting that while whole-cell protein samples for the anti-PilM, anti-PilN and anti-PilP Western blots were diluted either 200-fold or 100-fold prior to SDS-PAGE, samples for the anti-PilO were diluted 5-fold because the anti-PilO antibody we have recognizes PilO weakly.
Figure 5.1 Expression of *pilE* and *pilMNOP* from pETDuet vectors, pETDuet1 and pCDFDuet-1 in *E. coli*.

(A) Western blotting using anti-PilE antibody on uninduced and IPTG-induced whole protein extracts prepared from BL21 (DE3) cells transformed with pETDuet *pilE* and pCDFDuet *pilE*. Samples were diluted 200-fold prior to SDS-PAGE.  

(B) Western blotting using anti-PilM, anti-PilN, anti-PilO and anti-PilP antibodies on uninduced and IPTG-induced whole protein extracts prepared from BL21 (DE3) cells transformed with pETDuet *pilMNOP* and pCDFDuet *pilMNOP*. Prior to SDS-PAGE, whole protein samples were diluted as follows: for the detection of PilM and PilP, samples were diluted 200-fold. For the detection of PilN from pET-Duet vector, samples were diluted 200-fold, while from pCDFDuet they were diluted 20-fold. For the detection of PilO, samples were diluted 5-fold.
Next, the optimized pilF gene was digested from its pMK-RQ derivative using BspHI and NotI and subcloned into the MCS1 of both pETDuet pilMNOP and pCDFDuet pilMNOP, digested with the same enzymes. Expression of PilF, PilM, PilN, PilO and PilP proteins from pETDuet pilF-pilMNOP and pCDFDuet pilF-pilMNOP was assessed as described above, by inducing protein expression in transformed E. coli BL21 (DE3) cells with 1 mM IPTG and analyzing non-induced and induced whole-cell protein samples using both Coomassie staining (not shown) and Western blotting. This showed good expression of all proteins in both plasmids, apart from PilO in pETDuet pilF-pilMNOP, which could not be detected (Figure 5.2). Nonetheless, it was not clear whether the absence of PilO was because it was not expressed at all or whether it was due to the weak anti-PilO antibody being incapable of detecting the low amount of PilO expressed (Figure 5.2). Also, in order to obtain a PilN signal, samples for PilN detection had to be diluted 5-fold prior to SDS-PAGE, instead of 100-fold as previously. This indicated that introduction of PilF in the plasmids caused a drastic decrease in expression of the rest of the proteins (Figure 5.2).
Figure 5.2 Expression of pilF and pilMNOP from pETDuet pilF-pilMNOP and pCDFDuet pilF-pilMNOP in E. coli.

Western blotting using anti-PilF, anti-PilM, anti-PilN, anti-PilO and anti-PilP antibodies on uninduced and IPTG-induced whole protein extracts prepared from BL21 (DE3) cells transformed with pETDuet pilF-pilMNOP and pCDFDuet pilF-pilMNOP. Prior to SDS-PAGE, whole protein samples were diluted as follows: for the detection of PilF and PilM, samples were diluted 100-fold. For the detection of PilN and PilO, samples were diluted 5-fold. For the detection of PilP, samples were diluted 200-fold.
PilD was the last protein to be cloned into the pETDuet vectors. Unlike the rest of the optimized genes, pilD could not be directly subcloned into the pETDuet vectors, because in the purchased construct it was cloned in-frame to the pilG gene and thus it lacked the 5’ restriction site. Therefore, pilD was amplified from pMK-RQ pilGD to introduce a flanking BspHI site on its 5’ end, cloned into pCR8/GW/TOPO and sequenced. A gene with the correct sequence was selected, digested with BspHI and NotI, and subcloned into the MCS1 of both pETDuet pilE and pCDFDuet pilE plasmids digested as well with the same enzymes. Expression of PilD and PilE from pETDuet pilD-pilE and pCDFDuet pilD-pilE was assessed in E. coli. As shown, in Figure 5.3, there was good PilD and PilE expression from all plasmids.

**Figure 5.3 Expression of pilD and pilE from pETDuet pilD-pilE and pCDFDuet pilD-pilE in E. coli.**

Western blotting using anti-PilD and anti-PilE antibodies on uninduced and IPTG-induced whole protein extracts prepared from BL21 (DE3) cells transformed with pETDuet pilD-pilE and pCDFDuet pilD-pilE. Prior to SDS-PAGE, whole protein samples were diluted as follows: for the detection of PilD, samples were diluted 20-fold. For the detection of PilE, samples were diluted 200-fold.
To confirm that PilD is functional and cleaves PilE in *E. coli*, IPTG-induced samples prepared from *E. coli* cells transformed with the different pETDuet *pilE* and *pilD-pilE* plasmids, were compared using Western blotting. This showed that in the absence of PilD, PilE ran slightly higher on the SDS-PAGE gel compared to PilE in the presence of PilD (Figure 5.4). This indicates that PilE (prepilin) is successfully cleaved by PilD to generate the mature slightly smaller protein that runs lower on the SDS-PAGE gel.

**Figure 5.4 Western blotting analysis of PilE.**

Detection of PilE in the presence of PilD and in the absence of PilD. Western blotting using anti-PilE antibody on IPTG-induced whole protein extracts prepared from BL21 (DE3) cells transformed with pETDuet *pilE*, pETDuet *pilD-pilE*, pCDFDuet *pilE* and pCDFDuet *pilD-pilE*. 
In total, four different constructs were produced (pETDuet pilD-pilE and pCDFDuet pilD-pilE, pETDuet pilF-pilMNOP and pCDFDuet pilF-pilMNOP) resulting in two different possible plasmid combinations: 1) pETDuet pilD-pilE/pCDFDuet pilF-pilMNOP and 2) pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP.

The next step was to test the expression of all proteins by co-transforming the *E. coli* BL21 (DE3) strain with the two plasmid pairs and inducing protein expression using 1 mM IPTG as described above. In the pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP combination there was good protein expression of all proteins (Figure 5.5). In contrast, in the pETDuet pilD-pilE/pCDFDuet pilF-pilMNOP combination, expression of PilM and PilO was lost as no signal could be detected and also PilN expression was lower compared to the expression from the other plasmid pair (Figure 5.5). It is worth mentioning that colonies formed by *E. coli* cells co-transformed with the two different plasmid combinations were significantly small and they grew extremely slowly in culture, indicating some level of toxicity. Moreover, it is interesting that while in pETDuet pilF-pilMNOP PilO could not be detected, in the pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP plasmid combination PilO was detected. In an attempt to increase protein expression, this was repeated by re-transforming *E. coli* BL21 (DE3) cells with the two different plasmid combinations and inducing protein expression at different temperatures, however the results were always consistent with the original results.

Expression of all proteins from the pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP plasmid combination was approximately as good as when the proteins were co-expressed with another protein from a single pETDuet vector (Figure 5.2 and 5.3). This conclusion is reached because the dilutions used for all the Western blots remained about the same for all proteins, and the signals detected were similar.

Consequently, based on the Western blots the best plasmid combination to attempt to reconstitute a minimal Tfpa system in *E. coli* is the pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP combination.
Figure 5.5 Expression of *pilD*, *pilE*, *pilF* and *pilMNOP* from pETDuet *pilD*-pilE/pCDFDuet pilF-pilMNOP and pCDFDuet *pilD*-pilE/pETDuet pilF-pilMNOP plasmid combinations in *E. coli*.

Western blotting using anti-PilD, anti-PilE, anti-PilF, anti-PilM, anti-PilN, anti-PilO and anti-PilP antibodies on uninduced and IPTG-induced whole protein extracts prepared from BL21 (DE3) cells transformed with both pETDuet *pilD*-pilE/pCDFDuet pilF-pilMNOP and pCDFDuet *pilD*-pilE/pETDuet pilF-pilMNOP plasmid combinations. Prior to SDS-PAGE, whole protein samples were diluted as follows: for the detection of PilD, samples were diluted 20-fold. For the detection of PilE, PilF, PilM, and PilP, samples were diluted 100-fold. For the detection of PilN and PilO, samples were diluted 5-fold.
5.3 pilD, pilE, pilF, pilM, pilN, pilO and pilP co-expression by constructing a unique synthetic operon

The second strategy involved constructing a unique synthetic pilDEFMNOP operon using the optimized genes, as illustrated in Figure 5.6. This was performed in several steps, in which each gene was first cloned individually in a pET vector and then they were inserted sequentially by subcloning in the same pET vector in alphabetical order, to finally generate the pilDEFMNOP operon.

In brief, firstly the optimized pilD, pilE, pilF and pilMNOP genes were amplified using suitable primers to introduce an NdeI restriction site at their 5’ ends and NheI and XhoI restriction sites at their 3’ ends (Figure 5.6A). Then, they were cloned individually into pCR8/GW/TOPO, verified by sequencing and subcloned into the NdeI and XhoI restriction sites of the pET-21b vector. Subsequently, each pil gene was sequentially inserted in alphabetical order into pET-21b pilD, which is the primary vector for successive ligations, to form a unique synthetic operon, as follows (Figure 5.6). The pilE gene, including its upstream ribosomal binding site, was excised from pET-21b by XbaI and XhoI and subcloned into the NheI and XhoI sites of pET-21b pilD (Figure 5.6A). The cohesive ends generated by the two different endonucleases, XbaI and NheI, are compatible, and hence could be ligated creating a new vector site, which could not be cleaved by either of the two enzymes. In the same way, pilF was introduced into pET-21b pilDE, and lastly pilMNOP into pET-21b pilDEF to generate the ultimate pET-21b pilDEFMNOP construct. Consequently, this cloning method strategically placed the entire synthetic operon downstream of a T7lac promoter and each pil gene downstream its own ribosomal binding site (Figure 5.6B).
Figure 5.6 Construction of a unique synthetic pilDEFMNOP operon.

(A) Step by step procedure to construct the unique synthetic pilDEFMNOP operon. (B) The resulting pilDEFMNOP operon. Each synthetic pil gene is placed downstream its own ribosomal binding site, while the entire operon is downstream of a T7lac promoter.
Expression of all proteins from every consecutive construct generated was assessed in *E. coli* BL21 (DE3) strain. This was performed as described in the previous section using 1 mM IPTG to induce protein expression, and non-induced and induced whole-cell protein samples were analyzed by Western blotting. As shown in Figure 5.7, 5.8 and 5.9, there was good expression of all proteins from the resulting pET-21b derivatives: pET-21b *pilD*, pET-21b *pilE*, pET-21b *pilF*, pET-21b *pilMNOP*, pET-21b *pilDE* and pET-21b *pilDEF*.

The colonies of the *E. coli* BL21 (pET-21b *pilDEFMNOP*) transformants were considerably smaller than the colonies of other transformants with the precursor constructs, and they also grew slowly in culture. This showed that upon inserting more *pil* genes in pET-21b, there was increased toxicity that impaired cell growth. In agreement with this observation, the expression of all proteins decreased gradually as they were co-expressed with an increasing number of Pil proteins. This is evident from the anti-PiIE Western blots, as when PiIE was expressed alone the whole protein extracts were diluted prior to SDS-PAGE 100-fold (Figure 5.7). However, when PiIE was expressed together with the rest of the six Pil proteins, the whole protein extracts had to be diluted 10-fold and 4-fold, as a higher dilution failed to produce a PiIE signal (Figure 5.10).

Moreover, as shown in Figure 5.10 when all seven proteins were co-expressed from the pET-21b *pilDEFMNOP*, only the expression of PiIE increased upon induction using IPTG. In contrast, expression of PiIN decreased, while the expression of PiID, PiIF, PiIM, PiIP remained unchanged and PiIO could not be detected. This indicated that there was still some basal expression of the proteins even though the pET-21b vector had a T7lac promoter, which tightly controls protein expression in the absence of IPTG. Consequently, since protein expression was toxic to the cells, prolonged incubation of the cultures in the presence of IPTG resulted in impaired cell growth and decreased protein expression. Also, for an unknown reason, attempts to use the BL21 pLysS (DE3) strain to further control the protein basal expression proved lethal. Moreover, the absence of a PiIO signal does not necessarily mean that expression of PiIO was completely lost. Instead PiIO expression probably decreased from the pET-21b *pilDEFMNOP* construct compared to its expression from the pET-21b *pilMNOP*,

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which is consistent with the above observation with PilE, and since the anti-PilO antibody is poorly sensitive it was not detectable.

It is worth noting that expression of all proteins from the pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP plasmid combination (section 5.2) was better than the expression of proteins from the pET-21b pilDEFMNOP plasmid. This is evident from the dilutions used of the Western blot whole-cell protein samples, as significantly lower dilutions were used for the samples prepared from E. coli BL21 (pET-21b pilDEFMNOP) transformants, than for the samples prepared from E. coli BL21 (pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP) (Figure 5.5 and Figure 5.10).
Western blotting using anti-PilD, anti-PilE, anti-PilF, anti-PilM, anti-PilN, anti-PilO and anti-PilP antibodies on uninduced and IPTG-induced whole protein extracts prepared from BL21 (DE3) cells transformed with pET-21b pilD, pET-21b pilE, pET-21b pilF and pET-21b pilMNOP, respectively. Prior to SDS-PAGE, whole protein samples were diluted as follows: for the detection of PilD, samples were diluted 20-fold. For the detection of PilE, PilF, PilP, samples were diluted 100-fold. For the detection of PilM and PilN, samples were diluted 50-fold. For the detection of PilO, samples were diluted 4-fold.

Figure 5.7 Expression of pilD, pilE, pilF and pilMNOP from pET-21b in E. coli.
Figure 5.8 Expression of pilD and pilE from pET-21b pilDE in E. coli.

Western blotting using anti-PilD and anti-PilE on uninduced and IPTG-induced whole protein extracts prepared from BL21 (DE3) cells transformed with pET-21b pilDE. Prior to SDS-PAGE, whole protein samples were diluted as follows: for the detection of PilD, samples were diluted 20-fold. For the detection of PilE, samples were diluted 50-fold.

Figure 5.9 Expression of pilD, pilE and pilF from pET-21b pilDEF in E. coli.

Western blotting using anti-PilD, anti-PilE and anti-PilF on uninduced and IPTG-induced whole protein extracts prepared from BL21 (DE3) cells transformed with pET-21b pilDEF. Prior to SDS-PAGE, whole protein samples were diluted as follows: for the detection of PilD, samples were diluted 20-fold. For the detection of PilE, samples were diluted 4-fold. For the detection of PilF, samples were diluted 20-fold.
Figure 5.10 Expression of pilD, pilE, pilF and pilMNOP from pET-21b pilDEFMNOP in E. coli.

