

**Development of DNA-encoded cell-free biosensors
for the detection of bacterial infections**

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Submitted for the Degree of Doctor of Philosophy
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Imperial College London
September 2016

Declaration of Originality

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Acknowledgements

I would like to firstly thank my supervisor Paul Freemont for his unwavering support of this project and constant encouragement throughout my PhD.

I am particularly grateful to Dr. Kirsten Jensen for always being available, despite her own PhD and copious other responsibilities, to provide both practical and philosophical help.

I would like to thank Prof. Jane Davies for supporting this project and sharing her wealth of experience in cystic fibrosis with us. I would also like to thank Dr. David Bell for his unlimited patience and expertise with mass spectrometry. It has been a pleasure working together and learning from him. My thanks also go to Dr. Yonek Hleba for his assistance and for helpful discussions about subjects beyond my understanding. I wish to thank Alain Filloux and the members of his group as well for welcoming me into their lab.

I would like to thank everyone in the Freemont group, as well as the MSF group for making it such a friendly place to work. In particular, I am grateful to my fellow PhD student Nicolas Kylilis for sharing our experiences throughout this time; Loren Cameron for her enthusiasm and dedication; Dr. Richard Kelwick and Dr. Alex Webb for their help and kind advice.

Finally, my grateful thanks to my family who have always supported me, my friends who have put up with me, and to TA for believing in me.

Abstract

This thesis describes the development of cell-free biosensors for the detection of quorum-sensing molecules in human clinical samples. The biosensor devices were comprised of a transcription factor and a regulated GFP reporter output that was expressed in the presence of the target molecule. The genetic circuit was encoded on a DNA plasmid and expressed in a cell-free system, consisting of the basic transcription and translation machinery of *Escherichia coli* in an open reaction. A biosensor device specific for the 3-oxo-C12-HSL quorum-sensing molecule from *Pseudomonas aeruginosa* was implemented in cell-free systems and used to measure the presence of acyl homoserine lactones in sputum samples collected from cystic fibrosis patients. Mass spectrometry analysis of sputum samples was also conducted to validate the biosensor results. It was found that not all samples from *Pseudomonas*-infected patients had detectable levels of AHL, and that the detection limit of the biosensor was greater than the concentration of AHL measured in many of the analysed clinical samples. Optimisation of the sample preparation and cell-free assay improved the ability of the biosensor to detect 3-oxo-C12-HSL in cystic fibrosis sputum. The limitations of the assay were identified so that the biosensor can be further developed into a functional tool for the detection of AHLs in clinical samples. In addition to the clinical testing, two new biosensors were constructed for the detection of the AHL C8-HSL and the intracellular signalling molecule c-di-GMP, and both devices were shown to be functional in cell-free systems.

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1.3 Abbreviations

AHL	N-acyl homoserine lactone
AI-2	Autoinducer-2
AIP	Auto-inducing peptide
ASL	Airway surface liquid
BAL	Bronchoalveolar lavage
Bcc	<i>Bulkholderia cepacia</i> complex
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
DCM	Dichloromethane
DGC	Diguanylate cyclase
DKP	Diketopiperazines
DMSO	Dimethyl sulfoxide
E-AB	Electrochemical aptamer-based
EA	Ethyl acetate
ELISA	Enzyme-linked immunosorbent assays
EPS	Extracellular polymeric substances
FI/OD	Fluorescence over absorbance
FPLC	Fast protein liquid chromatography
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HPLC	High-performance liquid chromatography
HSL	Homoserine lactone
IDT	Integrated DNA Technologies
iGEM	International Genetically Engineered Machine competition
IQS	2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
m/z	Mass to charge ratio

MB	Methylene blue
mRNA	Messenger RNA
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NHQ	2-nonyl-4-quinolone
OD600	Optical density at 600 nm
OP	Oropharyngeal
OPH	Organophosphorus hydrolase
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
pGpG	5'-phosphoguanylyl-(3'-5')-guanosine
PQS	2-heptyl-3-hydroxy-4-quinolone
Ps+/-	<i>Pseudomonas aeruginosa</i> positive/ negative
PURE	Protein synthesis using recombinant elements
Q-ToF	Quadropole time-of-flight
QS	Quorum sensing
RBS	Ribosome binding site
SD	Standard deviation
SELEX	Systematic evolution of ligands by exponential enrichment
SPR	Surface plasmon resonance
TX-TL	Transcription-translation cell-free system

2 Introduction

2.1 Synthetic Biology

Synthetic Biology aims to apply engineering principles to the design and construction of new biological systems, or the modification of existing ones, at the level of pathways, networks, and chromosomes. A major motivation for this is the ability to create biological systems that can have applied functions, such as devices for medical, environmental, or industrial purposes. In order to create such devices it is critical to be able to design systems with predictable behaviour, which can be difficult due to the complexity of biological networks and organisms. Therefore a number of foundational tools and methods have been developed to enable this aim, which draw on the more established fields of molecular, micro- and systems biology (Kelwick, MacDonald, Webb, & Freemont, 2014).

The principle of standardisation highlights the requirement to have a library of available parts - generally defined as an individual genetic element such as promoters or genes – that have been well characterised so that their function and behaviour is fully understood (Canton, Labno, & Endy, 2008). The most well-known library is the Parts Registry by the BioBricks Foundation, which is composed of parts that have been submitted by the competitors of the annual International Genetically Engineered Machines (iGEM) competition. The registry currently has over 20,000 parts and is a valuable resource (<http://parts.igem.org>). However, because many of the parts are not fully characterized before submission, alternate libraries for professional use have been proposed, such as at the BIOFAB facility (Mutalik et al., 2013). The principle of modularisation entails that these genetic elements can be connected together to form functional devices, and in particular that they can be easily exchanged for other parts and re-purposed to create new devices. Many DNA assembly methods have been developed to enable this aim, with the rapid assembly of multiple parts being a key supporting technology (Casini, Storch, Baldwin, & Ellis, 2015). The design of devices with complex behaviour, such as the classic genetic oscillator and toggle switch, require mathematical modeling to predict and identify key parameters for the device to produce predictable behaviour (Arpino et al., 2013). Once designed and assembled, these devices are then implemented in a so-called “chassis” or host system, with the most well-known ones currently in use being model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* as well as non-living systems such as cell-free protein synthesis reactions.

Beyond the ability to design and create devices with complex functions from biological elements, proponents of synthetic biology have emphasised the transformative potential of this approach to create technologies that have applications beyond research. The diversity of biological mechanisms that exist in nature to, for example, produce chemical and materials, sense environmental elements or metabolise toxic substances is unparalleled and can be harnessed into useful biological devices. Many of the proposed technologies have focused on biomedical applications. For example, metabolic engineering advances enabled the synthesis of artemisinic acid, a precursor of the antimalarial artemisinin, through introduction of a biosynthetic pathway in *S. cerevisiae* (Paddon et al., 2013). Sophisticated sense-and-response circuits, which are able to release a treatment output when triggered by disease signals, have also been proposed for various diseases such as cancer (Forbes, 2010). Anderson et al. engineered *E. coli* to invade tumour cells by expressing a gene for invasins in response to triggers of hypoxia, cell density, and chemical induction (J. C. Anderson, Clarke, Arkin, & Voigt, 2006). One of the most popular areas of research has been the development of biosensors to measure the presence of target molecules, which takes advantage of the wide diversity and sensitivity of environmental monitoring mechanisms found in nature.

2.2 Biosensor designs and applications

2.2.1 An overview of the biosensor field

Biosensors are devices that measure the presence of a target molecule using biological detection elements. They are typically comprised of a recognition component that provides the specificity to the analyte of interest, and a transducer that can convert the detected biological interaction to a recordable signal (Zhou, Son, Liu, & Revzin, 2015). This broad definition encompasses a large category of devices, ranging from sophisticated research equipment to cheap disposable tests for single use. The field of biosensors has grown enormously since the 1980s, with a global market estimated to be worth over USD 12 billion in 2010 (Turner, 2013).

By far the most well-known and successful device developed is the blood glucose monitor for the control of diabetes, which makes up 85% of the global market in biosensors (Turner, 2013). The invention of a glucose oxidase electrode for the detection of glucose by Clark and Lyon in the 1960s (Clark & Lyons, 1962) is widely regarded as the origin of the modern biosensor field (Wang, 2008). Today these are usually found as disposable printed electrode strips paired with a control meter to calculate the blood glucose concentration. Further improvements include the development of implantable sensors that enable continuous monitoring, and the ultimate goal is considered to be non-invasive techniques that do not require blood sampling (Turner, 2013). Besides enzyme-based assays, antibodies are the next most common biological recognition element in biosensors. The technology of enzyme-linked immunosorbent assays (ELISA) consists of surface-attached antibodies that capture target antigens which are then labelled with secondary antibodies. This has been used to develop biosensor devices such as the at-home pregnancy test, which detects the presence of human chorionic gonadotropin hormone in urine (Braunstein, 2014; Vaitukaitis, Braunstein, & Ross, 1972). The method can also be used to capture cells as well as individual proteins, allowing the monitoring of cytokines secreted by the cells. An example of this in use as a diagnostic is the T-spot.TB, which measures interferon- γ produced by blood mononuclear cells (Meier, Eulenbruch, Wrighton-Smith, Enders, & Regnath, 2005). On the other end of the spectrum, in terms of cost and sophistication of technology, are research tools such as surface plasmon resonance (SPR), a form of label-free detection where a thin layer of metal or semiconductor material is functionalized with a biological recognition element. The

binding of analyte to the recognition element changes the refractive index at the surface, which can be measured by an optical reader. SPR is commonly used to measure protein-ligand interactions in research settings, and the detection of a wide range of analytes from hormones to bacterial cells have been proposed (Homola, 2008). However the high cost of the equipment largely precludes their use as biosensors *in situ*.

Besides enzymes and antibodies, there are also other recognition elements that can be used to develop new biosensors. Nucleic acids are an ideal example because they are easily designed to capture target complementary sequences. This has been exploited for the development of hybridization probes such as molecular beacons, a hairpin structure that is conjugated with fluorescence resonance energy transfer (FRET) pair on the ends of the stem (Tyagi & Kramer, 1996). When the target sequence binds to the complementary region in the loop domain, the beacon unfolds and fluorescence can be measured. Molecular beacon biosensors have been designed to sense pathogens by creating probes for *Pseudomonas aeruginosa* and *Legionella pneumophila*-specific sequences (Liu et al., 2011; Rai et al., 2012). Aptamers are DNA or RNA molecules that expand the target range from other nucleic acids to proteins and whole cells. They can be determined through a process known as systematic evolution of ligands by exponential enrichment (SELEX) (Ellington & Szostak, 1990). They function similarly to antibodies, but have certain advantages such as increased temperature stability and production *in vitro* without the need for animal lines.

An example of aptamers used for biosensing is the work done by the Plaxco group. They first developed thrombin biosensors by attaching aptamers labelled with the redox tag methylene blue (MB) to electrodes (Xiao, Lubin, Heeger, & Plaxco, 2005; Xiao, Piorek, Plaxco, & Heeger, 2005). Binding of the aptamer to thrombin changes the position of the label relative to the electrode surface and produces a measurable electronic response. The sensors were configured to either result in loss of signal if the binding between aptamer and target inhibits electron transfer between MB and electrode, or to switch on a signal if binding causes a conformation change that brings MB closer to the electrode surface. The researchers argued that this technology, named electrochemical aptamer-based sensors (E-AB), has advantages over conventional molecular beacon systems because the electronic output avoids common problems with optical detection such as photobleaching and masking of the signal by contaminants. They demonstrated that it is a versatile system that can be modified to detect

further protein targets, such as platelet-derived growth factor (Lai, Plaxco, & Heeger, 2007), as well as lead (Xiao, Rowe, & Plaxco, 2007) and small molecules including cocaine and the antibiotic kanamycin (Baker et al., 2006; Ferguson et al., 2014). Significantly, all of these sensors are highly sensitive; for example, the lead sensor had a detection limit of 62 parts per billion which is far below the 100 – 400 parts per million limit set for lead in soils by the US Environmental Protection Agency (Xiao et al., 2007). They are also capable of discerning the intended target even in complex conditions such as human blood serum (Lai et al., 2007) and saliva (Baker et al., 2006). A number of improvements to the original technology were further developed, including incorporation into microfluidics to enable continuous real-time detection in blood serum (Swensen et al., 2009) and optimising the probe density on the gold electrode surface to increase sensitivity (White, Phares, Lubin, Xiao, & Plaxco, 2008). The group found that the aptamer architecture could be modified as well to increase the distance between the MB tag and the electrode and thus reduce the background signal, either by introducing a sandwich sequence between binding regions of the aptamer or by linking three aptamers together to create a poly-aptamer (White & Plaxco, 2009).

Non-catalytic proteins are other biological molecules that can be used as capture probes, such as for the detection of proteases. Matrix metalloproteases, secreted enzymes that are associated with cancer cell migration, could be detected with a biosensor composed of an electrode functionalized with cleavable peptides (D. S. Shin et al., 2013). Finally, detection at the single-molecule resolution is an aim that is pushing the boundaries of biosensor ability. This has been proven to be possible in the case of DNA nanopores, which are composed of transmembrane proteins (such as α -hemolysin from *Staphylococcus aureus*) that have an aperture of a few nanometres (Kasianowicz, Brandin, Branton, & Deamer, 1996). The ion current across the pore can be monitored to detect single nucleotides passing through the pore, which has now been developed as a commercial DNA sequencing technique (Bayley, 2015).

In this overview, various examples of biosensor devices have been discussed showing the breadth and diversity of the field. Despite the variety of targets and capture methods, most share a common configuration of biological recognition elements physically linked to electrochemical or optical detection methods. A different class of biosensors is whole cell devices, where both detection and signal output elements are encoded within living cells.

2.2.2 Whole cell biosensors

Living cells are capable of monitoring their surrounding conditions and processing a response, which makes them suitable for adapting into biosensor devices. Due to the huge variety of metabolic processes that exist, there is a catalogue of natural biological recognition elements both at the DNA and protein level that can be exploited. These can be linked to output elements such as the expression of fluorescent or luminescent proteins, catalytic enzymes, pH modifiers, or growth-limiting factors.

Commercial systems utilizing whole-cell biosensors tend to be toxicity assays that rely on constitutive expression of a reporter output, which will be reduced when the cells are exposed to toxic compounds. For example, the Microtox® assay uses the naturally bioluminescent *Vibrio fischeri* (Modern Water). However, genetically engineering microbes to express sensing and reporting elements greatly expands the range of targets that can be detected. Many whole cell biosensors are based on paired transcriptional regulators and promoters, which enable the coupling of target detection and signal output by placing a reporter gene under the regulation of the promoter. For example, to detect heavy metals the cadmium-sensitive protein CadC from *Staphylococcus* species and firefly luciferase gene under the control of the *cadC* gene was inserted into *Bacillus subtilis*, creating a biosensor that could detect cadmium as well as lead and antimony (Tauriainen, Karp, Chang, & Virta, 1998). A set of Gram-positive and Gram-negative sensor strains were similarly constructed to sense zinc, lead, mercury and cadmium contamination, with both positively- and negatively-regulated outputs (Ivask, Rõlova, & Kahru, 2009). Biosensor devices for organic chemicals have also been constructed, such as XylR and DmpR from *Pseudomonas* species, which detect xylene and phenols respectively (Willardson et al., 1998; Wise & Kuske, 2000). The AraC and P_{BAD} pair was used to detect L-arabinose in an *E. coli* biosensor (Shetty, Ramanathan, Badr, Wolford, & Daunert, 1999), and by engineering the AraC protein Tang et al. were able to change the selectivity of this biosensor for the stereoisomer D-arabinose instead (S. Y. Tang, Fazelinia, & Cirino, 2008). These systems are based on intracellular detection where the analyte must passively diffuse into the cell or be actively taken up. For targets where this is not possible or desirable, surface display mechanisms have also been devised. For example, enzymes have been anchored to the cell surface using outer membrane

proteins, such as organophosphorus hydrolase (OPH) for the detection of organophosphorus pesticides and xylose dehydrogenase for the detection of D-xylose (Liang, Li, Mascin, & Liu, 2012; Richins, Kaneva, Mulchandani, & Chen, 1997). In the case of OPH, it was shown that cell-surface display of the enzyme compared to intracellular expression led to a seven-fold increase in rate of substrate catalysis, demonstrating a further potential benefit of extracellular display.

Besides protein-based detection, nucleic-acid receptor elements can be used in whole cell biosensors. Aptamers, as described above, are RNA molecules that can bind to specific analytes and in nature they can be found as a component of riboswitches. These sequences typically contain an aptamer region and a ribosomal binding site (RBS) that is sequestered in the secondary structure (Topp & Gallivan, 2010). In the case of positive regulation, the riboswitch undergoes a conformational change upon binding to the target analyte that releases the RBS for ribosome binding and subsequent downstream expression of an open reading frame. Negative regulation occurs either because binding to the analyte leads to a conformational change that sequesters the RBS or because binding physically blocks the ribosome being able to attach. All of these mechanisms have been designed for thiamine (vitamin B1) sensing. A native riboswitch was initially identified in *E. coli* that bound to thiamine pyrophosphate, a product of thiamine metabolism, and sequestered the RBS in the bound state (Winkler, Nahvi, & Breaker, 2002). This was then engineered to be positively regulated by creating a library of riboswitch variants and placing them upstream of the antibiotic resistance marker *tetA*, and applying consecutive rounds of selection. The selected riboswitches were then cloned upstream of reporter genes to create a whole cell biosensor that would activate expression in the presence of thiamine (Nomura & Yokobayashi, 2007). Another study constructed a negatively-regulated biosensor, by placing a theophylline aptamer in the 5' proximal coding region of GFP such that binding of theophylline would change the conformation of the RNA and prevent ribosome binding (Jo & Shin, 2009).