Western blotting using anti-PilD, anti-PilE, anti-PilF, anti-PilM, anti-PilN, anti-PilO and anti-PilP antibodies on uninduced and IPTG-induced whole protein extracts prepared from BL21 (DE3) cells transformed with pET-21b pilDEFMNOP. Prior to SDS-PAGE, whole protein samples were diluted as follows: for the detection of PilD, samples were diluted 10-fold. For the detection of PilE, samples were diluted 10-fold and 4-fold as indicated. For the detection of PilF, samples were diluted 50-fold. For the detection of PilM and PilN, samples were diluted 50-fold and 10-fold, as indicated. For the detection of PilO, samples were diluted 4-fold. For the detection of PilP, samples were diluted 100-fold.
5.4 Assessing piliation of *E. coli* BL21 (DE3) strains co-expressing *pilD*, *pilE*, *pilF* and *pilMNOP*

Piliation of the two *E. coli* strains containing the pCDFDuet *pilD*-pilE/pETDuet *pilF*- *pilMNOP* plasmid combination and pET-21b *pilDEFMNOP* was assessed in the presence of IPTG by IF microscopy using the anti-Tfp 20D9 antibody that is specific to the *N. meningitidis* 8013 strain’s Tfp. However, as any potential Tfp assembled were expected to be trapped in the periplasm, prior to IF microscopy the *E. coli* cells were converted to sphaeroplasts by subjecting them to a cold osmotic shock treatment to release the periplasmic content and thus expose potential Tfp.

Also, the general IF protocol used for the detection of Tfp in *N. meningitidis* required optimization for use in *E. coli*. This is because in *E. coli*, the antibody 20D9 generated a high background even in the control *E. coli* BL21 cells not expressing any Pil proteins (not shown), which made it impossible to be able to detect potential intra-periplasmic Tfp, that were expected to be short. Therefore, different blocking agents (0.2% gelatine, 1% gelatine, 3% BSA and 5% milk) were tested to attempt to eliminate the non-specific antibody binding and out of all 5% milk was found to be the best. Also, to further minimize the high background, the secondary goat anti-mouse antibody conjugated with Alex Fluor 488 that binds to 20D9 was used at 1/1,000 dilution instead of 1/100 that was originally used.

Piliation of the two *E. coli* strains was tested in two different conditions; by growing the cells on plates and also in cultures. When grown on plates, sphaeroplasts were prepared from a loopful of bacteria grown overnight at 37°C in the presence of IPTG. While, cultures were grown at 37°C to an OD₆₀₀ of 0.5-0.6, at which point IPTG was added and the cultures were further grown for 3 hours, before sphaeroplasts were prepared.
5.4.1 Assessing piliation of *E. coli* co-expressing *pilD*, *pilE*, *pilF*, *pilM*, *piN*, *pilo* and *pilP* genes from the pCDFDuet *pilD*-*pilE*/pETDuet *pilF*-*pilMNOP* plasmid combination

As controls, the *E. coli* BL21 (WT) strain and *E. coli* (pCDFDuet *pilE*) strain were included. To avoid misinterpreting high level of background staining for pili, samples visualized were diluted considerably; therefore for each different *E. coli* strain two images are displayed to give a more representative outlook of how they appeared. As shown in Figure 5.11A, few very short filaments could be detected in *E. coli* expressing all *pil* genes (pCDFDuet *pilD*-*pilE*/pETDuet *pilF*-*pilMNOP*), and only when grown on plates. These filaments were accepted to be Tfp, as such structures were not observed in the control strains. Also, these could not be detected in the *E. coli* (pCDFDuet *pilD*-*pilE*/pETDuet *pilF*-*pilMNOP*) strain grown in culture, indicating that growth of bacteria on plates is the best condition for the formation of Tfp (Figure 5.11B).

Furthermore, the sphaerooplasts of *E. coli* (pCDFDuet *pilE*) and *E. coli* (pCDFDuet *pilD*-*pilE*/pETDuet *pilF*-*pilMNOP*) strains prepared from cells grown in cultures appeared burst, while the sphaero plast of the control *E. coli* BL21 strain had a normal round morphology (Figure 5.11B). This indicated that growth of cells in culture along with protein expression stressed the cells even more causing their rupture.

Also, it is interesting to note, that even though the antibody 20D9 is specific for the Tfp structure, it seems to recognize the pilin subunits as well since there was a high background in the control *E. coli* (pCDFDuet *pilE*) strain (Figure 5.11). To further investigate the capacity of 20D9 to recognize the pilin subunits, I performed a Western blot using the 20D9 antibody, on *E. coli* BL21 cells expressing PilE from the pCDFDuet *pilE* plasmid and on *E. coli* BL21 cells not expressing any proteins (not shown). However, the antibody failed to produce any signal, indicating that since it was directed against the filaments of *N. meningitidis* 8013 stain, it can only recognize PilE in its native, non-denatured form.
Figure 5.11 Piliation as assessed by immunofluorescence microscopy in *E. coli* sphaeroplasts expressing *pilD, pilE, pilF, pilM, pilN, pilO* and *pilP* genes from the pCDFDuet *pilD-pilE*/*pETDuet pilF-pilMNOP* plasmid combination.

*E. coli* sphaeroplasts were prepared by subjecting cells to a cold osmotic shock treatment. Tfp (green) were detected with a monoclonal mouse antibody 20D9, which is specific for *N. meningitidis* 8013 filaments (Pujol *et al.*, 1997). Bacteria (red) were stained with propidium iodide. The *E. coli* BL21 (WT) strain and *E. coli* (pCDFDuet *pilE*) strain were included as controls. The white arrows indicate short intra-periplasmic Tfp. Scale bars represent 10 µm. (A) *E. coli* sphaeroplasts were prepared from cells grown on plates. (B) *E. coli* sphaeroplasts were prepared from cells grown in cultures.
5.4.2 Assessing piliation of *E. coli* co-expressing *pilD, pilE, pilF, pilM, pilN, pilO* and *pilP* genes from pET-21b *pilDEFMNOP*.

As controls, the *E. coli* BL21 (WT) strain and *E. coli* (pET-21b *pilE*) strain were included. Tfp were not detected in the *E. coli* (pET-21b *pilDEFMNOP*) strain when grown either on plates or in culture (Figure 5.12). Moreover as observed in Figure 5.12, there was limited background staining of 20D9 antibody in *E. coli* cells expressing all seven *pil* genes (plasmid pET-21b *pilDEFMNOP*), which indicated that expression of PilE was low, and agrees with the anti-PilE Western blot (Figure 5.10). In contrast, there was higher background staining in the control *E. coli* BL21 (pET-21b *pilE*) strain, as expression of *pilE* from pET-21b *pilE* plasmid was good (Figure 5.7).

Moreover, the sphaeroplasts of *E. coli* (pET-21b *pilE*) and *E. coli* (pET-21b *pilDEFMNOP*) strains prepared from cells grown in cultures appeared ruptured as well, which further demonstrates that the best way to prepare sphaeroplasts expressing proteins is from bacteria grown on plates (Figure 5.12).

Based on these results the best strategy favoring the reconstitution of a minimal Tfpa system is by co-expressing *pilD, pilE, pilF, pilM, pilN, pilO* and *pilP* from the pETDuet vectors and growing the cells on plates. To conclude, though the groundwork presented here for the reconstitution of a minimal Tfpa system in *E. coli* is not compelling, it appears promising and requires significant improvements.
Figure 5.12 Piliation as assessed by immunofluorescence microscopy in *E. coli* sphaeroplasts expressing *pilD*, *pilE*, *pilF*, *pilM*, *piN*, *piO* and *pilP* genes from pET-21b *pilDEFMNOP*.

*E. coli* sphaeroplasts were prepared by subjecting cells to a cold osmotic treatment. Tfp (green) were detected with a monoclonal mouse antibody 20D9, which is specific for *N. meningitidis* 8013 filaments (Pujol *et al.*, 1997). Bacteria (red) were stained with propidium iodide. The *E. coli* BL21 (WT) strain and *E. coli* (pET-21b *pilE*) strain were included as controls. Scale bars represent 10 µm. (A) *E. coli* sphaeroplasts were prepared from cells grown on plates. (B) *E. coli* sphaeroplasts were prepared from cells grown in cultures.
Chapter 6: Discussion, advances made and future perspectives
6.1 Identification of a complex interaction network between Pil proteins

As I have discussed in the Introduction, systematic genetic studies in many organisms identified all the pil genes involved in Tfp biology and characterized their corresponding mutants. These studies significantly revealed that though Tfp are primarily composed of one protein, the major pilin, they are complex machineries requiring a large set of proteins, which are understood to exert their function within a supramolecular interaction complex. Our group performed such systematic studies in *N. meningitidis* and identified 15 Pil proteins dedicated to the biogenesis of Tfp (Carbonnelle *et al.*, 2005, Carbonnelle *et al.*, 2006). These proteins were shown to act at different stages of biogenesis, which was resolved into four distinct steps: 1) pilus assembly, 2) functional maturation, 3) counteraction of PilT-mediated pilus retraction and 4) emergence of Tfp on the surface (Carbonnelle *et al.*, 2006). Additionally, 7 Pil proteins were identified that are dispensable for Tfp biogenesis but play significant roles in Tfp biology (Carbonnelle *et al.*, 2005, Brown *et al.*, 2010).

Consequently, the next step to expand our understanding of the molecular mechanisms underlying the biogenesis and functionality of these widespread organelles was to define how these proteins interact. Prior to this work, large-scale studies of interactions between Tfp proteins have only been performed in the Tfpb-expressing organism EPEC (Ramer *et al.*, 2002, Hwang *et al.*, 2003). These showed that Bfp proteins form a macromolecular complex spanning the periplasmic space that consists of two topologically distinct subcomplexes: an outer membrane complex built on the secretin BfpB multimers, that serve as a channel for the growing Tfp, and an inner membrane complex consisting of the pilin BfpA, pilin-like proteins BfpI, BfpJ and BfpK, and the integral membrane protein BfpE. Nonetheless, these results could not be extended to Tfpa-expressing bacteria that represent the majority of bacteria that express Tfp as many proteins are not shared between the two systems.

Here, I performed for the first time a systematic analysis to identify interactions between Tfpa Pil proteins of *N. meningitidis*. To do that, I opted for BACTH because it
was previously shown by Karimova et al. (2005) to be particularly suitable for the study of membrane-localized protein machineries. The only restriction of this system was that proteins analyzed need to have at least one terminus in the cytoplasm. Thus, out of the 18 Pil proteins that could be analyzed, as predicted by a bioinformatic analysis on their sub-cellular localizations, I selected 11 for identification of their binary interactions. These include six out of the seven proteins predicted to be involved in assembly (PilD, PilE, PilF, PilM, PilN and PilO), all traffic ATPases (PilF, PilT, PilT2 and PilU), the core integral protein PilG, and the cytoplasmic protein of unclear function PilZ.

This analysis was very successful as it identified 20 different binary protein-protein interactions, many of which are novel. This represents the most complex interaction network between Pil proteins reported to date and can be resolved into two subcomplexes: 1) a cytoplasmic subcomplex consisting of the four traffic ATPases and 2) an inner membrane subcomplex almost exclusively of proteins predicted to be involved in assembly (Figure 6.1). Remarkably, out of all proteins tested, only one the prepilin peptidase PilD did not yield any interactions with other proteins. This was unexpected because given the role of PilD in processing the leader peptide of pilins, it was expected to interact with at least PilE, as shown by other studies (Nunn & Lory, 1991, Aly et al., 2013). However, this lack of interactions is probably due to the membrane topology of PilD being incompatible with the BACTH system. Bioinformatic analysis using the membrane topology server TMHMM, showed that most likely both the N- and C-terminus of PilD are on the periplasmic side of the inner membrane. Therefore, in the future if the BACTH is extended to the remaining seven pilin-like proteins (ComP, PilH, PilI, PilJ, PilK, PilV and PilX), PilD should be excluded. Nonetheless, since each studied gene is cloned into four different vectors (pKT25, pKNT25, pUT18C and pUT18), this would represent an exhaustive effort with the testing of 756 additional plasmid combinations. Following this large-scale study, I subsequently performed a functional analysis of the PilM-PilN-PilO-PilP subcomplex identified to be essential in assembly (Figure 6.1).
Therefore, I will discuss results from the interaction studies in three sections, corresponding to the two subcomplexes: the traffic ATPases, the assembly machinery and the core integral protein PilG.

Figure 6.1 Schematic representation of the interactions between the proteins of the Tfpa machinery as determined in this study.

For the sake of clarity, the proteins in this cartoon are not drawn to scale.
6.1.2 Traffic ATPases: PilF, PilT, PilT2 and PilU

Traffic ATPases are dynamic hexameric toroidal assemblies that hydrolyze ATP. PilF, PilT, PilT2 and PilU belong to the family of Type II/IV secretion system ATPases and are defined by four signature motifs: the Walker A box, the atypical Walker B box, the His box, and the Asp box (Misic et al., 2010). In Tfp biology, the energy generated by the ATPases is used to drive two antagonistic processes: pilus assembly driven by PilF and pilus retraction driven by PilT (Jakovljevic et al., 2008). The other two ATPases which are PilT paralogues, PilU and the Neisseria-specific PilT2 seem to modulate the pilus dynamics (Brown et al., 2010).

Using BACTH, I successfully identified the homotypic PilF-PilF and PilT-PilT interactions, which were expected (Satyshur et al., 2007, Misic et al., 2010). Nonetheless, these interactions importantly proved that BACTH is a highly reliable system. Also, PilT2-PilT2 interactions were identified, which suggested that this recently discovered ATPase, possibly forms hexamers as well. However, for an unknown reason for now, I did not identify PilU-PilU interactions.

Strikingly, I identified interactions between different ATPase monomers: PilF-PilT2, PilT-PilT2, PilU-PilT and PilU-PilT2. This interaction network suggests three intriguing settings on how these ATPases modulate their functions in Tfp biology. Firstly, it is possible that there is a higher-order interaction between different homohexamers that controls the dynamics of Tfp. Secondly, unlike what is currently thought there might be formation of heterohexamers. Thirdly, PilT2 may act as the hub of the interaction network, because it is the only ATPase found to interact with all the others. This is a remarkable finding as it suggests that in vivo the two antagonistic ATPases PilF and PilT share a common interacting partner along with PilU, which would mean that assembly and retraction are tightly regulated by a common mechanism.

Focusing on the last two scenarios, it is possible that pilus retraction is fine-tuned by PilT-PilT2, PilT-PilU and PilT-PilT2-PilU heterohexamers. This strengthens the original assumption made by Brown et al. (2010), that PilT and PilU are unlikely to form separate retraction motors, as upon overexpressing them they were incapable of
substituting PilT. Importantly, such an organization is consistent with the phenotypic defects in the meningococcal pilT2 and pilU mutants that were suggested to result from altered pilus dynamics. As previously discussed (section 1.2.5), the meningococcal pilT2 mutant showed increased piliation, which means that PilT2 is an antagonist of Tfp assembly/stability, and suggested that it probably participates in retraction synergistically to PilT (Brown et al., 2010). The role of PilT2 in pilus dynamics is also demonstrated in the gonococcus, as it has been shown that the speed of motility is strongly reduced in the gonococcal pilT2 mutant (Kurre et al., 2012). Moreover, PilU, which in this analysis was not found to interact with itself like the rest of the ATPases, in the meningococcus it seems to alter the dynamics of bacterial adhesion to HUVEC and the formation of microcolonies (Brown et al., 2010, Eriksson et al., 2012). Thus, it is conceivable that PilT2 provides the platform on which heterohexamers are built, and PilU exerts its actions only when associated with other ATPases. Additionally, another factor supporting the existence of heterohexamer is the highly dynamic nature of extension and retraction. Up to now, it is commonly assumed that these opposing processes are mediated by two different homohexamers switching at the base of the pilus. However, this seems hardly compatible with the extremely rapid switches between extension and retraction of Tfp. Therefore a single heterohexameric motor, of which the net composition could vary, provides a more plausible mechanism to rule both of these processes.

Another important player in this fine-tuning of pilus dynamics is the cytoplasmic protein PilZ. This protein is dispensable for piliation in the meningococcus but the phenotypic defects of the corresponding meningococcal pilZ and pilZ/T mutants suggest that PilZ is involved in counterbalancing PilT-mediated retraction (Brown et al., 2010). The identified PilF-PilZ interaction in BACTH, was previously described between the PilF and PilZ orthologues in X. campestris (Guzzo et al., 2009). Nonetheless, it is further strengthens the possibility that PilF and PilZ associate to work synergistically in antagonizing PilT.

Taken together, these results indicate that, though pilus assembly and retraction are solely mediated by PilF and PilT respectively, the pilus dynamics are fine-tuned by
multiple interactions between the four ATPases and the cytoplasmic protein PilZ. In the future, these interactions could be further investigated biochemically using co-immunoprecipitation studies to confirm they exist in vivo. Also the existence of the proposed traffic ATPase heterohexamer, provides an intriguing project for future structural research, that would solve the mystery regarding the arrangements of these ATPases.

6.1.2 Tfp assembly machinery: PilM-PilN-PilO-PilP

Based on studies in *N. meningitidis*, seven proteins are dedicated to the assembly of Tfp: the major pilin PilE, the prepilin peptidase PilD, the assembly traffic ATPase PilF and four other proteins of unclear function PilM, PilN, PilO and PilP (Carbonnelle *et al.*, 2006). My PhD project was predominantly focused on the last four proteins, which, as mentioned previously, are encoded by the *pilMNOPQ* operon and possibly form the actual machinery essential for the mechanical assembly of Tfp. PilM, PilN, PilO and PilP are extremely conserved in Tfpa-expressing bacteria which signifies their importance in Tfp biology, however they are not found in Tfpb-expressing bacteria (Pelicic, 2008). Interestingly though, a recent structural study has shown that one of them, PilM structurally resembles the cytoplasmic region of a Tfpb inner-membrane protein, BfpC (Yamagata *et al.*, 2012). This suggested that possibly a common mechanism for Tfp assembly exists in all bacteria, and different proteins perform similar functions. Therefore, any advances made on these four proteins from the study presented here and studies in other Tfpa-expressing organisms, can perhaps be extended in the future to the less widespread Tfpb system.

While optimizing the BACTH technique, a study was published in *P. aeruginosa* that provided some evidence for the existence of the PilM-PilN-PilO-PilP complex, based on stability assays (Ayers *et al.*, 2009). In this study, Western blots showed that loss of PilM resulted in reduced levels of PilN, PilO and PilP, whilst PilM levels were unaffected by the absence of the other proteins. Also, the absence of either PilN or PilO markedly decreased the level of the other, which indicated that their stabilities are strongly interdependent. Additionally, the absence of either PilN or PilO led to reduced levels of PilP, whereas loss of PilP had negligible effects on the levels of PilN.
and PilO (Ayers et al., 2009). Thus, collectively the stabilities of all proteins, apart from PilM, were negatively affected by the absence of the other proteins, indicating possible interactions between them. Significantly, the second finding of our systematic analysis using BACTH was that five out of the seven assembly proteins interact in a highly ordered fashion: PilM-PilN-PilO-PilE, which suggested that they indeed form a subcomplex at the inner membrane. Also, although now we understand the reason why we did not find the pre pilin peptidase PilD within this complex, this remains unclear for PilF that powers the pilus assembly. However, several possible scenarios can explain this apparent paradox. Firstly, an interaction of PilF with any of the rest of the assembly proteins, might be too transient to be detected by BACTH. Secondly, PilF might interact with the mature form of PilE, formed once the full-length pre pilin has been processed by PilD (in this study, the pre pilin gene has been cloned in BACTH vectors). Thirdly, PilF might require more than one Pil partner to interact with the assembly subcomplex.

Having completed the systematic interaction study using BACTH, I performed a detailed functional analysis of the assembly machinery to unravel its architecture using a combination of different approaches. Firstly, to understand how these proteins interact within the subcomplex, it was important to determine the membrane topology of PilN and PilO proteins, which were the only proteins of the complex whose topology was unclear. To do that I employed a dual pho-lac reporter system, which showed that both proteins have a small N-terminal cytoplasmic domain, one transmembrane helix and a large C-terminal periplasmic domain (Karimova et al., 2009).

In BACTH, PilM was found to interact with itself and with PilN. Since I determined that PilN is a bitopic protein with its small N-terminal domain in the cytoplasm and the bulk of the protein in the periplasm, it could be deduced that PilN interacts with PilM through its N-terminus. Subsequently, I confirmed this by showing using BACTH that a truncated version of PilN consisting mainly of the short cytoplasmic domain and the transmembrane helix, was capable of interacting with PilM as strongly as full-length PilN. Additionally, the possibility that truncation altered the membrane topology of PilN was ruled out, by examining the topology of the truncated PilN as
well using the dual pho-lac reporter system, and this was found unchanged. Moreover, just before embarking on the BATCH study, another study was published in *P. aeruginosa* that identified a highly conserved cytoplasmic motif, INLLP, in PilN and hypothesized that it would be functionally important (Sampaleanu *et al.*, 2009). Thus, it was rational to next investigate whether this PilN motif plays a role in the PilM-PilN interaction. Using PilN point mutants in this INLLP motif (PilN<sub>N8A</sub>, PilN<sub>L9A</sub> and PilN<sub>P11A</sub>), we could confirm that it is essential for the protein’s function in the assembly process, as both PilN<sub>N8A</sub> and PilN<sub>L9A</sub> abolished the PilM-PilN interaction, demonstrated using BACTH, and piliation altogether in *N. meningitidis*. Remarkably, few months after, the 3D structure of *T. thermophilus* PilM in complex with the cytoplasmic domain of PilN was solved, which is the only study so far providing detail at the atomic level of an interaction between two Tfp biogenesis proteins (Karuppiah & Derrick, 2011) (Figure 6.2). In this study, high quality crystals of PilM could only be obtained in the presence of a synthetic peptide, corresponding to this highly conserved motif (Figure 6.2) (Karuppiah & Derrick, 2011). This revealed that PilM has a structure most similar to the actin-like protein FtsA, which consists of two domains each divided into 2 subdomains (1A/C and 2A/B) (Figure 6.2A) (Karuppiah & Derrick, 2011). Also, like FtsA, PilM binds ATP, however there was no evidence of ATP hydrolysis by PilM and data showed that under physiological conditions the ATP binding site is likely to be saturated (Karuppiah & Derrick, 2011). This interaction between PilM and PilN is likely to exist in all Tfpa-expressing organisms because, aside from the high conservation of the cytoplasmic PilN motif, the PilN binding site which is a narrow channel between the 1A and 1C subdomains of PilM, was shown to be lined with some well-conserved hydrophobic residues that are capable of forming hydrogen bonds with the main chain atoms of the PilN conserved sequence (Karuppiah & Derrick, 2011) (Figure 7B-C). In support, more recently the interaction between PilM and the conserved N-terminus of PilN was also demonstrated in the *P. aeruginosa* orthologues using pull-down assays (Tammam *et al.*, 2013). Interestingly, the *T. thermophilus* PilM was found as a monomer in solution, whereas the BACTH showed that the *N. meningitidis* PilM can interact with itself (Karuppiah & Derrick, 2011). One possible explanation for this, is that though PilM can self-polymerize, in the presence of PilN it preferably forms a PilM-PilN heterodimer. In T2S system a
systematic effort has been performed to determine the structure of all of its components. Remarkably, this showed that the cytoplasmic domain of GspL is similar to PilM, while its periplasmic portion, which consists of a circular permutation of the common ferredoxin fold (βαβ), is predicted to be similar to PilN whose structure is yet to be solved (Abendroth et al., 2004a, Abendroth et al., 2009, Karuppiah & Derrick, 2011, Sampaleanu et al., 2009). Thus, this indicates that the full-length GspL protein is structurally equivalent to the PilM-PilN complex.

Figure 6.2 Structure of *T. thermophilus* PilM with a PilN peptide.

(A) Ribbon plot of PilM (magenta), overlaid with PilN peptide (blue) and ATP. (B) Sequence conservation mapped onto the PilM structure using CONSURF. Maroon indicates high, white medium, and cyan low sequence conservation respectively. (C) Details of the PilM-PilN interaction; protein-peptide hydrogen bonds and key residues are indicated. PilM is shown in magenta and PilN in blue. From Karuppiah & Derrick (2011).
As shown by BACTH, PilN also interacts with PilO that has similar bitopic topology. The PilN-PilO interaction relies mainly on the periplasmic domains of these proteins, which was determined by repeating the BACTH analysis using truncated versions of PilN and PilO and comparing the resulting strength of the interactions to the strength of the interactions between the full-length proteins. This finding confirmed the report by Sampaleanu et al. (2009), published just before we started our BACTH analysis, which showed that when co-expressed in E. coli the periplasmic domains of P. aeruginosa PilN and PilO form a stable heterodimer. However, here we show that the transmembrane domains of PilN and PilO also contribute to this interaction. In the same study by Sampaleanu et al. (2009) the structure of the periplasmic domain of PilO (PilOΔ68) was determined, and was identified to be homologous to the T2S protein GspM (Abendroth et al., 2004b). This showed that it consists of two distinct structural domains (Figure 6.3A) (Sampaleanu et al., 2009). The N-terminal domain of the periplasmic domain of PilO is composed of two distinct antiparallel α-helices, denoted CC1 and CC2 (CC, coiled-coil), which form an extended arm (Figure 6.3A). In contrast, the C-terminal domain forms a more compact core domain consisting of two αββ-subdomains, which form a circular permutation of the ferredoxin fold, similar to the ferredoxin-like fold of GspM (Sampaleanu et al., 2009) (Figure 6.3A). PilO was shown to interact with itself by BACTH and based on the structure of the PilOΔ68 dimer, this dimerization is possibly facilitated by the CC domains of the two interacting monomers ‘wrapping’ around each other (Figure 6.3B) (Sampaleanu et al., 2009). In agreement with the results we present here, the T2S system PilM-PilN and PilO homologues, GspL and GspM interact with themselves and each other (Abendroth et al., 2004a, Abendroth et al., 2009, Abendroth et al., 2004b, Sandkvist et al., 1999).
Finally, we established that PilP, which could not be analyzed using BACTH, interacts with the PilM-PilN-PilO complex. This was concluded from stability assays, which showed that the absence of one of PilN/PilO/PilP proteins results in the instability/degradation of the other, similarly to what was previously observed by Ayers et al. (2009), and also from the co-immunoprecipitations studies which showed that PilP co-immunoprecipitates with PilM, PilN and PilO. Both of these assays are important, because they show that the identified binary BACTH interactions exist in vivo and impart further support for the existence of a PilM-PilN-PilO-PilP complex. It is important to mention that although our original aim was to also test whether PilM, PilN and PilO proteins co-immunoprecipitate from the different samples, this was unfortunately not possible because apart from the anti-PilP antibody, the other antibodies are not sufficiently sensitive for the purpose of this assay. Critically, in our paper of the work described in Chapter 3 and Chapter 4, Marta Castagnini ultimately confirmed the existence of the PilM-PilN-PilO-PilP subcomplex by showing that it can self-assemble in *E. coli* in the absence of any
other Pil proteins (Georgiadou et al., 2012). Moreover, though it is obvious that PilP and PilM cannot interact due to their topologies, it is difficult to predict whether PilP interacts with PilN, PilO or the PilN-PilO heterodimer. Co-immunoprecipitations of PilP with PilN in the absence of PilO or with PilO in the absence of PilN, which would have answered this question, were pointless since the stability assays revealed that PilN and PilO are strongly dependent on each other for stability. Nonetheless, the possibility that PilP interacts with the PilN-PilO heterodimer is supported by the dramatically reduced stability of both proteins in the N. meningitidis ΔpilP mutant. Also, a report that was published just after we identified the interaction of PilP with the PilM-PilN-PilO complex supports this possibility, as it showed that when a soluble version of P. aeruginosa PilP was co-expressed in E. coli with the periplasmic domains of PilN and PilO, they formed a stable heterotrimer (Tammam et al., 2011). Moreover, this report showed that PilP is structurally homologous to the T2S protein GspC, which like PilP has also been shown to interact with the N0 domain of the outer membrane secretin GspD (Tammam et al., 2011, Korotkov et al., 2011). Highlighting further the similarities between the Tfp and T2S systems, GspC has been shown to interact with the GspM-GspL complex (Possot et al., 2000, Gerard-Vincent et al., 2002, Robert et al., 2005). Therefore, PilP is the linking component between the outer membrane complex consisting of the secretin PilQ and PilW protein and the inner membrane platform consisting of the PilF, PilM, PilN and PilO proteins.