The essentially modular nature of biosensors, composed of separate recognition and output elements, makes them particularly suitable for the synthetic biology approach that emphasizes standardized and easily interchangeable DNA parts. Furthermore, detected signals can be integrated into more complex genetic circuits that perform computational operations. As such, there have been a large number of whole cell biosensors proposed in the

synthetic biology field over the years, with a few examples of successful detection in real world conditions. An arsenic biosensor, composed of an *E. coli* arsenic-responsive promoter and repressor pair coupled to LacZ and XylE reporter elements, produced a pH change that could be visualized by a colour indicator, and this was shown to be able to detect arsenic contamination in groundwater well samples (Aleksic et al., 2007; de Mora et al., 2011). Courbet *et al* developed digital amplifying switches to convert signal detection into a digital-like response. The presence of the target analyte induces expression of a serine integrase, which then inverts a transcriptional terminator sequence so that RNA polymerase can read through. Using these switches improved the robustness and sensitivity of biosensors that were tested with human clinical samples, and the researchers developed a glucose-sensitive switch that was able to measure glycosuria in diabetic patients' urine samples with a better sensitivity than urinary dipsticks (Courbet, Endy, Renard, Molina, & Bonnet, 2015). Extracellular detection was investigated by our group with the development of whole cell biosensors for the monitoring of *Schistosomiasis* parasites (Webb et al., 2016). Proteases are secreted by the parasites to facilitate their invasion of mammalian hosts, and a detection module was designed for these comprised of an anchor component in the cell membrane linked to a protease recognition motif and streptavidin-binding epitope tag exposed on the cell surface. In both *E. coli* and *B. subtilis* hosts, exposure to *S. mansoni* transformation fluid resulted in cleavage of the tag and concomitant loss of fluorescent signal.

Living cells have a number of advantages that makes them particularly attractive as biosensor devices. They are easy to produce because they can self-replicate, and do not require the same costly purification methods as enzymes (Raut, O'Connor, Pasini, & Daunert, 2012). They are capable of performing complex signal processing functions as described above. Furthermore, freeze drying cells or inducing sporulation enables them to be stored and transported easily (Date, Pasini, Sangal, & Daunert, 2010; Webb et al., 2016). However, the use of living cells outside of contained laboratory conditions also poses some serious challenges in terms of regulatory approval for genetically modified organisms. Within the European Union, devices have to be subject to extensive field-testing before being deemed safe for use, which can be time-consuming and costly (French et al., 2011). In cases where exposure to microbial cells is particularly undesirable, an alternative host such as cell-free systems may be preferred.

2.2.3 Cell-free biosensors

In recent years, cell-free systems have received renewed interest as a platform for the implementation of engineered biological systems. In principle, the term cell-free encompasses a large group of different systems, ranging in complexity from single enzymatic reactions such as Polymerase Chain Reaction (PCR) to multi-enzyme synthetic pathways. In practice it usually refers to coupled transcription and translation systems that are commonly used for cell-free protein synthesis. They are especially useful for the production of high value or toxic proteins; for example, they have been commercialized for the expression of cytokines and antibody fragments (Yin et al., 2012; Zawada et al., 2011). Cell-free systems are generally made up of three components: clarified cell lysate, energy buffer mix, and DNA template. Today the most common sources for cell lysate are *E. coli*, wheat germ, and rabbit reticulocytes (Katzen, Chang, & Kudlicki, 2005); there has also been work on developing *S. cerevisiae* extracts (Hodgman & Jewett, 2013). The “Protein synthesis Using Recombinant Elements” (PURE) system is an alternative form wherein each component is individually purified (Shimizu et al., 2001). However, due to the high cost and lower protein yield it is generally only used for specialised purposes.

The use of cell-free systems was pioneered by the work of Nirenberg and Matthaei, who first developed cell lysates from *E. coli* to unravel the mechanism of translation (Nirenberg & Matthaei, 1961). In subsequent years they were used primarily as a tool for protein production. A standard S30 protocol, named after the speed of the centrifugation step, was published in 1973 and provided a framework for further developments (Zubay 1973). Improvements to the protocol for preparing cell-free systems, such as the introduction of T7 RNA polymerase driven expression (Milligan, Groebe, Witherell, & Uhlenbeck, 1987) and the optimization of the growth medium and reaction buffer (Carlson, Gan, Hodgman, & Jewett, 2012), were mainly targeted towards optimizing the yield. Besides the function of pure protein synthesis, cell-free also gained attention as a simple and accessible system for studying the workings of genetic circuits. It was demonstrated that multiple elements could be expressed within a single reaction to produce transcriptional cascade, AND gate, and negative feedback loop behaviour (Noireaux, Bar-Ziv, & Libchaber, 2003; J Shin & Noireaux, 2012). In synthetic biology in particular, there is a demand for rapid test cycles of biological circuits and cell-free has been proposed to be an ideal prototyping platform that can be used to screen circuit designs before they are implemented in living systems

(Chappell, Jensen, & Freemont, 2013; Sun et al., 2013). With this resurgence in the use of cell-free, there was also an emerging interest in its suitability for applications beyond prototyping, and particularly in biosensing.

In 2014 Pardee and colleagues demonstrated that cell-free systems could be used to make paper-based biosensor devices (Pardee et al., 2014). Commercial *E. coli* S30 and PURE expression systems were applied to filter paper discs and freeze-dried, then activated by rehydration. A series of RNA-actuated circuits were constructed to activate gene expression in the presence of trigger mRNA, by designing a type of riboswitch known as toehold switches. In these the RBS sequence is sequestered in the loop of a hairpin structure, with a sensing sequence that is complementary to the target mRNA in the stem and 5' single-stranded domain. The sequence flexibility of these devices allowed the authors to design biosensors for a diverse range of targets, including antibiotic-resistance and Ebola nucleoprotein mRNA sequences that were able to distinguish between different strains of the virus. It was demonstrated that besides GFP the paper-based devices could also produce visual outputs by expressing β -galactosidase or chitinase. In a subsequent publication, the authors developed Zika-specific biosensors and demonstrated that detection was possible from viral cultures and plasma samples of infected macaque (Pardee et al., 2016). They optimized the sample preparation protocol with the addition of a boiling step to release the RNA targets from the viral host, and a 3-hour RNA amplification step to reach the detection limit of the biosensor device. The benefits of such systems are the low cost – estimated to be \$0.65 per sensor with commercial cell extracts and as low as \$0.04 if produced in house – and the ability to rapidly screen designed circuits for new targets (Pardee et al., 2014).

Previous work in our group has focused on developing biosensors for signalling molecules involved in biofilm formation. The following section will provide an overview of biofilms and the cellular signalling that plays a role in their regulation.

2.3 Biofilms

2.3.1 The survival benefits of biofilms

Microbial biofilms are co-operative cell consortiums that exist within a protective matrix and coordinate their behaviour and gene expression at the population level. In environments with limited availability of nutrients and other resources, biofilms have been found to be the predominant life form for bacteria (Hall-Stoodley & Stoodley, 2005). Although microbiological research has predominantly studied microbes in the planktonic state, the importance of understanding how they exist in a biofilm form is increasingly recognised. One of the first recorded observations of bacteria was in fact a biofilm, when Anthony van Leeuwenhoek observed a scraping of his own dental plaque under the microscope over 300 years ago (Dufour, Leung, & Lévesque, 2010). In the 1930s and 1940s, it was discovered that bacteria preferentially grew on surfaces rather than as free-floating cells in water (Henrici, 1933; Zobell, 1943). Since then, it has been found that almost all bacterial species can form biofilms (Van Houdt & Michiels, 2010). In natural environments, biofilms are involved in several key processes including the decomposition of organic matter and the nitrogen cycle, and have consequently been harnessed in industrial applications such as sewage treatment (Boyd & O'Toole, 2012). However, in other industrial settings such as drinking water systems or food processing they are a major source of contamination and can harbour reservoirs of pathogenic bacteria (Simoës, Simoës, & Vieira, 2010). Biofilms are also problematic in clinical settings, because they cause infections in multiple areas of the body, including urinary tracts, heart valves, sinus cavities, chronic wounds and cystic fibrosis lungs (G. G. Anderson, 2003; Bjarnsholt et al., 2009; Høiby, Döring, & Schiøtz, 1986; Kirketerp-Møller et al., 2008; Sanderson, Leid, & Hunsaker, 2006). They can also form dangerous infections on the surface of implanted medical devices such as catheters and joint prostheses (Darouiche, 2001).

The process of biofilm formation occurs in several defined stages (Lindsay & von Holy, 2006). Prior to cellular attachment, the relevant surfaces are pre-conditioned by adsorption of small molecules such as nutrients. The cells that initially adhere to this surface have loose attachments that can easily be disrupted. Sensing of this transient attachment then triggers production of extracellular polymeric substances (EPS), which form a more stable bond

between the surfaces and neighbouring cells. These matrix components include polysaccharides, extracellular DNA, pili, and fimbriae (Pamp, Gjermansen, & Tolker-Nielsen, 2007). An intricate structure begins to emerge once a surface has been colonised, with microcolonies of living cells linked together by less dense channels that enable transport and storage of resources. Other cells may also become trapped in the matrix at this point, enabling the development of heterogeneous multi-species biofilms. The final stage of biofilm formation involves the release of cells from the mature complex. This can be initiated by dispersal mechanisms, such as cells secreting enzymes to dissolve the matrix, or by changing their own cellular phenotype to exit the biofilm lifestyle.