One of the most significant results from the BACTH analysis, novel at the time it was identified, was that the main pilus constituent, PilE, interacts strongly with PilO and more weakly with PilN, which is consistent with the role of these proteins in assembly. What is intriguing, however, is that these interactions occur in the absence of PilD and hence with the prepilin. Thus, although it is widely believed that the prepilin is first cleaved by PilD and then loaded on the pilus assembly machinery, it is not difficult to imagine the reverse order of events, which would agree with our finding. Moreover, based on the observation that PilE (prepilin) interacts strongly with PilO and weakly with PilN, one possible hypothesis could be that PilO first interacts with the prepilin and localizes it to the assembly machinery to be cleaved by PilD, and subsequently PilN recognizes the mature PilE and orchestrates its
assembly into filaments. Therefore, an obvious next step of this project could be investigating the chain of events during Tfp assembly. At the moment, the interaction between the major pilin and proteins of the assembly machinery has been recently demonstrated in *P. aeruginosa* as well, using pull-down experiments (Tammam *et al.*, 2013). In this report, *P. aeruginosa* expressing hexahistidine-tagged PilQ were subjected to nickel affinity chromatography, in which PilN, PilO, PilP and the major pilin (PilA) were captured along with PilQ, indicating that they all form a complex (Tammam *et al.*, 2013). Additionally, in T2S an interaction between the corresponding PilE and PilM-PilN homologues, GspG and GspL, has been established using *in vivo* cross-linking and co-immunoprecipitation studies (Gray *et al.*, 2011). However unlike our findings, prior processing of GspG by the prepilin peptidase was essential for the GspG-GspL interaction to occur, which might be one of the divergent features between the two systems (Gray *et al.*, 2011).

Based on the many architectural similarities between the Tfp and T2S system mentioned above, it is tempting to make some predictions regarding the arrangement of the traffic ATPase PilF within the assembly machinery, as so far no interaction between PilF and the other proteins involved in assembly could be detected. In T2S system, the cytoplasmic traffic ATPase GspE interacts with the cytoplasmic domain of GspL (that is homologous to PilM), which results in the association of GspE with the cytoplasmic membrane, and also stimulates its activity by 2-fold when compared to the activity of GspE alone (Sandkvist *et al.*, 1995, Camberg *et al.*, 2007). Hence by analogy, PilF might be recruited to the inner membrane to power Tfp assembly by interacting with PilM. Interestingly, the crystal structure of *T. thermophilus* PilM showed a stretch of conserved residues on the opposite site of the PilN binding groove, which implied recognition of other Tfp proteins and could thus be possibly implicated in the binding of PilF (Karuppiah & Derrick, 2011). Consequently, similarly to GspL, the structurally homologous PilM-PilN complex might provide the link between the major pilin PilE and the ATPase PilF.

In conclusion, the results presented here confirmed that PilM, PilN, PilO and PilP proteins form an inner membrane subcomplex, PilM-PilN-PilO-PilP, which we expect to form the Tfp assembly machinery and also to transduce the energy generated by
ATP hydrolysis to pilus assembly. Also, here we present novel evidence showing that PilN and PilO proteins of the assembly machinery interact with the actual substrate, the main pilus constituent PilE, which provides a snapshot of the assembly subcomplex in action. However, further experiments are essential to further unravel the mechanism of assembly. Since here we have employed a functional approach, in which we firstly identified binary protein-protein interactions, subsequently established in vivo the existence of the PilM-PilN-PilO-PilP subcomplex, and finally attempted to reconstitute a Tfpa minimal assembly system (section 6.2), the next step would be to determine the structure of the entire complex in the presence of PilE. Such a structure of the assembly machinery would ultimately delineate how these proteins act within the complex to bind PilE, extrude it from the inner membrane and finally polymerize, in the same way the crystal structure of the usher FimD bound to the FimC-FimF-FimG-FimH, chaperone-tip fibrillum complex, unravelled how the pilin subunits are incorporated within the Type I pili (Geibel et al., 2013). Also, something more feasible but interesting and essential would be to investigate the chain of events in Tfpa assembly process, mentioned above. Since it has already been demonstrated in a cell free system that PilD and PilE work on their own without requiring any other Tfpa proteins, it would be interesting to investigate the interactions of PilE with the assembly machinery in vivo in the presence and absence of PilD. This could be performed by immunoprecipitating PilE and subsequently assessing if PilN and PilO co-immunoprecipitate with it, in both N. meningitidis WT and ΔpilD mutant strains. Also, further interaction studies are essential to identify the assembly partner of PilF. One approach would be to replace PilF with a hexahistidine-tagged version of itself, chemically cross-link the complex and subsequently affinity purify PilF, which would pull along with it the entire cross-linked complex. However, care should be taken on the position of the tag, as it could disrupt protein interactions. Moreover, since both PilF and PilM are cytoplasmic, it would likely be relatively easy to purify them and test the potential interaction using biochemical methods, such as Biacore. This would be highly informative because Biacore employs surface plasmon resonance to monitor molecular interactions in real time and provides quantitative information on specificity, kinetics, and affinity between interacting proteins.
6.1.3 The core integral protein PilG

PilG is a one of the most enigmatic proteins essential for Tfp biogenesis. Although, PilG is one of the core proteins conserved across Tfp and T2S system, which entailed that it is an essential component of Tfp biogenesis, paradoxically the systematic genetic analysis in *N. menigitidis* showed that it is dispensable for Tfp assembly and is involved in counteracting PilT-mediated retraction, since the pilG/T meningococcal mutant was piliated (Carbonnelle *et al.*, 2006, Pelicic, 2008). This finding conflicted with the general hypothesis that PilG is the platform on which Tfp biogenesis occurs. Thus, considering its role in counter-retraction and studies in Tfpb and T2S systems, which showed that PilG interacts with the ATPases, it is hinted that PilG is possibly implicated in regulating the energy generated by PilT to support pilus disassembly (Ramer *et al.*, 2002, Hwang *et al.*, 2003, Arts *et al.*, 2007a, Milgotina *et al.*, 2011).

Notably, our BACTH findings concerning PilG are consistent with the original finding that PilG is dispensable for pilus assembly. This is because PilG interacted with only three other proteins, which for example is less than the number of interactions identified for the PilT2 ATPase that is dispensable for Tfp biogenesis. Hence, this is not in favour of the role of PilG as a platform protein, which would be expected to interact with most if not all of the Pil proteins in order to construct the entire biogenesis machinery. In support of its role in pilus retraction, two of the proteins PilG was found to interact with are the traffic ATPase PilT2 that probably works synergistically with PilT, and the major pilin PilE. Also, I found that PilG interacts with PilO too, and remarkably both interaction of PilG with the assembly proteins (PilE and PilO) are very strong. Furthermore, unsurprisingly BACTH demonstrated that PilG interacts with itself, which is in accord with the structural data showing that PilG is a tetramer (Collins *et al.*, 2007).

In complete disagreement with our results, very recent work published in *P. aeruginosa* supports that PilC (homologue of PilG) is essential, whilst PilM, PilN, PilO and PilP are dispensable for pilus biogenesis (Takhar *et al.*, 2013). These results are based on the levels of extracellular PilA (homologue of PilE) obtained after shearing, which showed that the pilC/T mutant lacked surface pili while the pilM/N/O/P-pilT
double mutants had surface pili (Takhar et al., 2013). Two possibilities can justify these conflicting results on PilG function; either the Tfp assembly mechanism is different in *P. aeruginosa* and *N. meningitidis* or the problem lies in the experimental procedures performed. In my opinion, it is highly unlikely these discrepancies are due to the former possibility, because the extreme conservation of *pilG* and *pilMNOP* genes in Tfpa-expressing bacteria implies a universal mechanism in Tfp biogenesis. Thus, I believe that technical problems are responsible for the disagreement regarding the role of PilG. It is worth pointing out, that the experimental procedures employed in both species to draw conclusions on the function of PilG are based on loss of function mutations. Therefore, until direct evidence is provided by the formation of pili in a heterologous organism in the presence or absence of PilG, the function of PilG will remain speculative. Our early attempts in reconstituting such a minimal Tfpa assembly system in *E. coli* provided some evidence that PilG is dispensable in Tfp assembly, since few Tfp filaments could be observed in *E. coli* expressing the seven assembly proteins: PilD, PilE, PilF, PilM, PilN, PilO and PilP, in the absence of PilG (see section 6.2). Nevertheless, these results are not clear-cut and the experiment requires significant advances, hence it is too soon to make any conclusions on the function of PilG based on this study. At the moment, what is critical would be to confirm the results in *P. aeruginosa* using another method, such as IF microscopy, which would provide direct evidence on whether pili are formed in the *pilC/T* and *pilM/N/O/P-pilT* double mutants, by their visualization on the bacterial surface.

Apart from these conflicting results, the *P. aeruginosa* study also showed that the N-terminal domain of PilC interacts with PilB (homologue of PilF) (Takhar et al., 2013). This was performed by incubating the purified N-terminal domain of PilC (hexahistidine-tagged) with *P. aeruginosa* cell lysates, and subsequently the PilC along with bound PilB were co-purified by nickel affinity chromatography (Takhar et al., 2013). Also, similarly to the study in the Tfpb system (described in section 1.2.4.3.2) in which BfpE was overexpressed and led to phenotypes exhibited by loss of pilus retraction, in *P. aeruginosa* overexpression of the C-terminal domain of PilC reduced twitching motility, which suggested an interaction between PilC and PilT.
(Takhar et al., 2013). Thus, these two interactions of PilC-PilB and PilC-PilT, further strengthen the hypothesis that the function of PilG in counter-retraction is mediated through interactions with ATPases. Even though in BACTH, I did not identify an interaction between PilG and the assembly ATPase PilF, it is not impossible that they do interact, especially considering the existence of a heterohexameric motor.

Taken together, these results are particularly interesting, however further experiments are necessary to determine the exact role of PilG in Tfp biology. These could include co-immunoprecipitation studies to search for any other partners of PilG and determining the structure of the entire Tfp biogenesis supramolecular complex. Moreover, it is essential to investigate whether PilG is dispensable or essential for Tfp assembly in more species and hence clarify if the differences observed in P. aeruginosa are species-specific. Finally, in the future successful reconstitution of a Tfp minimal assembly system in a heterologous organism would clearly elucidate the role of PilG.

6.2 Attempts at reconstituting a Tfpa minimal assembly system in E. coli

The last step of the functional analysis of the assembly machinery was to attempt to reconstitute a minimal Tfpa assembly system in a heterologous non-piliated organism. As mentioned above, this system could be instrumental in Tfp biology, because it would unambiguously confirm that PilD, PilE, PilF, PilM, PilN, PilO and PilP are sufficient for pilus assembly and also it would provide a simple model for characterization of the underlying molecular mechanisms of pilus assembly.

The general method in creating the Tfpa minimal system involved co-expressing the pilD, pilE, pilF genes, together with the pilMNOP operon in the E. coli BI21 (DE3) strain, which is non-piliated, and then assessing piliation of the cells by IF microscopy. To avoid protein production problems caused by biased codon usage, the seven genes were synthesized and optimized for E. coli expression by GeneArt. In order to co-express the seven optimized genes, I employed two different strategies. In the first strategy, genes were cloned into two compatible pETDuet
vectors, pETDuet-1 and pCDFDuet-1, to produce four different constructs (pETDuet pilD-pilE, pCDFDuet pilD-pilE, pETDuet pilF-pilMNOP and pCDFDuet pilF-pilMNOP), resulting in two different plasmid combinations: 1) pETDuet pilD-pilE/pCDFDuet pilF-pilMNOP and 2) pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP. In the second strategy, a unique synthetic pilDEFMNOP operon was synthesized by inserting sequentially pil genes into pET-21b, in alphabetical order.

The expression of the seven proteins from the different constructs was next tested by Western blotting. I could confirm that expression of proteins from the pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP combination was good. In contrast, expression of most proteins from the pETDuet pilD-pilE/pCDFDuet pilF-pilMNOP combination was dramatically low. However, this was not surprising as constructing pCDFDuet pilMNOP was problematic all along. Thus, I continued working with the pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP plasmid combination. Also, I confirmed that all proteins could be expressed from the pET-21b pilDEFMNOP construct. However, it was evident that expression of all Pil proteins decreased progressively as they were co-expressed with an increasing number of proteins from the operon. Out of the two strategies, proteins were co-expressed best from the pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP plasmid combination than from the pET-21b pilDEFMNOP. Nonetheless, the colonies of both strains, E. coli (pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP) and E. coli (pET-21b pilDEFMNOP), were considerably smaller than WT untransformed cells, and they grew slowly in culture indicating some level of protein toxicity.

I assessed piliation of the two E. coli BL21 strains, containing the pCDFDuet pilD-pilE/pETDuet pilF-pilMNOP plasmid combination and pET-21b pilDEFMNOP, using IF microscopy, in two different conditions; by growing the cells on plates and in cultures. However, prior to IF microscopy E. coli cells were converted to sphaeroplasts by subjecting them to a cold osmotic shock treatment, to release their periplasmic content. This was essential because the pilQ gene was not included in the experiment to allow the surface exposure of possible fibres, thus any potential Tfp assembled were expected to be trapped in the periplasm. Additionally, the customary IF protocol used for detection of Tfp in N. meningitidis required significant
optimization for use in *E. coli*, because the anti-Tfp 20D9 antibody generated a really high background, even in *E. coli* cells not expressing any Pil proteins. On testing many different blocking agents, I found that out of all 5% milk was better than the rest. Additionally, the secondary goat anti-mouse antibody conjugated with Alex Fluor 488 was used at a much lower dilution than previously, to minimize photobleaching and the high background.