In the case of infections, forming a biofilm provides a protective and stable environment for cells to survive attacks from the host immune system and antibiotic treatment. Biofilms have been shown to be up to 1000-fold more resistant to antibiotics than planktonic cells (Govan & Deretic, 1996). Various mechanisms contribute to this enhanced antibiotic resistance. The EPS matrix forms a physical barrier against antimicrobial agents so that antibiotics diffuse slower through the matrix and kill only the cells at the surface. It is thought that this is mediated through the binding and sequestration of antibiotics to components of the matrix, such as DNA and alginate (Chiang et al., 2013; Hentzer et al., 2001). Furthermore, there is a physiological heterogeneity within biofilm structures, with cells in the interior having a decreased metabolic rate compared to cells in the outer part (Stewart & Franklin, 2008; Werner et al., 2004). This is related to the theory of persister cells, a sub-population of dormant cells within biofilms that are particularly resistant to antimicrobials (Lewis, 2010). Reduced metabolic activity makes these cells less susceptible to antibiotics targeting the growth and replication stages. Enhanced expression of antimicrobial tolerance genes has also been observed in biofilm cultures versus planktonic ones. For example, the alternate sigma factor RpoS which controls stress response is regulated by quorum-sensing in *P. aeruginosa* (Schuster, Hawkins, Harwood, & Greenberg, 2004), and efflux pumps are upregulated in *P. aeruginosa* and *Burkholderia cenocepacia* biofilms to resist the effects of antibiotics (Buroni et al., 2014; Liao, Schurr, & Sauera, 2013).

Forming a biofilm is a behaviour that provides benefits at the population level, and it requires cells to switch on certain functions that are only a good use of resources when they are performed as a group (such as the expression of EPS matrix components) (Schuster, Joseph

Sexton, Diggle, & Peter Greenberg, 2013). In order to be able to co-ordinate such functions, cells engage in a process known as quorum sensing.

2.3.2 Mediating intercellular communication with quorum sensing

Quorum sensing (QS) is a mechanism of cell-to-cell communication that enables cells to monitor cell density and regulate their behaviour in response. Cells synthesise extracellular signalling molecules (also known as autoinducers), release them into the surrounding environment, and detect them with intracellular response elements (Miller & Bassler, 2001). Since the concentration of signalling molecule in the environment increases as the population density rises, cells can activate certain processes in a collective manner. Examples of such population-level processes are the activation of bioluminescence and the production of virulence factors (Bassler & Losick, 2006).

Gram-negative and Gram-positive bacteria have different types of QS. Gram-positive bacteria use a family of small oligopeptides, also known as autoinducing peptides (AIPs), which are synthesised intracellularly and transported out of the cell (often undergoing further maturation during secretion) (Miller & Bassler, 2001). The first quorum-sensing molecule ever identified was an AIP from *Streptococcus pneumoniae*, which was named the competence-stimulating peptide (Tomasz, 1965). Signalling peptides are detected in the extracellular environment by binding to membrane-localised sensor kinases from two-component systems. An example of this is the *agr* system from *Staphylococcus aureus*, which consists of a cyclic AIP that activates its cognate AgrC histidine kinase (Thoendel, Kavanaugh, Flack, & Horswill, 2011). In addition to sensing peptides extracellularly and transmitting the signal through a phosphorylation cascade, there are also pathways that actively transport signal peptides back into the cell where they can interact with cytoplasmic receptors directly. A group of such receptor proteins known as RRNPP (Rgg/Rap/NprR/PlcR/PrgX) – encompassing proteins found in *Bacillus*, *Enterococcus*, and *Streptococcus* species – share a structural motif of tetratricopeptide (TPR)-like repeats that enables the protein-peptide interaction (Parashar, Aggarwal, Federle, & Neiditch, 2015). The receptors have different mechanisms when induced, including phosphorylation, transcriptional activation and repression, and these regulate a diverse set of behaviours such as sporulation, plasmid conjugation, and virulence (Monnet & Gardan, 2015).

Gram-negative bacteria generally use N-acyl homoserine lactones (AHLs) that can diffuse through the cell membrane and are detected by cytoplasmic transcription regulators. These systems were first identified when studying the cell-density dependent control of luciferase expression in *Vibrio fischeri* (Eberhard et al., 1981; Nealson, Platt, & Hastings, 1970). They are usually arranged in a positive feedback loop where detection of AHL upregulates expression of the synthase and leads to production of more signalling molecule, thus spreading the signal throughout a population. Both groups also use autoinducer-2 (AI-2) molecules, synthesised by the LuxS protein or homologs, as a third class of signalling (Pereira, Thompson, & Xavier, 2013). This class is thought to be responsible for inter-species communication, rather than the species-specific communication of AHLs and AIPs.

In *P. aeruginosa*, there are two known AHLs involved in QS, N-3-oxododecanoyl-homoserine lactone (3-oxo-C12-HSL) and N-butyryl-homoserine-lactone (C4-HSL), as well as the non-AHL signalling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS). There are also less well-studied compounds that have been isolated from *P. aeruginosa* and are proposed to play a role in intercellular signalling, namely 2,5-diketopiperazines (DKPs) and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) (Campbell, Lin, Geske, & Blackwell, 2009; Holden et al., 1999; J. Lee et al., 2013).

AHLs are synthesised by LuxI-type synthases, LasI for 3-oxo-C12-HSL and RhII for C4-HSL, and detected by LuxR-type transcription factors, LasR and RhIR, which together control expression of up to 10% of the bacterial genome (Schuster & Peter Greenberg, 2006). PQS is synthesised by the *pqsABCDH* genes and detected by the PqsR receptor (Déziel et al., 2004; E C Pesci et al., 1999). The members of the LuxR-type receptor family all have an N-terminal ligand binding and C-terminal DNA-binding domain, which contains a helix-turn-helix motif. They are stable when bound to their cognate AHLs and dimerise to bind to DNA at target sequences containing so-called *lux* boxes (Engelbrecht, Nealson, & Silverman, 1983; Stevens, Dolan, & Greenberg, 1994; Zhang et al., 2002). Many of the annotated LuxR-type receptors appear to not have a paired synthase protein, including QscR from *P. aeruginosa*, which has been shown to be non-specifically activated by both short- and long-chain AHLs (J. H. Lee, Lequette, & Greenberg, 2006). This has been hypothesized by Lee et al. to be a mechanism for detecting other species in the same environment.

The QS regulation in *P. aeruginosa* is arranged in a hierarchical fashion, with LasR at the top of the network (Figure 1). When bound to 3-oxo-C12-HSL, LasR controls the expression of a large number of genes, including the LasI synthase and both the *rhlRI* and *pqsR/ABCDH* pairs (Latifi, Foglino, Tanaka, Williams, & Lazdunski, 1996; Everett C Pesci, Pearson, Seed, & Iglewski, 1997; Seed, Passador, & Iglewski, 1995). The RhlR and C4-HSL complex then activates its own regulon as well as inducing expression of RhlI (Latifi et al., 1996). The PQS and C4-HSL systems are closely intertwined, because PqsR will upregulate RhlRI while RhlR in turn inhibits PqsH and PqsABCD expression (McKnight, Iglewski, & Pesci, 2000; Papenfort & Bassler, 2016).

The key processes that QS regulates in *P. aeruginosa* are biofilm formation and virulence factor expression. In a study of *P. aeruginosa* strains with loss-of-function QS mutations, the knock-out strains grew into flat undifferentiated biofilms rather than the multi-dimensional mushroom shape of wild-type strains, and were susceptible to SDS treatment unlike the wild-type (D. G. Davies, 1998). Many genes implicated in biofilm formation have been found to be controlled by QS, including the expression of rhamnolipids (Davey, Caiazza, & O'Toole, 2003; Ochsner & Reiser, 1995), lectins (Tielker et al., 2005; Winzer et al., 2000), fimbriae (Vallet, Olson, Lory, Lazdunski, & Filloux, 2001), and siderophores (Banin, Vasil, & Greenberg, 2005; Patriquin et al., 2008). QS by PQS also appears to regulate the production of extracellular DNA, a component of the EPS matrix, through a process of controlled cell lysis (Allesen-Holm et al., 2006; Yang, Nilsson, Gjermansen, Givskov, & Tolker-Nielsen, 2009). There is some overlap between factors described above which contribute to both biofilm formation and virulence, but additional factors that are under QS control and increase the pathogenicity of *P. aeruginosa* include elastases, proteases, and exotoxin A (Antunes, Ferreira, Buckner, & Finlay, 2010; Wagner, Gillis, & Iglewski, 2004).

The process of biofilm formation is not only regulated by QS, however. The intracellular signalling molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) has been recognized as playing a key role in controlling initiation of this process. Studies have found that these signalling networks may be interconnected in various species, including *P. aeruginosa* (Deng et al., 2012; Ueda & Wood, 2009), and it is proposed that this may be a way for the various inputs of QS to be converted into a general cellular signal (Papenfort & Bassler, 2016).