Remarkably, I could detect few very short filaments only in *E. coli* (pCDFDuet *pilD*-pilE/pETDuet *pilF*-pilMNOP) strain grown on plates. These structures were perceived to be Tfp because I did not observe anything similar in the control strains: *E. coli* BL21 (WT) and *E. coli* (pCDFDuet *pilE*). Importantly, the IF pictures clearly reflected the expression of the proteins in the two different strains. The sphaeroplasts of the *E. coli* (pET-21b pilDEFMNOP) strain, in which expression of all proteins was significantly lower than the *E. coli* (pCDFDuet *pilD*-pilE/pETDuet *pilF*-pilMNOP) strain, resembled the sphaeroplasts of the control WT strain. Therefore, it is probable that the reason this strain failed to express Tfp is because the low expression of PilE was limiting the assembly of Tfp. Likewise, in the *E. coli* (pCDFDuet *pilD*-pilE/pETDuet *pilF*-pilMNOP) strain, it is possible that only few pili could be detected because the high background caused by the good expression of PilE, obscured the rest of the pili from being detected. Also, it is imaginable that another reason behind this low abundance of pili is because they are too unstable and require the presence of the pilin-like proteins PilH, PilI, PilJ and PilK for their stability. As a result, in the absence of these proteins, pili might be prone to dissociation during the osmotic shock procedure. Additionally, the IF pictures revealed that growth of cells expressing all Pil proteins in cultures stressed them further which resulted in their rupture. Consequently, the best strategy supporting the reconstitution of a minimal Tfpa system in *E. coli* is by co-expressing *pilD, pilE, pilF* and *pilMNOP* from the pETDuet vector and growing the cells on plates.

Taken together, the preliminary results presented here for the reconstitution of a minimal Tfpa system in *E. coli* are not persuasive, however they represent a promising groundwork for significant improvements. The osmotic shock procedure could be optimized to release the periplasmic content and hence expose Tfp in a
more gentle way, with anticipation to minimize the high background caused by the concurrent release of the membrane/Tfp-bound PilE subunits. Furthermore, a better microscopy technique might be essential to visualize the short Tfp, such as immunogold transmission electron microscopy (TEM) that is significantly more resolutive. Also, non-shocked *E. coli* expressing the seven proteins could be examined using TEM for the presence of bulges, similar to the membranous protrusion containing coiled Tfp that were observed in the *N. gonorrhoeae pilQ/T* mutants (Wolfgang *et al.*, 2000). While this approach does not aim for the visualization of Tfp extending from the bacterial surface, it might be less problematic because it would omit the conversion of cells into sphaeroplasts, which possibly leads to the disruption of the fibres. Additionally, one possible attempt to improve protein expression from the synthetic operon would be to alter the order of the *pil* genes within the operon. Such a relationship between operon organization and gene expression has been demonstrated by Lim *et al.* (2011) using synthetic operons in *E. coli*, which showed that the expression of a given gene increases with the length of the operon and as its position moves farther from the end of the operon (Lim *et al.*, 2011). Even though, we observed an inverse relationship between the expression of a given gene and the length of the operon, which is opposite to what was shown in the above paper, it is worth examining if placing *pilE* at the end of the operon increases its expression to optimal levels that could allow the assembly of Tfp. Finally, different plasmid/strain combinations can be tested to obtain the best balance of PilE expression; that is high enough to allow pilus assembly, but low enough to minimize background staining. A candidate strain to control protein expression would be the Tuner (DE3) in which expression of proteins is tightly regulated by the IPTG-concentration. Therefore, protein expression from the synthetic operon could be increased using higher levels of IPTG, whereas protein expression from the pETDuet vectors could be slightly decreased to minimize the PilE background by using lower levels of IPTG.
6.3 Final conclusions and perspectives

Here we present a systematic study using the *N. meningitidis* Pil proteins, which identified the most complex interaction network between Pil proteins of Tfpa machinery to date. This unveiled many novel interactions and provided an outline of the architecture of the Pil multiprotein complex spanning the inner membrane. This subsequently allowed an in depth functional characterization of the assembly machinery, in which we proved that PilM, PilN, PilO and PilP, form an inner-membrane subcomplex and also demonstrated an interaction of this assembly machinery with its substrate PilE. Furthermore, we determined the topology of two proteins PilN and PilO, mapped the interaction domains between PilE, PilM, PilN and PilO, and showed that a conserved N-terminal motif in PilN is critical for the protein’s function in the pilus assembly process by mediating the PilM-PilN interaction. Thus, these results provide a more detailed picture of the Tfpa assembly machinery and additionally reinforce the original idea that Tfpa and the evolutionary related T2S are assembled by a related mechanism.

Now, this work on the assembly machinery paves the way to further studies that would elucidate the order of events in pilus assembly, the stoichiometry of proteins within the assembly complex and the molecular mechanisms of how PilE is manipulated within the complex to form pili. Out of all the studies proposed above, the most informative would be to solve the structures of the following complexes PilN-PilO-PilE and PilM-PilN-PilO-PilP-PilE, to provide detail at the atomic level of these interactions. Understandably, this task would be extremely difficult, however the data obtained will be invaluable as by superimposing the solved structures of the proteins on their own (apart from PilN, that remains to be solved) would provide details on the structural changes the assembly machinery undergoes to initiate pilus assembly and subsequent elongation and extrusion from the membrane. Hence, this approach could determine the function of each protein within the machinery and potentially lead to the proposal of a Tfpa assembly model. Moreover, these structural advances are likely to have major practical impact for the design of new drugs that could inhibit the assembly of Tfpa, which could have a broad spectrum due to the widespread nature of these filaments in pathogenic bacteria.
Regarding the association of PilF with the assembly machinery that remains to be elucidated, additional interaction studies are necessary. Furthermore, studies in other Tfpa-expressing species should be performed to determine the function and interaction partners of PilG that is the centre of much dispute (Carbonnelle et al., 2006, Takhar et al., 2013).

To provide a complete picture of the Tfp machinery the remaining pilin-like proteins PilH, PilI, PilJ, PilK, ComP, PilV and PilX, could also be tested using BACTH. This would provide insights into the mechanism of their incorporation into the fibers, and it would be interesting to see if they do so by interacting with PilN and PilO, similarly to PilE.

Significantly, in the future successful reconstitution of a minimal Tfpa assembly system, which was attempted here, apart from unambiguously determining the set of proteins that are required for Tfp assembly, it will provide a useful tool for characterization of the underlying molecular mechanisms of assembly. This will allow the contribution of each protein in pilus assembly to be studied without being influenced by the presence of the other accessory Pil proteins. Also, similarly to the study performed by Cisneros et al. (2012b), each Tfp assembly protein could be replaced by its T2S homologue, to test if they are functionally interchangeable in pilus assembly. This would be considerably faster and easier to perform in E. coli, since working with N. meningitidis requires tedious work in a CL3 laboratory. Most importantly, the function of each Pil protein dispensable to Tfp assembly could be systematically studied by co-expressing them along with the assembly Pil proteins and performing phenotypic analysis of the fibres for the two functions that are retraction-independent: aggregation and adhesion. This would be particularly interesting as it provides an alternative ‘gain-of-function’ approach in characterizing these proteins, which was so far based on the phenotypes of their corresponding pil and double pil/T mutants. Moreover, it would be exciting to assess if the pilin-like proteins PilH, PilI, PilJ and PilK actually make the Tfp filaments more stable as suggested above.
Overall, this study provides a picture with unprecedented detail of the macromolecular machinery at play in Tfp biology, which improved our understanding of its composition and organization, as well as extended our appreciation for the complexity of the system.
Bibliography


Appendix
Large-scale study of the interactions between proteins involved in type IV pilus biology in *Neisseria meningitidis*: characterization of a subcomplex involved in pilus assembly

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Summary

The functionally versatile type IV pili (Tfp) are one of the most widespread virulence factors in bacteria. However, despite generating much research interest for decades, the molecular mechanisms underpinning the various aspects of Tfp biology remain poorly understood, mainly because of the complexity of the system. In the human pathogen *Neisseria meningitidis* for example, 23 proteins are dedicated to Tfp biology, 15 of which are essential for pilus biogenesis. One of the important gaps in our knowledge concerns the topology of this multiprotein machinery. Here we have used a bacterial two-hybrid system to identify and quantify the interactions between 11 Pil proteins from *N. meningitidis*. We identified 20 different binary interactions, many of which are novel. This represents the most complex interaction network between Pil proteins reported to date and indicates, among other things, that PilE, PilM, PilN and PilO, which are involved in pilus assembly, indeed interact. We focused our efforts on this subset of proteins and used a battery of assays to determine the membrane topology of PilN and PilO, map the interaction domains between PilE, PilM, PilN and PilO, and show that a widely conserved N-terminal motif in PilN is essential for both PilM–PilN interactions and pilus assembly. Finally, we show that PilP (another protein involved in pilus assembly) forms a complex with PilM, PilN and PilO. Taken together, these findings have numerous implications for understanding Tfp biology and provide a useful blueprint for future studies.

Introduction

The hair-like filaments known as pili (or fimbriae) that extend from the surface of numerous species are arguably bacteria favourite colonization factor (Sauer *et al.*, 2000). In pathogenic species, pili mediate adhesion to host cells and the extracellular matrix, and play a central role in the establishment of infection. Therefore, pili continue to be intensively studied as they represent primary targets for the development of new therapies against bacterial pathogens that impose a heavy burden on human health and economy by infecting mankind, livestock and crops. Among the multiple types of pili that have been identified, none are as widespread as type IV pili, Tfp (Pelicic, 2008). Tfp might be present in 150 different species spanning most bacterial phyla and are the only pilus present in both Gram-negative and Gram-positive bacteria. This is likely a consequence of their functional versatility since in addition to their role in promoting attachment to a variety of biotic and abiotic surfaces, Tfp often mediate bacterial aggregation, uptake of DNA during transformation and twitching motility (Mattick, 2002). This versatility results from a remarkable capacity to retract and thereby generate mechanical force (Merz *et al.*, 2000; Maier *et al.*, 2002).

Type IV pili are morphologically similar in different species, i.e. they are thin, long and flexible filaments that often interact laterally to form bundles, and they share a number of sequence and structural characteristics (Craig *et al.*, 2004). They are predominantly polymers of one protein named pilin (PilE in *N. meningitidis* nomenclature used throughout this manuscript). Pilins, which are synthesized as preproteins, have a conserved N-terminus encompassing a leader peptide that is cleaved by a prepilin peptidase, PilD (Strom *et al.*, 1993). Although the lengths of the leader peptide and mature protein define two distinct pilus subtypes named type IVa (Tfpa) and type IVb (Tfpb), the first of which is by far the most widespread (Pelicic, 2008), all pilins have similar ‘lollipop’ structures with a globular head and a stick formed by an
extended N-terminal α-helix (Craig and Li, 2008). This hydrophobic α-helix represents the major assembly interface between subunits and is packed within the interior of the filament in a helical fashion (Craig and Li, 2008).

Intensive efforts for more than two decades, mainly in human pathogens such as enteropathogenic Escherichia coli (EPEC), Neisseria gonorrhoeae, N. meningitidis, Pseudomonas aeruginosa and Vibrio cholerae, have resulted in the identification of probably all the proteins dedicated to Tfp biology (Pelicic, 2008). However, the molecular mechanisms underlying Tfp biogenesis and most Tfp-mediated functions are still to be elucidated. This is mainly due to the complexity of the system, with between 10 and 18 proteins necessary for Tfp biogenesis in V. cholerae and P. aeruginosa respectively, and several other proteins that modulate Tfp-linked functions. For example, a systematic analysis in N. meningitidis has shown that 15 proteins are essential for Tfp biogenesis (PilC1/PilC2, PilD, PilE, PilF, PilG, PilH, PilL, PilJ, PilK, PilM, PilN, PilO, PilP, PilQ, PilW), while seven (ComP, PilT, PilT2, PilU, PilV, PilX and PilZ2) are dispensable for pilation but fine-tune Tfp-linked functions (Carbonnelle et al., 2005; Brown et al., 2010). The 15 proteins essential for Tfp biogenesis are conserved in sequence and genomic organization in bacteria expressing Tfp, even in phylogenetically distant species, which suggests that a common mechanism is involved (Pelicic, 2008). Although mutants in the corresponding pil genes are invariably non-piliated, studies in Neisseria species have demonstrated that these proteins act at different stages of pilus biogenesis (Wolfgang et al., 1998; 2000; Carbonnelle et al., 2005). In N. meningitidis, pilation could be restored in the absence of eight of the above 15 proteins when pilus retraction is abolished by a concurrent mutation in pilT that encodes the traffic ATPase powering disassembly of pilins from Tfp (Carbonnelle et al., 2006). Therefore, eight Pil proteins are dispensable for pilus assembly per se, indicating that pilus assembly is simpler than expected and may require ‘only’ PilD, PilE, PilF, PilI, PilM, PilN, PilO and PilP.

The exact function of an overwhelming majority of the Pil proteins is still to be determined. The elucidation of the structure of some of them, e.g. PilE (Parge et al., 1995), has improved our understanding of several aspects of Tfp biology. However, it is widely accepted that most of these proteins exert their action within a large multiprotein complex. Therefore, further advances in our understanding of Tfp biology necessitate the characterization of this machinery by identifying the underlying protein–protein interactions. Systematic studies to unravel these interactions have been conducted in EPEC that express Tfpb known as bundle-forming pili (Bfp). This has been carried out: (i) by determining stability of every Bfp protein by immunoblotting in mutants harbouring in-frame deletions in each bfp gene (the rationale being that the absence of one Bfp protein might result in instability/degradation of interacting partners) (Ramer et al., 2002) and (ii) by chemical cross-linking and affinity purification of a large protein complex and identification of all the interacting partners by immunoblotting (Hwang et al., 2003). Unfortunately, due to the important differences between the two Tfp subtypes (Pelicic, 2008), these results cannot be easily extrapolated to Tfpb-expressing bacteria where less is known about Pil–Pil interactions and the topography of the resulting machinery. Indeed, no similar systematic studies have been conducted in bacteria expressing Tfp where only a handful of Pil–Pil interactions have been identified by a variety of approaches including: (i) decreased stability of one protein in the absence of others, i.e. PilW–PilQ and PilM–PilN–PilO–PilP (Carbonnelle et al., 2005; Ayers et al., 2009), (ii) yeast two-hybrid, i.e. PilZ–PilF (Guzzo et al., 2009), (iii) co-purification of recombinant proteins, i.e. PilN–PilO and PilN–PilO–PilP (Sampaleanu et al., 2009; Tammam et al., 2011) and (iv) co-crystallization, i.e. PilM–PilN (Karuppiah and Derrick, 2011). Interestingly, some of these studies have confirmed the important similarities with the type II secretion machinery, a system that mediates the passage of folded proteins through the outer membrane in Gram-negative bacteria, which is evolutionarily related to Tfp biogenesis and is thought to function by a similar mechanism (Ayers et al., 2010).

Extending the frontiers of knowledge in Tfpb biology necessitates a better understanding of the composition and organization of this multiprotein machinery. Therefore, in the present study, we have addressed this issue first by identifying multiple interactions between 11 N. meningitidis Pil proteins using a bacterial two-hybrid system and then by performing a detailed functional analysis of a subcomplex involved in pilus assembly using a combination of approaches.