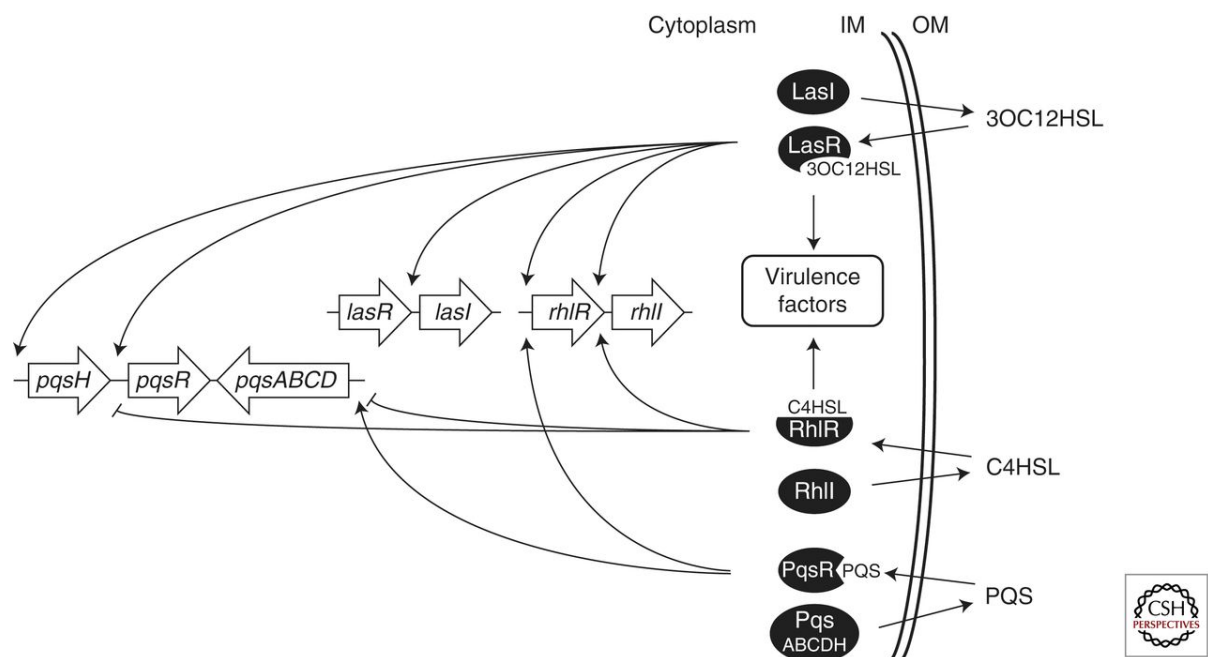


Figure 1 The QS network in *P. aeruginosa*. The QS molecules 3-oxo-C12-HSL, C4-HSL and PQS are synthesised by the *lasI*, *rhII* and *pqsABCDH* genes and detected by the *lasR*, *rhIR* and *pqsR* genes respectively. Reproduced from Rutherford and Bassler 2012. © Cold Spring Harbor Laboratory Press 2012

2.3.3 Intracellular signalling with C-di-GMP

Bacterial cells can switch from a planktonic to ‘sessile’ state, often triggered by environmental cues such as iron levels, osmolarity, and carbon availability (O’Toole, Gibbs, Hager, Phibbs, & Kolter, 2000; O’Toole & Kolter, 1998; Sauer et al., 2004; Pradeep K Singh, Parsek, Greenberg, & Welsh, 2002). There appears to be a myriad of different biofilm formation mechanisms, but one aspect that is shared among bacterial species undergoing this process is the production of the intracellular secondary messenger c-di-GMP.

This signal molecule was first discovered to regulate cellulose synthesis in *Glucanobacter xylinus* (Ross et al., 1986). Since then it has been found to be nearly ubiquitous as an intracellular secondary messenger across bacterial species. It is synthesised by a class of proteins known as diguanylate cyclases (DGCs), which have a conserved GGDEF amino acid domain (Paul et al., 2004). Two molecules of GTP are converted to one molecule of c-di-GMP by these proteins. Degradation is carried out by phosphodiesterases (PDEs), which either have an EAL domain, or more rarely a HD-GYP domain (Christen, Christen, Folcher, Schauerte, & Jenal, 2005; Ryan et al., 2006). This either degrades them to 5’-phosphoguanylyl-(3’-5’)-guanosine (pGpG) or guanosine monophosphate (GMP), respectively. The EAL and HD-GYP domains are often positioned in a protein together with another regulatory domain, so that the synthesis or degradation of c-di-GMP is controlled by an upstream signal, such as phosphorylation (Rao et al., 2009).

Whole-genome sequencing analysis has revealed that a single species can contain multiple DGCs and PDEs. For example, *E. coli* has 29 predicted ones; *P. aeruginosa* has 41, and *Vibrio cholerae* has 72 (Povolotsky & Hengge, 2012). This strongly suggests that c-di-GMP controls a complex regulatory network, and two different modes of regulation have been proposed: either there are localised concentration gradients in which specific DGCs are coupled to effectors, or there is integration of multiple synthesis pathways to regulate a single output.

C-di-GMP is able to work through a variety of different effectors to regulate functions via transcription, translation, and post-translation. The PilZ domain is a widely-studied motif that binds c-di-GMP: it is often found in cellulose synthases, such as the Alg44 gene of *P. aeruginosa* that synthesises alginate (Merighi, Lee, Hyodo, Hayakawa, & Lory, 2007). A wide variety of c-di-GMP binding domains have been identified besides PilZ, including the W[F/L/M][T/S]R motif in *Vibrio* species (Ferreira, Chodur, Antunes, Trimble, & McCarter,

2012; Krasteva et al., 2010), the RxxD motif in *Caulobacter crescentus* and *P. aeruginosa* (Duerig et al., 2009; V. T. Lee et al., 2007), and two riboswitch classes (K D Smith, Shanahan, Moore, Simon, & Strobel, 2011; Sudarsan et al., 2008). These operate through a number of different mechanisms, including modifying protein-protein interactions, upregulating transcription or removing repression, inhibition of DNA-binding, and regulation of mRNA splicing. The cellular outputs that they regulate are equally diverse, including motility, virulence, biofilm formation, expression of fimbriae, and synthesis of EPS (Boyd & O'Toole, 2012). Overall, due to the widespread presence of c-di-GMP, as well as its key role in regulating the transition to a biofilm state, c-di-GMP could be a useful indicator to determine whether bacteria are in a motile or biofilm lifestyle.

2.4 Cystic Fibrosis

2.4.1 General overview

Cystic fibrosis is an autosomal recessive disease caused by mutations in the CF transmembrane regulator (CFTR) gene. It is the most common genetic disorder in Caucasians, with a frequency of 1 in every 3000 live births (O'Sullivan & Freedman, 2009), and affects 70000 people worldwide (Ciofu, Tolker-Nielsen, Jensen, Wang, & Høiby, 2015). The CFTR gene encodes a chloride channel protein found in epithelial cells. There are nearly 150 different mutations that have been found to cause CF (Ratjen et al., 2015), which are grouped into five classes based on how the CFTR protein is affected, such as decreased production, failure to localise to the cell membrane, or lack of functional regulation (Figure 2). The F508del mutation (deletion of phenylalanine at position 508), which belongs to class II, is the most common allele mutation in north American and north European populations (O'Sullivan & Freedman, 2009). Besides chloride and bicarbonate ion transport, CFTR has also been found to play a regulatory role in other forms of transport, including the epithelial sodium channel, outwardly rectifying chloride channel and ATP channels (Ismailov et al., 1996; Schwiebert et al., 1995; Sugita, Yue, & Foskett, 1998). Disruption of CFTR function has global effects on the body, most notably affecting the gastrointestinal, reproductive, and respiratory organs. For example, pancreatic insufficiency is found in a majority of patients and can result in vitamin deficiencies and malnutrition, the major cause of early mortality among CF infants until treatments with pancreatic enzyme replacements were developed (O'Sullivan & Freedman, 2009). Cystic fibrosis-related diabetes is another disease caused by obstructions in intrapancreatic ducts as a result of CFTR dysfunction (Moran et al., 2010). In men, the vas deferens is affected by non-functioning CFTR and male CF patients are generally sterile (Jarvi et al., 1995). The effect of CFTR disruption on lung function is the most clinically significant, however, because pulmonary disease is the cause of mortality for over 90% of CF patients (Jane C Davies, 2002).

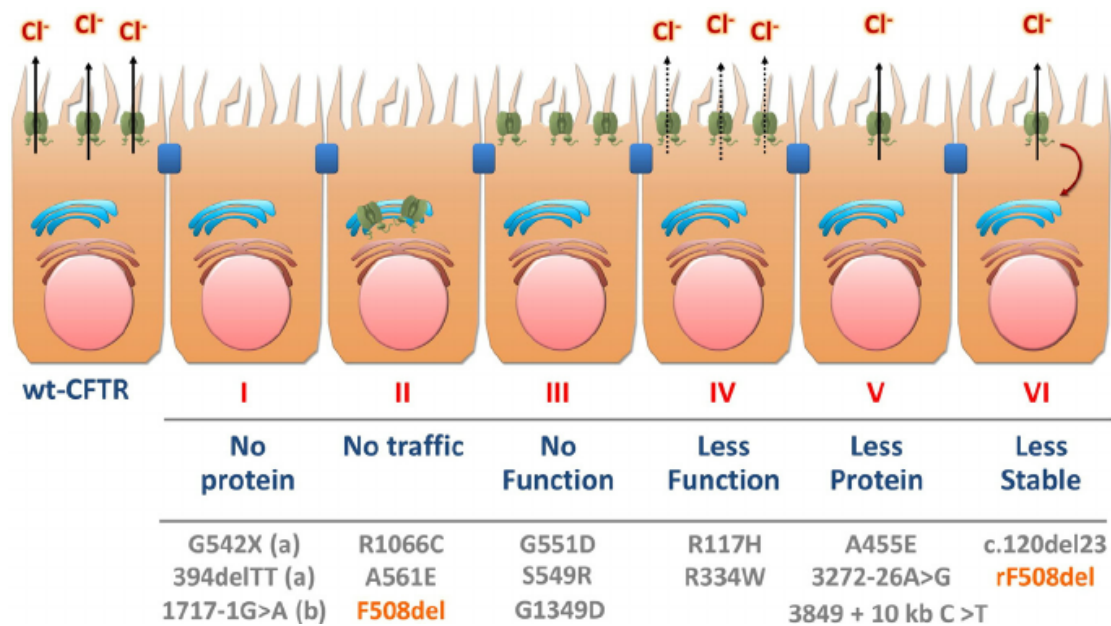


Figure 2 Classes of CFTR mutations according to their effect on function. Class I mutations result in no protein production, class II mutations lead to errors in protein processing, class III mutants have defects in ion gating, class IV mutants have decreased ion flow, class V mutants have less functional protein e.g. due to abnormal splicing, and class VI mutants are instable in the cell membrane. Examples of mutations in these classes are listed on the bottom row. Reproduced from Bell et al. 2015.