Results

Identification and quantification of protein–protein interactions between 11 N. meningitidis Pil proteins

Although two-hybrid methodology can identify protein–protein interactions on a large scale and help charting protein networks involved in virtually any biological process (Uetz and Hughes, 2000), it has not been used systematically in Tfp biology. We opted for the bacterial adenylate cyclase two-hybrid (BACTH) system in which studied proteins are coexpressed in a E. coli cya mutant as fusions with one of two fragments (T18 and T25) from the catalytic domain of Bordetella pertussis adenylate cyclase (Karimova et al., 1998). Interaction of two-hybrid proteins results in a functional complementation between T18 and T25 leading to cAMP synthesis, and transcript-
tional activation of the lactose or maltose operons that can be easily detected on agar plates. We chose this system because many of the Pil proteins are in the inner membrane and BACTH is particularly appropriate for studying interactions among membrane proteins, as demonstrated by the systematic characterization of the interaction network between proteins involved in cell division in *E. coli* (Karimova *et al.*, 2005). The only limitation of this system is that cAMP needs to be produced in the cytoplasm, precluding the analysis of proteins that have no cytoplasmic domain (e.g. proteins localized in the periplasm or outer membrane).

Of the 18 *N. meningitidis* Pil proteins that could be analysed by BACTH (the localization of PilC1/PilC2, PilP, PilQ and PilW preclude their analysis), we selected 11 (PilD, PilE, PilF, PilG, PilM, PilN, PilO, PilT, PilT2, PilU and PilZ) for a systematic identification of their binary interactions. For each protein, four different plasmids were generated by cloning the full-length corresponding gene into appropriate BACTH vectors to create fusions with the N- or C-terminus of T18 and T25. The nomenclature that was used directly reflects the nature of the engineered fusion, e.g. T18–PilD and PilID–T18 indicate that the T18 domain has been fused to the N- and C-terminus of PilID respectively. All the possible pairs of T18 and T25 plasmids, 484 in total, were co-transformed in BTH101, an *E. coli* cya mutant. Functional complementation between T18 and T25 was determined by plating transformants on selective MacConkey/maltose plates and observing the coloration of the colonies after 40–48 h of prolonged incubation, 45 (9.3%) yielded coloured colonies (Fig. 1) with coloration varying between light pink and purple. In 11/45 cases (24.4%), only a fraction of the colonies were coloured. Importantly, only one protein (PilD) yielded more than twofold higher than the activity measured for the negative control. Twenty-nine interactions were provisionally classified as strong (β-galactosidase activity > 1000 U mg⁻¹), while 14 were weaker.

In summary, we have identified 43 interactions between 10 Pil proteins using BACTH. Since some interactions were identified multiple times (e.g. the PilZ–PilF interaction has been identified with six different plasmid combinations), this analysis identified 20 different Pil–Pil interactions and outlines the most complex interaction network between Pil proteins to date. A graphical representation of the topology of this network (Fig. 2 inset) reveals interesting features. It appears that there are two subcomplexes that are linked through the PilT2–PilG interaction. The first subcomplex consists of the four traffic ATPases (PilF, PilT, PilT2 and PilU) and PilZ that specifically interacts with PilF. The possibility that traffic ATPases form heteromultimers might have important implications for Tfp biology. The second subcomplex consists almost exclusively of proteins that are thought to be involved in pilus assembly [only Pil4 acts after that step (Carbonnelle *et al.*, 2006)], which interact in a highly ordered fashion: PilM–PilN–PilO–PilE. Since little is known about the molecular mechanisms of pilus assembly, we focused our further analysis on this subcomplex.

**Determination of the membrane topology of PilN and PilO**

To better understand the topology of the submachinery involved in pilus assembly, it is necessary to know the topology of each of its components. Since the topology of PilE is known (i.e. when not part of a pilus, PilE is a bitopic inner membrane protein with its C-terminal globular head in the periplasm) and PilM is cytoplasmic, it was necessary to experimentally determine the topology of PilN and PilO. Indeed, although all bioinformatic tools we have tested agree that these proteins have one transmembrane domain and are therefore bitopic proteins in the inner membrane, they predict different topologies (data not shown). We therefore experimentally determined the membrane topology of PilN and PilO using a dual reporter pho–lac system (Karimova *et al.*, 2009). The full-length pilN and pilO genes were cloned in frame with a dual reporter encoding an *E. coli* alkaline phosphatase.
fragment (PhoA22–472) and the α-peptide of E. coli β-galactosidase (LacZ4–60). After introducing the resulting plasmids into E. coli DH5α, transformants were streaked on agar plates containing the chromogenic substrate of alkaline phosphatase, X-Phos. A periplasmic location of the reporter is revealed by high alkaline phosphatase activity and hence blue colour, whereas a cytosolic location results in no coloration. As controls directing the reporter to the periplasm or the cytoplasm, we used two previously published fusions with the E. coli YmgF polytopic protein (Karimova et al., 2009). As can be seen in Fig. 3A, both PilN–PhoLac and PilO–PhoLac exhibited a blue phenotype, indicating a periplasmic location of the reporter and hence of the C-terminus of PilN and PilO. PilN1–50–PhoLac and PilO1–50–PhoLac, in which the reporter was fused with the first 50 residues in both PilN and PilO (that encompass the predicted transmembrane segment) gave similar results (Fig. 3A). This confirms that PilN and PilO have a similar topology (Fig. 3B). Based on our results and TMHMM predictions (Krogh et al., 2001), PilN and PilO have a short N-terminal segment of 20–27 aa in the cytoplasm, one transmembrane helix and the C-terminal main part of the protein (154 of 199 aa for PilN and 174 of 215 aa for PilO) in the periplasm.

**Mapping of the interaction domains between PilE, PilM, PilN and PilO**

Next, we further examined the interactions between PilE, PilM, PilN and PilO by mapping the domains critical for protein–protein interaction using BACTH. We generated truncated versions of PilE, PilN and PilO corresponding to the first 39 to 50 residues of these proteins (PilE1–39, PilN1–50 and PilO1–50), which consist mainly of the short cytoplasmic domain and the transmembrane helix. Our rationale was that this would help determine the contribution of the C-terminal periplasmic domains of these proteins to the interactions identified above. These shorter
Quantification of Pil–Pil interaction identified by BACTH. The efficiency of functional complementation between the indicated hybrid proteins was quantified by measuring β-galactosidase activities. As a positive control, we used a strain co-transformed with pUT18C–zip and pKT25–zip, in which the T18–Zip and T25–Zip hybrid proteins interact through a leucine zipper motif (Karimova et al., 1998). As a negative control, we used a strain co-transformed with pUT18C and pKT25 plasmids containing no inserts. Results are expressed as units of β-galactosidase activity per milligram of bacteria (dry weight) and are the mean ± standard deviation of at least three independent experiments. The red line indicates the background β-galactosidase activity measured in the negative control. The inset is a graphical representation of the protein network that was identified. The thickness of the edges between nodes is proportional to the number of times that link has been identified (between one and six times).

versions were fused to T18 and T25 as above and the corresponding plasmids were then co-transformed in E. coli BTH101. Functional complementation between T18 and T25 was further quantified by measuring β-galactosidase activities in liquid culture (Fig. 4).

The first interaction we examined was PilM–PilN, which was identified in two combinations (T18–PilM/T25–PilN and T18–PilN/T25–PilM). Due to the topology of PilN (Fig. 3B) and the cytoplasmic localization of PilM, it was expected that the interaction between these two proteins would rely on the short cytoplasmic fragment of PilN. Our analysis showed that PilN1–50 interacts with PilM as well as the full-length version of this protein (Fig. 4), and this was observed in both the above combinations. Interestingly, for a reason that remains unknown, the T18–PilM/T25–PilN1–50 interaction (787 ± 112 U mg⁻¹) was even slightly stronger than the original T18–PilM/T25–PilN (450 ± 13 U mg⁻¹). These results demonstrate that PilM interacts with the N-terminus of PilN, which is the only domain of this latter protein critical for the interaction.

Next, we examined the PilN–PilO interaction, which was also identified in two combinations (T18–PilO/T25–PilN and T18–PilN/T25–PilO). Unlike T18–PilO1–50/T25–PilN, in which there was no functional complementation between T18 and T25, the T18–PilO/T25–PilN1–50 combination yielded significant β-galactosidase activity (609 ± 36 U mg⁻¹) that was approximately five times higher than the negative control (127 ± 25 U mg⁻¹) (Fig. 4). However, this activity was reduced when compared to that of the original T18–PilO/T25–PilN (1049 ± 129 U mg⁻¹). In the second combination, T18–PilN/T25–PilO, functional complementation between T18 and T25 was abolished with shorter versions of the proteins. Taken together, these results indicate that the PilN–PilO interaction relies mainly on the globular periplasmic domains of these proteins, but that the N-terminus of PilN contributes to this interaction since T25–PilN1–50 was still capable of interacting with T18–PilO.

Finally, we examined the PilO–PilE interaction, which was again identified in two combinations (T18–PilO/T25–PilE and T18–PilE/T25–PilO). In the first combina-
tion, T18–PilO/T25–PilE, no functional complementation between T18 and T25 was detected with shorter versions of the proteins. In the second combination, while no functional complementation occurred with T18–PilE/T25–PilO 1–50, the T18–PilE 1–39/T25–PilO plasmids yielded significant β-galactosidase activity (493/1100689 ± 1) (Fig. 4). However, this activity was approximately three times lower than that measured with full-length proteins T18–PilE/T25–PilO (1448/11006350 ± 1). These results indicate that the PilO–PilE interaction is mediated mainly by the globular periplasmic domains of these proteins, but that the N-terminus of PilE contributes to this interaction since T18–PilE1–39 was capable of interacting with T25–PilO.

Taken together, these results give a clear picture of the topology of the subcomplex involved in Tfp assembly. In brief, PilM interacts with the N-terminus of PilN, which interacts with PilO along the whole length of the two proteins. PilO then interacts with PilE along the whole length of the two proteins.

Assessment of the functional importance of a conserved N-terminal motif in PilN

As described previously (Sampaleanu et al., 2009; Karuppiah and Derrick, 2011), the cytoplasmic portion of PilN contains a short motif INLLPY (residues 7 to 12) that is highly conserved even in phylogenetically distant species, which suggests that it could be functionally important. Since we found that the cytoplasmic portion of PilN is critical for the interaction between PilM and PilN (see Fig. 4), we postulated that the INLLPY motif might play a role in this interaction. This was tested by constructing variants of PilN in which three invariant residues in the above motif were individually changed to alanines by site-directed mutagenesis (PilNN8A, PilNL9A and PilNP11A) and the effect on the functional complementation between T18 and T25 observed in the T18–PilM/T25–PilN and T18–PilN/T25–PilM combinations was quantified by measuring the corresponding β-galactosidase activities (Fig. 5A).

In both combinations, no functional complementation occurred with the PilNN8A and PilNL9A variants, while the PilNP11A variant was still able to interact with PilM as well as PilNWT (Fig. 5A). Importantly, the absence of functional complementation occurred with the PilNN8A and PilNL9A variants, while the PilNP11A variant was still able to interact with PilM as well as PilNWT (Fig. 5A). Importantly, the absence of functional complementation with the PilNN8A variant was not due to a lack of production and/or major instability since these variants were able to interact with PilO in the T18–PilO/T25–PilN and T18–PilN/T25–PilO combinations (Fig. 5A) and were expressed as well as PilNWT as demonstrated by immunoblotting (data not shown). It should be noted, however, that the β-galactosidase activities with the PilNN8A variant were reduced when compared to that measured with PilNWT (456 ± 29 U mg⁻¹ versus 1212 ± 468 U mg⁻¹ in the T18–PilO/T25–PilN combination, and 439 ± 36 U mg⁻¹ versus 1049 ± 114 U mg⁻¹ in the T18–PilN/T25–PilO combination), which suggests that
Mapping interacting domains between PilE, PilM, PilN and PilO by BACTH. Truncated variants of PilE, PilN and PilO fused to T18 and/or T25 fragments were constructed. The strength of each interaction was quantified by measuring β-galactosidase activities and compared to the strength of the interaction with the full-length protein (where not indicated, both proteins are full-length). Results are expressed as units of β-galactosidase activity per milligram of bacteria (dry weight) and are the mean ± standard deviation of at least three independent experiments. The red line indicates the background β-galactosidase activity measured in the negative control.

Next, we tested whether these PilN variants were functional in *N. meningitidis* by assessing whether they were able to restore piliation in a *pilN* mutant. The different *pilN* alleles constructed by site-directed mutagenesis were cloned under the control of an IPTG-inducible promoter, and they were again demonstrated by immunoblotting to be expressed as well as PilNWT (data not shown), and integrated ectopically in the genome of a non-polar ΔpilN meningococcal mutant. Piliation in the presence of IPTG was then assessed by immunofluorescence microscopy, using the 20D9 monoclonal antibody that is specific for the Tfp of strain 8013 (Pujol et al., 1997). As can be seen in Fig. 5B, piliation was restored in the ΔpilN/pilNWT strain at levels indistinguishable from those observed in the ΔpilN/pilNWT complemented mutant, which indicates that PilNWT is functional with respect to Tfp biogenesis. In contrast, the ΔpilN/pilN1A and ΔpilN/pilN9A strains are non-piliated, even though they produce the corresponding PilN variants as verified by immunoblotting (data not shown), indicating that PilN1A and PilN9A are unable to promote Tfp biogenesis.

Taken together, these data confirm that the highly conserved N-terminal motif in PilN is crucial for this protein’s function, most probably by mediating the PilM–PilN interaction within the subcomplex involved in pilus assembly.

**Further characterization of a complex between PilM, PilN, PilO and PilP**

As mentioned above, one of the proteins predicted to be involved in pilus assembly, PilP (Carbonnelle et al., 2006), could not be analysed using BACTH because it is a lipoprotein that does not possess a cytoplasmic portion (Golovanov et al., 2006). Therefore, to further improve our understanding of the composition of the pilus assembly machinery, we decided to test interactions between PilP and the PilM, PilN and PilO proteins by determining (by immunoblotting) the stability of every protein in *N. meningitidis* non-polar deletion mutants in each corresponding gene and by using a biochemical approach, i.e. by performing co-immunoprecipitations.