2.4.2 Infectious disease in CF

Pulmonary disease in CF is caused by sustained inflammation and infection, which eventually leads to severe and irreversible lung damage. The origin and progression of these events is still not fully understood, although many individual mechanisms by which CFTR mutations affect lung function directly have been discovered. Without normal CFTR activity the transport of chloride and bicarbonate ions is reduced, which leads to dehydrated airway surface liquid (ASL) that impairs mucociliary clearance (Boucher, 2007). In addition, hyperviscous mucus is produced that adheres tightly to the epithelial cells and again obstructs clearance (Quinton, 2008). The deregulated ion transport across the apical membrane also results in ASL with an abnormally low pH (Coakley et al., 2003), which disrupts normal airway defence mechanisms such as defensins (Pezzulo et al., 2012). It is not yet clear whether inflammation in the lungs occurs solely in response to infection or directly as a result of CFTR mutations, with evidence supporting both hypotheses. On the one hand, studies of CF lungs of infants before infection found no signs of inflammation (Bedrossian, Greenberg, Singer, Hansen, & Rosenberg, 1976; Oppenheimer & Esterly, 1975), and foetal tracheal xenograft models showed normal cytokine levels that became elevated after infection (Tirouvanziam et al., 2000). However, transgenic mice models have indicated that the

abnormal ASL in the CF lung promotes an immune response even in the absence of infection (Livraghi-Butrico et al., 2012). Inflammation is mainly mediated by excess neutrophils, which can contribute to ASL dehydration as well as producing elastase (Mott et al., 2012; Sly et al., 2013). In combination with other proteases from inflammation or infection they cause severe damage to lung tissue. The effects of poor mucociliary clearance, inflammation and infection are therefore all contributors to the decreased lung function in CF.

Infections in CF are caused by a number of common species. In infancy and childhood *Haemophilus influenzae* and *Staphylococcus aureus* are the most common, and *P. aeruginosa* follows this initial colonisation (O'Sullivan & Freedman, 2009). By adolescence, up to 85% of patients in the UK are infected with *P. aeruginosa* (Davies 2002). Other infections that affect a smaller number of patients include *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia*, the *Burkholderia cepacia* complex, nontuberculous mycobacteria, and fungal *Aspergillus* species (Ratjen et al., 2015). In the US, methicillin-resistant *S. aureus* (MRSA) is often found with prevalence up to 30% among patients between 10 and 20 years old (Cystic Fibrosis Trust, 2014). Infections are typically diagnosed by conventional culture of respiratory samples, which can mean species that are not easily cultured (such as anaerobes) are missed during diagnosis (Tunney et al., 2008; Zemanick & Hoffman, 2016).

The CF-altered lung environment facilitates colonisation by bacteria, in particular *P. aeruginosa*, in three main ways. Firstly, bacterial cells are able to bind to the epithelium more easily than in a healthy lung. *P. aeruginosa* expresses adhesins, including pili and flagella, which bind to receptors on epithelial cells. Mutant pili-deficient *P. aeruginosa* strains have been shown to cause less severe disease in mouse models (H. Tang, Kays, & Prince, 1995). It has been shown that *P. aeruginosa*, as well as other pulmonary pathogens, can bind to the disaccharide GalNAc β 1-4Gal, a component of asialylated glycolipids which are found on the surface of epithelial cells (Krivan, Roberts, and Ginsburg 1988). CFTR mutant cells have an increased concentration of these glycolipids on the cell surface, which may be responsible for the increased attachment seen in mutant cells versus healthy cells (Davies et al. 1997; Saiman and Prince 1993). *Pseudomonas* species also produce a neuraminidase enzyme that can cleave the glycolipids, and could be involved in enhancing attachment by increasing availability of the GalNAc β 1-4Gal receptor (Saiman & Prince, 1993). Binding to the receptor is known to set off a signalling cascade response in epithelial cells which eventually leads to

increased levels of interleukin-8 and recruited neutrophils to the area (Ratner et al., 2001). This particular attachment mechanism is found in both *P. aeruginosa* and *S. aureus*, and could explain why these two species are preferentially found in CF versus others. Secondly, bacterial cells that have entered the lung are less likely to be cleared due to the defects in innate defence mechanisms. The cilia of epithelial cells are unable to beat normally and bacteria remain trapped in the thicker ASL rather than moved out of the lung (H Matsui et al., 1999). Interestingly however, other diseases with impaired mucociliary clearance do not lead to a similar incidence of *P. aeruginosa* infections, suggesting that this effect is contributory rather than the main factor for CF lung infections (Davies 2002). Lastly, the mucous plaques in CF lungs enable bacterial survival and growth, by limiting the diffusion of antimicrobials and promoting the formation of macrocolonies in hypoxic conditions (Hirotohi Matsui et al., 2006; Worlitzsch et al., 2002).

After initial attachment and colonization, bacteria can enact a series of adaptations to survive in the CF lung. One study found that *P. aeruginosa* isolates were hypermutable in nearly 40% of CF patients tested (Oliver et al., 2000). This enables them to switch on and off mechanisms to evade attacks from innate immune defence, for example with the expression of protease and siderophore virulence factors (Haas, Kraut, Marks, Zanker, & Castignetti, 1991; Klinger, Straus, Hilton, & Bass, 1978), as well as from continuous antibiotic treatments (Hancock & Speert, 2000). The alginate overproducing mucoidy phenotype is an example of a mutation that is often encountered after initial colonisation, and acquisition of mucoid isolates is associated with a decline in pulmonary function (Pritt, O'Brien, & Winn, 2007). There is significant evidence that *P. aeruginosa*, as well as other CF pathogens such as *A. xylosoxidans*, exist in biofilm structures within the CF lung; this has been shown with *in vitro* studies (Hirotohi Matsui et al., 2006), microscopy (Bjarnsholt et al., 2009; Ciofu et al., 2015; Hansen et al., 2009; P K Singh et al., 2000), and direct detection of quorum-sensing molecules (Barr et al., 2015; Chambers, Visser, Schwab, & Sokol, 2005; Erickson et al., 2002; Favre-Bonté et al., 2002; Middleton et al., 2002; P K Singh et al., 2000; Struss et al., 2013; Ward et al., 2003). In long-term chronic infections, however, *P. aeruginosa* strains have often been found to have quorum-sensing loss-of-function mutations, which suggests it may be a phenotype that is advantageous for early acquisition but deleterious for long-term survival (Hoffman et al., 2009; Jorth et al., 2015; R. S. Smith & Iglewski, 2003). Spatial

separation also causes divergent evolution of bacterial populations within regions of the lung, resulting in a collection of strains with diverse virulence phenotypes (Jorth et al., 2015).

2.4.3 Detection and treatments for CF infections

In addition to treatment for the other defects caused by CFTR mutation, such as pancreatic enzyme supplementation, there is a diverse range of interventions to target pulmonary function in particular. Clearance of airways, aided by chest physical therapy, is a standard treatment (Ratjen et al., 2015). Inhaled recombinant human DNase digests the extracellular DNA from dead neutrophil cells to reduce mucus viscosity (Fuchs et al., 1994), as well as reducing the antibiotic tolerance of biofilms (Alipour, Suntres, & Omri, 2009). Macrolide antibiotics such as azithromycin are also commonly administered by inhalation since they have both antibiotic and immunosuppressive effects. To combat inflammation in the lungs, treatments such as corticosteroids and ibuprofen are sometimes used, although they can have negative side effects (O'Sullivan & Freedman, 2009). Inhalation of saline treats the underlying defect rather than symptom suppression, as higher salt concentrations leads to increased water in the airways through osmosis and improves mucociliary clearance. Mannitol is used for similar effect (Aitken et al., 2012). There are also numerous drug and gene therapy treatments in clinical trials to restore CFTR activity, with the potentiator drug ivacaftor having been approved for use in patients with class III mutations (where CFTR is expressed but ion gating is defective) (Ramsey et al., 2011). Once severe pulmonary disease develops, drastic intervention in the form of lung transplantation is necessary. Therefore, the emphasis is on preventing or delaying the onset of infection as much as possible.

When infection is detected at an early stage, eradication is possible through the application of inhaled antibiotics. In Europe this is often paired with systemic drugs either through oral or intravenous means (Lund-Palau et al., 2016). Prophylactic use of antibiotics to prevent the onset of infection is not recommended, however (Mogayzel et al., 2013). *P. aeruginosa* infections in early childhood were found to be successfully eradicated in 90% of cases, yet within three years nearly half of patients were re-infected (Kidd et al., 2015). When infections eventually become chronic, the aim of treatment changes to suppression in order to limit inflammation damage, rather than aggressive attempts at eradication. A constant therapy of inhaled antibiotics is usually administered (Lund-Palau et al., 2016). During this period, patients may experience pulmonary exacerbations, episodes when a sudden increase in

symptoms and decrease in lung function are observed after previously stable condition. Intravenous antibiotics are the standard treatment, although the exact cause of exacerbations is still debated and both viral and fungal infections are contributors in some cases (Amin, Dupuis, Aaron, & Ratjen, 2010; Wat et al., 2008). In 25% of patients, the initial baseline level of lung function is not recovered after treatment which makes exacerbations a serious concern for CF health (Sanders et al., 2010).

Due to the relative success of early intervention compared to treatment of chronic disease, accurate diagnosis of infections is crucial. Microbiological culture of patient samples is the gold standard and routinely used. Young patients cannot easily produce sputum samples for culture, and can be tested instead using oropharyngeal (OP) or bronchoalveolar lavage (BAL) sampling. OP cultures can produce variable results, however, while BAL is not suitable for continuous monitoring due to the invasiveness of the procedure (Lund-Palau et al., 2016). A few other methods have developed that can improve detection and identification of CF pathogens. Antibodies against *P. aeruginosa* antigens in blood serum can be monitored, but they were not found to be useful at detecting early infection in a study of CF children (Douglas et al., 2010). Sequencing of 16s ribosomal RNA offers the potential to vastly improve identification of bacterial pathogens, especially for hard-to-culture species, and analysis of CF sputum samples have revealed a wide diversity of microbes not expected to be in the lung microbiome (Salipante et al., 2013). Due to high cost and expertise needed, however, it still is not widely used as a diagnostic method (Clarridge & Alerts, 2004). Mass spectrometry for the identification of pathogens has received a great deal of interest and there are now multiple commercial systems available for clinical diagnostics (Havlicek, Lemr, & Schug, 2013). However, although it can improve the accuracy of species identification, a preliminary sample culture step is still required and therefore, it does not provide a quicker turn-around than standard culture methods.

The process of bacterial infection in CF through the stages of initial colonisation, eradication, re-infection, and finally chronic establishment by *P. aeruginosa* is still an area of active research and far from fully understood. Better monitoring and detection of bacterial species (ideally through non-invasive means) can lead to significant improvements in early detection and subsequently treatment outcomes, as well as providing better understanding of how bacteria are living, adapting, and responding to treatment in the CF lung.

2.5 Project motivation and aims

2.5.1 Previous work in developing biosensors for *P. aeruginosa*

Construction of cell-free biosensors targeting quorum-sensing molecules from *Pseudomonas aeruginosa* was the focus of previous work in our group. This thesis builds on those constructs, which are briefly summarised here.

Two biosensors were developed, psB1A2:LasRV and pSB1A2:RhIR, comprising the LasR and RhIR transcription factors regulated by 3-oxo-C12-HSL and C4-HSL, respectively (Chappell, 2013). First, a range of putative inducible promoter sequences were selected from the *P. aeruginosa* genome and tested to identify promoters directly regulated by the transcription factors. Three inducible promoters for 3-oxo-C12-HSL and one for C4-HSL were found. Next, the promoters were placed in genetic circuits to regulate the expression of the reporter gene GFPmut3b, with the transcription factors constitutively expressed on the same plasmid (Figure 3). The constructs were characterised in *E. coli* and shown to be able to detect AHLs from *P. aeruginosa* cultures. The functionality of the biosensors *in vitro* using commercial cell-free extracts was then also demonstrated (Chappell, 2013; Chappell et al., 2013).

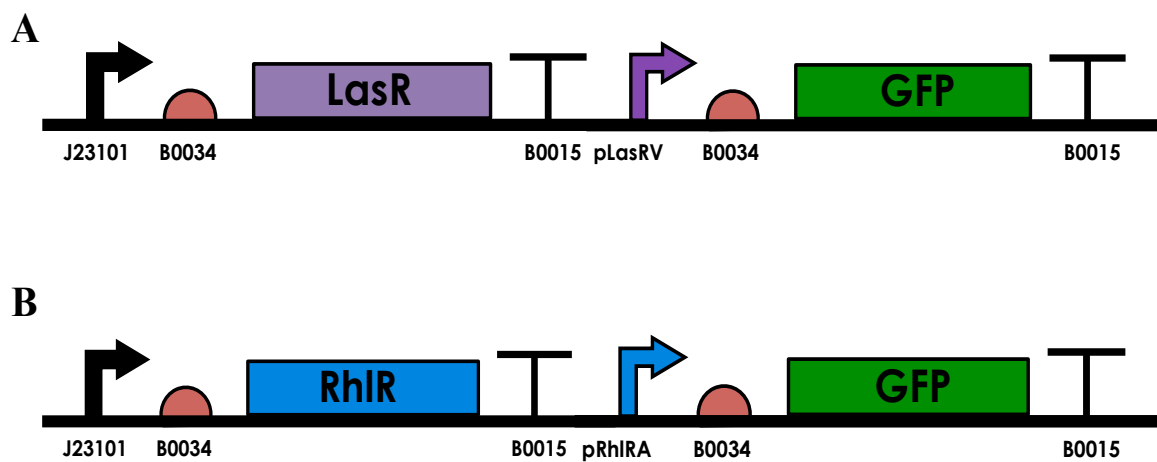


Figure 3 Architecture of biosensors previously developed for *P. aeruginosa* AHLs. (A) 3-oxo-C12-HSL-detecting psB1A2:LasRV biosensor. The LasR transcription factor is expressed constitutively under control of J23101 promoter and B0034 RBS. GFP is under control of the LasR-regulated promoter pLasRV. **(B)** C4-HSL-detecting pSB1A2:RhIR biosensor. The RhIR transcription factor is expressed constitutively under control of J23101 promoter and B0034 RBS. GFP is under control of the RhIR-regulated promoter pRhIR.

2.5.2 Motivation

The early detection of CF lung infections, especially by the dominant pathogen *P. aeruginosa*, is a critical requirement for improving the prognosis of patients as discussed above. It is known that QS is an adaptive behaviour of bacteria which plays a role in establishing biofilm colonies, the presumed state of bacteria in chronic infections, as well as regulating virulence. Therefore it is hypothesised that detection of QS molecules within the CF lung can provide a better insight into how bacteria survive and adapt within this unique environment. While there have been studies to measure these signals in clinical samples, it remains a time-consuming process that requires expensive equipment and cannot be widely used. The aim of this thesis was to determine if cell-free biosensors could be a viable tool for measuring QS molecules in clinical samples of CF patients and further develop the biosensor assay for diagnostic purposes.

2.5.3 Aims

- Expand the cell-free biosensor assay to include additional targets in CF pathogens involved in biofilm formation
- Determine if cell-free biosensors could measure AHLs in CF clinical samples
- Identify the key bottlenecks and optimise the biosensor assay for diagnostic testing

3 Materials and Methods

3.1 Materials

Table 1 Bacterial strains

Name	Genotype	Use	Source
<i>E. coli</i> NEB 5α	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Plasmid stocking	New England Biolabs (#C2988J)
<i>E. coli</i> NEB 10β	<i>Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (Str^R) rph spoT1 Δ(mrr- hsdRMS-mcrBC)</i>	Cloning	New England Biolabs (#C3019H)
<i>E. coli</i> BL21 Gold (DE3)	<i>E. coli B F– ompT hsdS(r – m –) dcm+ Tetr gal λ(DE3) endA Hte</i>	<i>In vivo</i> characterisation, protein production	Agilent Technologies (#230132)
<i>P. aeruginosa</i> PA01	Wild-type	Biofilm studies	Alain Filloux group
<i>P. aeruginosa</i> PA14	Wild-type	Biofilm studies	Alain Filloux group
<i>P. aeruginosa</i> PAK	Wild-type	Biofilm studies	Alain Filloux group

For additional materials, see Supplementary Methods in Appendix 8.3 (p. 150)

3.2 DNA manipulation

Table 2 DNA plasmids

Plasmid name	Insert parts
pSB1A2:VpsT ₀₉₁₆	p0916_B0034_GFPmut3b_B0015_J23101_B0034_VpsT_B0015
pSB1A2:VpsT ₀₉₁₇	p0917_B0034_GFPmut3b_B0015_J23101_B0034_VpsT_B0015
pSB1A2:VpsT ₀₉₂₈	p0928_B0034_GFPmut3b_B0015_J23101_B0034_VpsT_B0015
pSB1A2:VpsT ₀₉₃₄	p0934_B0034_GFPmut3b_B0015_J23101_B0034_VpsT_B0015
pSB1A2:VpsT ₁₅₈₅	p1585_B0034_GFPmut3b_B0015_J23101_B0034_VpsT_B0015
pSB1A2:VpsT _{VpsL}	pVpsL_B0034_GFPmut3b_B0015_J23101_B0034_VpsT_B0015
pSB1A2:LasRV	J23101_B0034_LasR_B0015_pLasV_B0034_GFPmut3b_B0015
pSB1A2:RhIR	J23101_B0034_RhIR_B0015_pRhIA_B0034_GFPmut3b_B0015
pSB1A2:CepRI319	B0015(RC)_CepR(RC)_B0034(RC)_J23101(RC)_pCepI319_GFPmut3b_B0015
pSB1A2:CepRI344	B0015(RC)_CepR(RC)_B0034(RC)_J23101(RC)_pCepI344_GFPmut3b_B0015
pSB1A2:GFP	J23101_B0034_GFPmut3b_B0015
pSB1A2:I13504	B0034_GFPmut3b_B0015

RC= reverse compliment

For DNA part sequences, see Appendix Table A8 (p. 153)

3.2.1 Restriction digestion

NEB enzymes were used according to the manufacturer's protocol. Generally, DNA digest reactions were made up of 500-1000 ng plasmid DNA, 10 U enzyme, 1X corresponding

buffer and water up to a final volume of 25 µl, then incubated at 37°C for 1 h. Digested fragments were analysed by gel electrophoresis.