We first generated rabbit antisera for these four proteins and used them to confirm that PilM, PilN, PilO and PilP were detected by immunoblotting in the WT strain and not in non-polar mutants in which the respective genes were cleanly deleted (Fig. 6A). As previously carried out in EPEC or *P. aeruginosa* (Ramer et al., 2002; Ayers et al., 2009), we performed further immunoblots to determine whether deletion of one of the above four proteins had a negative impact on the stability of the remaining three, which is considered as evidence that these proteins form a complex. As shown in Fig. 6A, while PilM levels were unaffected by the absence of and PilM had no impact on the levels of PilN, PilO and PilP, the latter three proteins showed mutually stabilizing effects. PilN and PilO were strongly dependent on each other for stability and the absence of either protein resulted in slightly reduced levels of PilP. In the absence of PilP, there was a dramatic decrease of levels of both PilN and PilO. We ruled out the possibility that the above effects were due to polarity since in each case stability of each protein was restored in complemented mutants in which a WT copy of the corresponding genes was expressed ectopically under the transcriptional control of an IPTG-inducible promoter (Fig. 6A).

Since most of the above proteins are membrane proteins, we performed protein extraction using B-PER that contains a mild, non-ionic detergent. After cross-linking of
the antibodies against PilM, PilN, PilO and PilP to protein A/G agarose, identical amounts of B-PER protein extracts were subjected to immunoprecipitations. Each antibody could immunoprecipitate the corresponding protein from the WT strain but not from mutants in which the respective genes were interrupted (data not shown). Precipitated samples were then subjected to immunoblotting using the PilP antiserum. As shown in Fig. 6B, PilP co-immunoprecipitates with PilM, PilN and PilO when using the antibodies raised against these proteins. Control experiments showed that PilP was not precipitated with the same antibodies when using B-PER extracts prepared from ΔpilM, ΔpilN, ΔpilO and ΔpilP mutants (Fig. 6B). These results show that the PilM, PilN, PilO and PilP proteins involved in pilus assembly form a multimolecular subcomplex in the inner membrane of *N. meningitidis.*
Identification of interactions between PilP and PilM, PilN and PilO by determining stability of each protein by immunoblotting in non-polar \( \Delta \text{pilM} \), \( \Delta \text{pilN} \), \( \Delta \text{pilO} \) and \( \Delta \text{pilP} \) mutants and/or by performing co-immunoprecipitations.

A. PilM, PilN, PilO and PilP were detected by immunoblotting in whole-cell protein extracts of non-polar \( \Delta \text{pilM} \), \( \Delta \text{pilN} \), \( \Delta \text{pilO} \) and \( \Delta \text{pilP} \) mutants and \( \Delta \text{pilM}/\Delta \text{pilM}_{\text{WT}} \), \( \Delta \text{pilN}/\Delta \text{pilN}_{\text{WT}} \), \( \Delta \text{pilO}/\Delta \text{pilO}_{\text{WT}} \) and \( \Delta \text{pilP}/\Delta \text{pilP}_{\text{WT}} \) complemented strains. The WT strain was included as a positive control. For each blot, equal amounts of whole cell extracts were loaded in each lane.

B. Identical amounts of B-PER protein extracts (500 μg) from \( N. \) meningitidis WT strain or \( \Delta \text{pilM} \), \( \Delta \text{pilN} \), \( \Delta \text{pilO} \) and \( \Delta \text{pilP} \) non-polar mutants were immunoprecipitated using anti-PilM, anti-PilN, anti-PilO and anti-PilP antibodies. Ten microlitres of precipitates were subsequently probed for the presence of PilP by immunoblotting using an anti-PilP serum. It should be noted that since the signal was much stronger in the precipitates of the WT strain obtained using anti-PilP and anti-PilO antibodies, these have been diluted prior SDS-PAGE 100- and 50-fold respectively.

C. B-PER protein extracts from an \( E. \) coli BL21 (pACYCDest pilMNOP) strain engineered to coexpress PilM, PilN, PilO and PilP were immunoprecipitated using anti-PilM, anti-PilN, anti-PilO and anti-PilP antibodies. Ten microlitres of precipitates were subsequently probed for the presence of PilP by immunoblotting.

Discussion

Now that all the genes involved in Tfp biology have been identified and the corresponding mutants systematically characterized, the next step to better understanding of the mechanisms governing the assembly and functionality of these widespread virulence organelles is defining the way the numerous corresponding proteins interact to form what is expected to be an intricate machinery.

Large-scale studies of interactions between proteins involved in Tfp biology have only been performed in the Tfpb-expressing organism EPEC. Similar studies have also been performed for the evolutionarily related type II secretion machinery (Ayres et al., 2010). In EPEC, using comprehensive collections of in-frame deletion mutants and antibodies against the corresponding proteins, Ramer et al. found that the stability of 11 of the 12 Bfp proteins necessary for pilus biogenesis depends on the presence of at least one other Bfp protein, which was taken as (indirect) evidence that these proteins interact (Ramer et al., 2002). Together with the experimental localization of these proteins in different cellular fractions, it was inferred that two topographically distinct subcom-
plexes exist: one in the outer membrane centred on the secretin multimers that serve as a channel for the growing Tfp, and one at the inner membrane consisting of the pilin, pilin-like proteins and inner membrane proteins. Direct evidence that at least 10 of these Bfp proteins physically interact was obtained by immunoblotting after affinity purification of a chemically cross-linked oligomeric protein complex (Hwang et al., 2003). Unfortunately, owing to the extensive differences between the two Tfp subtypes (Pellicic, 2008), these results cannot be easily extrapolated to Tfpa-expressing bacteria that represent the vast majority of the bacteria that harbour Tfp. This prompted us to initiate this large-scale identification of the binary interactions (which remain for the most part uncharted in the above studies) between Tfpa Pil proteins using the human pathogen N. meningitidis as a model. We opted for BACTH because it has proven invaluable for the study of complex membrane-localized protein machineries (Karimova et al., 2005).

We decided to focus our efforts on the putative subcomplex at the inner membrane where most of the divergence between Tfpa and Tfpb systems reside (Pellicic, 2008). Of the 18 proteins having a predicted topology a priori compatible with BACTH analysis, we selected 11, including six out seven proteins (PilD, PilE, PilF, PilM, PilN and PilO) predicted to be essential for pilus assembly (Carbonnelle et al., 2006), all the traffic ATPases (PilF, PilI, PilT2 and PilU), the universally conserved inner membrane protein PilG and a cytoplasmic protein of unclear function (PilZ). Strikingly, only the prepilin peptidase PilD yielded no interactions, which is perhaps surprising given its role in processing the leader peptide of prepilins and prepilin-like proteins (Strom et al., 1993). However, a subsequent prediction of its topology by TMHMM (Krogh et al., 2001) indicates that this is most likely because both the N- and C-terminus of PilD, to which the T18 and T25 fragments have been fused, might be on the periplasmic side of the inner membrane and therefore incompatible with BACTH analysis. Therefore, if this BACTH analysis is to be extended in the future to the remaining seven pilin-like proteins (ComP, PilH, PilI, PilU, PilK, PilV and PilX), PilD should be excluded. Nevertheless, since each studied gene is cloned in four different vectors, this would still represent a very substantial effort with the testing of 756 additional combinations of T18 and T25 plasmids.

The first important finding in this study, which identified the largest interaction network between proteins involved in Tfp biology, is that multiple interactions occurred between the four traffic ATPases present in the meningococcus. Traffic ATPases, which have been extensively studied, form toroidal homohexamers that convert the energy from ATP hydrolysis into mechanical energy (Satyshur et al., 2007; Savvides, 2007), which in Tfp biology is used to power pilus assembly (PilF) or retraction (PilI). Therefore, the homotypic PilF–PilI and PilI–PilI interactions were not unexpected, and the PilI2–PilI2 interactions suggest that this recently discovered parologue of PilI (Brown et al., 2010) might form hexamers as well. The reason we did not identify PilI–PilU interactions is unclear at this time. Strikingly, we found evidence that different traffic ATPases interact with each other as evidenced by the PilI–PilI2, PilI–PilI2, PilI–PilU and PilI–PilI2 interactions. PilI2 appears to be a hub as it interacts with all the other traffic ATPases. Although a higher-order interaction between different homohexamers cannot be excluded, it is possible that heterohexamers exist (Fig. 7). Such heterohexamers could have important roles in Tfp biology. For example, it is possible that pilus retraction is fine-tuned by PilI2–PilI2, PilI–PilU and PilI–PilI2–PilU heterohexamers, which would strengthen our earlier assumption that PilI paralogues in the meningococcus are unlikely to form separate retraction motors based on the finding that when overexpressed PilI2 and PilU cannot substitute for PilI (Brown et al., 2010). Furthermore, such a possibility is consistent with the phenotypic defects in meningococcal pilI2 and pilI2 mutants that were suggested to result from altered pilus dynamics (Brown et al., 2010). Another important player in this fine-tuning of pilus dynamics might be PilZ, which is dispensable for piliation in the meningococcus but plays an important role in Tfp biology (Brown et al., 2010), that interacts strongly and specifically with PilF. These findings strengthen a previous report describing an interaction between the PilZ and PilF orthologues in Xanthonomas campestris (Guzzo et al., 2009). Heterohexamers of traffic ATPases might also provide an elegant explanation to the question of how bacteria can switch between pilus extension and retraction. Rather than two different homohexameric motors switching at the base of the pilus, which is hardly compatible with the extremely rapid switches...
between extension and retraction, there could be a single heterohexameric motor the net composition of which could vary and govern extension or retraction of the pilus.

The second important finding in this study was that five out of seven proteins that were originally predicted to play a role in pilus assembly based on genetic studies (PilD, PilE, PilF, PilM, PilN, PilO and PilP) (Carbonnelle et al., 2006) indeed form a subcomplex at the inner membrane. There was only limited evidence for this complex so far in 
P. aeruginosa
in which the absence of one of the PilM, PilN, PilO and PilP proteins was shown to have a negative impact on the stability of the others (Ayers et al., 2009). While we now understand why the prepilin peptidase PilD was not found within this complex, this is less clear for PilF which powers pilus assembly. However, several scenarios might explain this apparent incongruity: (i) interaction of PilF with the pilus assembly subcomplex might be too transient to be detected by BACTH, (ii) PilF might interact with PilE only when this protein has been processed by PilD (the full-length prepilin gene has been cloned in BACTH vectors used in this study) or (iii) more than one Pil partner might be necessary for PilF to interact with the pilus assembly subcomplex. We have further unravelled the architecture of the above subcomplex (Fig. 7) by using a combination of different approaches. We have found that PilM, an ATP-binding cytoplasmic protein (Karuppiah and Derrick, 2011), interacts with itself and the N-terminus of the bitopic PilN protein that is on the cytoplasmic side of the inner membrane. This interaction is dependent on a short sequence motif in PilN that was found to be very conserved and predicted to be functionally important (Ayers et al., 2009), which we have demonstrated here. Point mutants in this INLLPY motif abolish the PilM–PilN interaction and piliation altogether, which validates the recently reported 3D structure of Thermus thermophilus PilM (Karuppiah and Derrick, 2011). Indeed, high-quality crystals of PilM could only be obtained in this study in the presence of a synthetic peptide corresponding to the N-terminus of PilN encompassing the above motif. PilN then interacts with the other bitopic inner membrane protein PilO. This interaction relies mainly on the periplasmic domains of these two proteins, which confirms a recent report showing that when coexpressed in E. coli the periplasmic domains of P. aeruginosa PilN and PilO form a stable heterodimer (Sampaleanu et al., 2009). However, we show here that the transmembrane domains of PilN and PilO also contribute to this interaction. Finally PilP, which could not be analysed by BACTH, was found to interact with PilM–PilN–PilO by showing that the absence of one of these proteins often results in instability/degradation of the others and/or by showing that they co-immunoprecipitate. This is an important result as it shows that the above binary BACTH interactions coexist in vivo and lends further support for the existence of a PilM–PilN–PilO–PilP complex. Although it is clear based on their predicted topologies that PilP cannot interact with PilM, it is difficult to predict whether it interacts with PilN, PilO or both proteins (Fig. 7). This latter possibility is supported by the dramatically reduced stability of both PilN and PilO in a ΔpilP mutant and by a very recent report showing that when a soluble version of P. aeruginosa PilP was coexpressed in E. coli with the periplasmic domains of PilN and PilO, these proteins formed a stable heterotrimer (Tammam et al., 2011). Another significant result was that the main pilus constituent, PilE, interacts strongly with PilO (and more weakly with PilN), which provides a snapshot of the subcomplex involved in pilus assembly in the presence of its actual substrate, the pilin.

Finally, our findings concerning the universally conserved inner membrane protein PilG whose role in Tfp biology is unclear are also notable. It seems unlikely that PilG is the inner membrane scaffold on which the entire pilus biogenesis protein machinery is built, as often postulated, because it interacts only with three proteins (which is less than the number of interactions identified for the PilT2 ATPase that is dispensable for Tfp biogenesis). This is consistent with our prior finding that PilG is dispensable for pilus assembly since a pilG/T meningococcal mutant is piliated (Carbonnelle et al., 2006). However, we found that PilG interacts strongly with PilE and PilO that are essential for pilus assembly, which suggests that further studies are needed to determine its exact role in Tfp biology.

In summary, our work provides a picture with unprecedented detail of the macromolecular machinery at play in Tfp biology in a model piliated organism, N. meningitidis. Moreover, by showing that the subcomplex dedicated to pilus assembly can self-assemble in E. coli, this study paves the way for a previously unexplored research avenue consisting in the reconstitution of a minimal Tfp assembly system in this host, which could have important consequences on our understanding of the biology of these fascinating organelles.

**Experimental procedures**

**Strains and plasmids**

*Escherichia coli* DH5α was used for cloning and topology determination experiments. *E. coli* BTH101 (Euromedex), which is a non-reverting cya mutant (F−, cya-99, araD139, galE15, galK16, rpsL1, hsdR2, mcrA1, mcrB1), was used for BACTH assays. *E. coli* BL21(DE3) was used for protein expression and purification experiments. Strains were routinely grown in liquid or solid Luria–Bertani (LB) medium (Difco) containing, when required, 100 μg ml−1 spectinomycin, 100 μg ml−1 ampicillin and 50 μg ml−1 kanamycin (all from Sigma). Ultra-competent cells were prepared as described elsewhere (Inoue et al., 1990). The WT strain of *N. meningitidis* used in this study is a recently sequenced and systemati-
cally mutagenized variant of the serogroup C clinical isolate 8013 (Geoffroy et al., 2003; Rusniok et al., 2009). *N. meningitidis* was grown on GCB agar plates (Difco) containing Kellogg’s supplements and, when required, 100 μg ml⁻¹ kanamycin and 3 μg ml⁻¹ erythromycin. Plates were incubated in a moist atmosphere containing 5% CO₂.

The plasmids used for BACTH assays were constructed as follows. The full-length *pilO*, *pilE*, *pilF*, *pilG*, *pilM*, *pilN*, *pilO*, *pilT*, *pilT2*, *pilU* and *pilZ* genes were amplified from strain 8013 genomic DNA (extracted with the Wizard genomic DNA purification kit from Promega) using *Pfu*Ultra II DNA polymerase (Agilent) and suitable primers (Table 1). PCR products were cloned directly in pCR8/GW/TOPO (Invitrogen) (Table 2). All the inserts were verified by sequencing to contain no errors. Each *pil* gene was then gel-extracted (using NucleoSpin Extract II from Macherey-Nagel) after *Bam*HI and *Kpn*I digestion and subcloned into each BACTH vector (*pUT18*, *pUT18C*, *pKT25* and *pKNT25*) cut with the same enzymes (Table 2). The same two-step cloning strategy was used to produce BACTH plasmids in which truncated versions of *pilE*, *pilN* and *pilO* amplified using suitable primers (Table 1) were fused to T18 and T25 (Table 2).

pTop, which contains a dual reporter pho–lac (Karimova et al., 2009), was used to determine the topology of *pil* and *pilO*. Full-length or truncated versions of the *pil* and *pilO* genes have been gel-extracted after *Bam*HI and *Kpn*I digestion of the corresponding pCR8/GW/TOPO derivatives and subcloned into pTop cut with the same enzymes (Table 2). The *pil* genes were thus fused in frame with the dual reporter. The resulting recombinant plasmids were transformed into competent *E. coli* DH5α cells, which were plated on LB plates supplemented with 80 μg ml⁻¹ X-Phos (5-bromo-4-chloro-3-indolyl phosphate disodium salt) (Sigma), 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Merck Chemicals), 50 mM phosphate buffer (pH 7.0) and 50 μg ml⁻¹ kanamycin. The plates were incubated overnight at 37°C and the coloration was scored.

*pilN* point mutant alleles were generated using the Quick-change site-directed mutagenesis kit (Stratagene) as previously described (Helaine et al., 2007), with *pYU61* used as DNA template and a series of complementary primers (Table 1). All mutant *pilN* alleles have been verified by sequencing before they were subcloned into BACTH vectors as above. The *pilN* mutant alleles were also amplified using suitable primers flanked by *Pac*I sites and subcloned in pGCC4 (Mehr et al., 2000) restricted with the same enzyme. This generated vectors that contain the mutant alleles under the transcriptional control of an IPTG-inducible promoter within a region of the gonococcal genome conserved in *N. meningitidis*. These vectors were first transformed into strain 8013 in which genome they integrated by allelic exchange, and the endogenous *pilN* copy was then interrupted by transforming these strains with genomic DNA extracted from a *ΔpilN* non-polar mutant (see below). The resulting strains were grown on GCB agar plates supplemented with 100 μg ml⁻¹ kanamycin, 3 μg ml⁻¹ erythromycin and 0.25 mM IPTG before they were analysed for piliation as described below. *N. meningitidis* non-polar *ΔpilM*, *ΔpilN*, *ΔpilO* and *ΔpilP* mutants have been constructed by splicing PCR as described elsewhere (de Berardinis et al., 2008). In brief, two sets of primers (F1/R1 and F2/R2) were used to amplify approximately 500 bp fragments upstream and downstream from each target gene respectively. The R1 and F2 primers were designed to delete the coding region of the mutated genes from the start codon to approximately 30 bp before the stop codon in order to preserve ribosomal binding sites used by downstream genes. Primers R1 and F2 contained 23-mer overhangs that are complementary to the *aphF* and *aphR* primers used to amplify the promoterless *aphA*-3 antibiotic selection cassette from start to stop codons respectively. Primers F1 and/or R2 contained 12-mer overhangs corresponding to the DNA uptake sequence that is necessary for DNA to be taken up by the meningococcus during natural transformation. In the first step, three PCR fragments were amplified using F1/R1, F2/R2 and *aphF*/*aphR*, and the high-fidelity Herculase II Fusion DNA polymerase (Agilent). These fragments were then combined and spliced together using the same enzyme and the F1 and R2 primers. The spliced PCR fragments were then directly transformed into *N. meningitidis* and mutants were selected on GCB agar plates supplemented with 100 μg ml⁻¹ kanamycin. For each mutant, at least two colonies were isolated and further verified by PCR using the F1 and R2 primers. These mutations were then transformed into strains in which genome the WT alleles under the transcriptional control of an IPTG-inducible promoter were previously integrated by allelic exchange (Carbonnelle et al., 2006).

To produce and/or purify antibodies against PilM, PilN, PilO and PilP (see below), we constructed a series of plasmids for expressing these proteins in *E. coli*. First, two plasmids designed to produce PilM and PilP fused to a polyhistidine tag were constructed as follows. Full-length *pilM* was amplified using suitable primers (Table 1) and cloned directly in pCR8/GW/TOPO (Invitrogen). The *pilM* insert, which was verified by sequencing to contain no errors, was then gel-extracted after *Nde*I and *Bam*HI digestion and subcloned in PET-14b cut with the same enzymes (Table 2). An internal fragment of the *pilP* gene (coding for residues 17–145 of the mature protein) was also cloned using a similar two-step cloning strategy in PET-20b cut with *Eco*RI and *Xho*I. Subsequently, to increase protein yields during purification, we designed a series of plasmids to produce PilM, PilN and PilO fused to the maltose-binding protein (MBP). The full-length genes were amplified using suitable primers (Table 1), cloned in pCR8/GW/TOPO and found to contain no errors by sequencing (Table 2). They were then gel-extracted after *Eco*RI and *Sal*I (*pilM*), *Eco*RI and *Pst*I (*pilN*), or *Eco*RI and *Pst*I (*pilO*) digestions and subcloned in the pMAL-c2x vector cut with the same enzymes (Table 2).

To engineer an *E. coli* strain that coexpresses PilM, PilN, PilO and PilP, we amplified the entire locus from strain 8013 using suitable primers (Table 1), gel-extracted it after *Nde*I and *Xho*I digestion and cloned it directly in pACYCDuet-1 vector (Novagen) cut with the same enzymes (Table 2).

**Bacterial adenylate cyclase two-hybrid procedures**

Competent BTH101 cells were co-transformed with 20 ng each of two recombinant plasmids encoding fusions to T18 and T25 respectively. Two hundred microlitres of the transformed cells was plated on MacConkey agar base medium supplemented with 0.5 mM IPTG, 1% maltose solution
Table 1. Primes used in this study.

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<td>CATTATACAAATACATGCCGCCCTCCCTACAGGGGAAGG</td>
<td>Site-directed mutagenesis of pilN</td>
</tr>
<tr>
<td>dir PilNAprA</td>
<td>CATTATACAAATACATGCCGCCCTCCCTACAGGGGAAGG</td>
<td>Site-directed mutagenesis of pilN</td>
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</tbody>
</table>

a. Lower case is used for overhangs. Restriction sites are underlined. Mismatched bases generating mutations are in bold.
Table 2. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description/purpose</th>
<th>Source/reference</th>
</tr>
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<tbody>
<tr>
<td>pCRII-TOPO</td>
<td>TA cloning vector for direct ligation of PCR products</td>
<td>Invitrogen</td>
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<td>This study</td>
</tr>
<tr>
<td>TOPO pilP</td>
<td>pilP fragment flanked by EcoRI + Xhol in pCRII-TOPO</td>
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<tr>
<td>PCR8/GW/TOPO</td>
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<td>Invitrogen</td>
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<tr>
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<td>This study</td>
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<td>Karimova et al. (2001)</td>
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<tr>
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<tr>
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<td>BACTH vector expressing PilF–T18</td>
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<td>pUT18 pilG</td>
<td>BACTH vector expressing PilG–T18</td>
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<td>BACTH vector expressing PilZ–T18</td>
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<td>pUT18C</td>
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<td>BACTH vector expressing T18–PilD</td>
<td>This study</td>
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<tr>
<td>pUT18C pilE</td>
<td>BACTH vector expressing T18–PilE</td>
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<td>pKT25</td>
<td>BACTH vector designed to express a protein fused in frame at its N-terminus with T25; p15 ori; Km&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>BACTH vector expressing T25–PilD</td>
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<td>pKT25 pilE</td>
<td>BACTH vector expressing T25–PilE</td>
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<td>BACTH vector expressing T25–PilI&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>pKT25 pilOshort</td>
<td>BACTH vector expressing T25–PilO&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
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</table>
(Sigma), 100 μg ml⁻¹ ampicillin and 50 μg ml⁻¹ kanamycin. Plates were incubated at 30°C and the colour of the colonies was scored after 40–48 h. In every assay, positive and negative controls, generating purple and white colonies respectively, were included. All the positive plasmid combinations, i.e. generating coloured colonies, were transformed again for confirmation of the phenotypes.

The efficiency of the functional complementation between T18 and T25 for the positive plasmid combinations, were quantified by measuring β-galactosidase activities in liquid culture (Karimova et al., 1998). Transformants to be assayed were grown at 30°C for 14–16 h in 5 ml of LB supplemented with 0.5 mM IPTG, 100 μg ml⁻¹ ampicillin and 50 μg ml⁻¹ kanamycin. At least three independent cultures were performed for each transformant to be tested. These were then diluted 1/5 in M63 broth and the OD₆₀₀ was recorded. Next, cells were permeabilized by adding 20 μl of chloroform and 20 μl of 0.1% SDS to 1.5 ml of bacterial suspension. Tubes were then subjected to vortexing for 10 s and incubated at 37°C in a shaking incubator for 40 min. For the enzymatic reactions, 10 μl of the permeabilized cells were added to 990 μl of PM2 (70 mM Na₂HPO₄, 12H₂O, 30 mM NaH₂PO₄

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H2O, 1 mM MgSO4, 0.2 mM MnSO4, pH 7.0) containing 100 mM β-mercaptoethanol. The tubes were placed in a heat block at 28°C for 5 min before the reaction was started by adding 0.25 ml of 0.4% O-nitrophenol-β-galactoside (ONPG) in PM2 buffer (without β-mercaptoethanol). The reaction was stopped by the addition of 0.5 ml of 1 M Na2CO3, which occurred after 20 min for positive samples and after 60 min for negative samples, at which point the OD420 and OD600 were recorded. The enzymatic activity A (in units per milliliter) was calculated using the following formula: A = 200 × (OD420 per minute of incubation) × dilution factor. The results were expressed as units of enzymatic activity per milligram of bacterial dry weight, where 1 unit corresponds to 1 nmol of ONPG hydrolysed per minute (Karimova et al., 1998), considering that 1 ml of culture at an OD600 of 1 corresponds to 300 μg bacteria (dry weight).

**SDS-PAGE, antisera and immunoblotting**

**Neisseria meningitidis** whole-cell protein extracts were prepared as previously described (Helaine et al., 2005) or by resuspending bacteria directly in Laemmli sample buffer (Bio-Rad) and heating for 10 min at 100°C. *E. coli* whole-cell protein extracts were prepared by centrifuging bacteria and resuspending pellets directly in Laemmli sample buffer. When needed, proteins were quantified using the Bio-Rad Protein Assay as suggested by the manufacturer. Separation of the proteins by SDS-PAGE and subsequent blotting to Amersham Hybond ECL membranes (GE Healthcare) was carried out using standard molecular biology techniques (Sambrook and Russell, 2001). Blocking, incubation with primary and/or secondary antibodies and detection using Amersham ECL Plus (GE Healthcare) were carried out following the manufacturer’s instructions. Alternatively, SDS-PAGE gels were stained using Bio-Safe Coomassie (Bio-Rad). Rabbit antisera were used at 1/2000 (anti-PiLO), 1/5000 (anti-PiLM and anti-PiLN) and 1/50 000 (anti-PiLP) dilutions. Amersham ECL-HRP-linked secondary anti-rabbit antibody (GE Healthcare) was used at a 1/10 000 dilution.

Antisera against PiLM, PiLN, PiLO and PiLP were produced in rabbits as follows. Two antisera (anti-PiLM and anti-PiLP) were produced against purified recombinant proteins. Anti-PiLM was produced against a recombinant polyhistidine–PiLM (full-length protein) that was purified from *E. coli* BL21 (pYU12) using Ni-NTA affinity resin (Qiagen). Anti-PiLP was produced against a recombinant *PiP17–145–polyhistidine* that was purified from *E. coli* BL21 (pET20–piP17–145) using Ni-NTA affinity resin. Anti-PiLO and anti-PiLP were produced by immunizing animals with a mixture of two different peptides using the Double-X strategy (Eurogentec). Peptides corresponding to residues 125–140 and 185–199 of PiLO, and 45–59 and 169–183 of PiLO were used for the immunizations. Anti-PiLM, anti-PiLN and anti-PiLO sera have been purified by immunoaffinity using MBP–PiLM, MBP–PiLN and MBP–PiLO recombinant proteins that were purified using amylose resin (New England Biolabs) from *E. coli* BL21 transformed with pYU42, pYU51 and pYU44 respectively.

**Detection of Tfp**

Tfp were visualized by immunofluorescence microscopy using a Nikon Eclipse E600 microscope as previously described (Helaine et al., 2005). The only minor differences consisted in the use of DAPI (4’,6-diamidino-2-phenylindol) (Invitrogen) at 100 ng ml⁻¹ for staining the bacteria, and the use of Aqua-Poly/Mount (Polysciences, Inc.) as mounting medium.

**Immunoprecipitations**

Immunoprecipitations were performed using the Crosslink immunoprecipitation kit (Pierce) following the manufacturer’s instructions. In brief, antibodies were first bound to Protein A/G Plus Agarose (5 μg of purified anti-PiLM, anti-PiLN and anti PiLO antibodies, and 8 μl of anti-PiLP serum) and then cross-linked using disuccinimidyl suberate (DSS). Protein extracts, prepared using the B-PER bacterial protein extraction reagent (Pierce), were then immunoprecipitated (500 μg per reaction) overnight at 4°C. After several washing steps, precipitated proteins were eluted in 50 μl of elution buffer and analysed by immunoblotting as described above.

**Acknowledgements**

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**References**