3.2.2 Dephosphorylation

DNA vectors linearised by restriction digests were pre-treated with Antarctic Phosphatase (NEB) before ligation to reduce number of background colonies. To 25 µl digest reactions, 2.5 U Antarctic Phosphatase was added and the reaction incubated at 37°C for 30 min. DNA fragments were then purified using agarose gel extraction.

3.2.3 Phosphorylation of 5' ends

The 5' ends of double-stranded linear DNA fragments were phosphorylated using T4 Polynucleotide Kinase (NEB), in reactions consisting of 100 ng DNA, 10 U Polynucleotide Kinase, and 1X T4 DNA Ligase buffer in a final volume of 25 µl. Reactions were then incubated at 37°C for 30 min.

3.2.4 Ligation

Purified DNA fragments were assembled using T4 DNA Ligase, consisting of 50 ng vector and typically 2:1 molar ratio of insert: vector DNA, 200 U ligase and 1X T4 DNA Ligase buffer in a 25 µl volume reaction. Reactions were incubated at room temperature for 1 h, or overnight at 16°C. 5 µl of ligation reaction was transformed into NEB 10β cells (section 3.2.9).

3.2.5 In-Fusion cloning

For assembly of PCR fragments, In-Fusion HD Cloning kit (Clontech) was used. PCR fragments were synthesized in PCR reactions with Phusion polymerase (section 3.2.7), and then purified either with PCR clean-up, or gel extraction if there were non-specific bands. DNA fragments (50 ng vector, 1:1 – 3:1 molar ratio insert: vector) were combined in 10 µl reactions containing 2 µl HD enzyme premix and incubated at 50°C for 15 min. 5 µl of assembly reaction was transformed into NEB 10β cells (section 3.2.9).

3.2.6 Inverse PCR and ligation

Linearised vectors were synthesised by PCR with Phusion polymerase (section 3.2.7). PCR reactions were treated with DpnI to digest template DNA, with 20 U enzyme directly added

to the PCR reaction and incubated at 37°C for 1 h. Reactions were then purified using the Qiagen PCR purification kit. Ligation reactions were set up with 50 ng phosphorylated DNA, 200 U T4 DNA ligase, 1X T4 DNA ligase buffer, and ddH₂O up to 25 µl, then incubated overnight at 16°C before transformation into NEB 10β cells.

3.2.7 Polymerase Chain Reaction (PCR)

To generate DNA fragments for assembly into plasmids, Phusion High-Fidelity DNA polymerase (NEB) was used. PCR reactions were made up with 1 U Phusion polymerase, 1X HF buffer or 1X GC buffer for fragments > 2 kB, 100 nM dNTPs, 200 nM each primer, and 1 ng template DNA in a total volume of 50 µl. PCR cycles were programmed as follows: denaturation at 95°C for 2 min, then 30 cycles of (i) 30 s at 95°C, (ii) 30 s at annealing temperature, (iii) extension at 72 °C for 15s/kb + 15s, followed by a final extension at 72°C for 5 min.

For colony PCRs, Taq DNA polymerase was used. 25 µl reactions were prepared with Taq DNA polymerase, Taq buffer, 100 nM dNTPs, 200 nM each primer, and 1 µl of template (picked colony dissolved in 20 µl water). PCR cycles were programmed as follows: cell lysis and denaturation at 95°C for 10 min, then 30 cycles of (i) 30 s at 95°C, (ii) 30 s at annealing temperature, (iii) extension at 68°C for 60s/kb, followed by a final extension at 68°C for 5 min. PCR products were analysed by gel electrophoresis.

3.2.8 DNA oligonucleotides

PCR primers were synthesised by IDT, and dissolved in water to a final concentration of 100 µM. Working solutions were made at 10 µM.

3.2.9 Transformation of *E. coli*

Bacterial cells (NEB 5α, NEB 10β, and BL21 Gold (DE3)) were chemically transformed. 50 µl aliquots of cells were mixed with DNA and incubated on ice for 30 min. Cells were heat-shocked at 42°C for 30 s, then put back on ice for 5 min. 450 µl SOC medium was added to cells and incubated with shaking at 37°C for 1 h, unless plasmids had ampicillin resistance in which case the recovery step was skipped. Cells were spread on LB agar plates containing selective antibiotic and incubated overnight at 37°C.

3.2.10 Isolation of plasmid DNA

For purification of plasmid DNA, the QIAprep miniprep kit or QIAfilter plasmid midi/maxiprep kit (Qiagen) was used according to manufacturer's protocol. For isolation of plasmid DNA to use in cell-free reactions, 200 ml cell culture was prepared and the purified DNA was re-suspended in sterile nuclease-free water (Sigma Aldrich).

3.2.11 Isolation of linear DNA

To isolate DNA fragments from agarose gels following electrophoresis, the Qiaquick Gel Extraction kit (Qiagen) was used according to manufacturer's protocol. For purification directly from PCR reactions, the Qiaquick PCR purification kit (Qiagen) was used following manufacturer's protocol.

3.2.12 DNA gel electrophoresis

Agarose gels were prepared with 1% w/v agarose, 1X TAE buffer, and either GelRed (Biotium) at 0.5X or SYBR® Safe (Thermo Fisher Scientific) at 1X. Samples were loaded with 1X loading buffer and gels were run in 1X TAE buffer, typically at 100 V for 30-45 min.

3.2.13 Quantification of DNA

The concentration of DNA solutions was measured using the NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific), or the NanoPhotometer® P-Class P300 (Implen GmbH) as instructed by the manufacturers.

3.2.14 Sequence verification of DNA

DNA plasmids were sequenced either by Source Bioscience (Nottingham, UK) or Eurofins Genomics (Ebersberg, Germany). For Source Bioscience, 5 µl plasmid DNA at a final concentration of approx. 100 ng/µl and 5 µl sequencing primers at 3.2 µM were submitted. For Eurofins Genomics, 15 µl plasmid DNA between 50-100 ng/µl was pre-mixed with 2 µl primer (starting concentration 10 µM) for submission.

3.3 Acyl homoserine lactones

Lyophilised AHLs were dissolved in ethyl acetate (acidified with 0.01% acetic acid) to a final concentration of 110 mM and serial dilutions were made in DMSO to create the stock solutions.

3.4 Cell-free systems

3.4.1 *E. coli* S30 cell-free system

The *E. coli* S30 Extract System for Circular DNA (Promega, #L1020) was used for expression of genetic circuits from DNA plasmids. The reactions were prepared with a few modifications from manufacturer's protocol. The total volume was scaled down from 50 µl to 25 µl. Each reaction was composed of 10 µl S30 Pre-Mix, 7.5 µl S30 cell extract, 1.25 µl amino acid mixture minus methionine, 1.25 amino acid mixture minus cysteine or leucine, 1 µg plasmid DNA (2 µg for pSB1A2:RhIR), 0.5 µl inducer if used, and nuclease-free water (Sigma Aldrich) up to 25 µl. If using inducer, stock solution was added directly before measurement. Reactions were transferred to a black clear-bottom 384-well plate (Greiner Bio-One) and measured either in the POLARstar or CLARIOstar plate reader (BMG Labtech). Plates were incubated at 30°C for 8 hours, and fluorescence (485 nm excitation, 520 nm emission) measured every 15 min with 10 s shaking at 200 rpm before each measurement for up to 8 hours.

3.4.2 Data analysis

Three cell-free reactions were measured for each sample. The GFP fluorescence (485 nm excitation, 520 nm emission) was measured at 15 min intervals for up to 8 hours, while incubated at 30°C. There is an initial decrease in fluorescence until the solution reached a stable temperature, with the lowest point reached at 30 min. The increase in fluorescence for GFP-expression plasmids would plateau at 240 min. Therefore gain in fluorescence was calculated as below:

$$Gain(X) = Fl_x t_{240\ min} - Fl_x t_{30\ min}$$

The fluorescence of samples from different experiments was normalised by calculating a relative output value. Positive control reactions were included in each plate reader

measurement and the mean fluorescence was used as standard value to normalise to. The relative output was calculated as follows:

$$Relative\ output\ (X) = \frac{Gain\ (X)}{Gain\ (std)}$$

Gain (X) is the gain in fluorescence for sample X and *Gain (std)* is the mean gain in fluorescence for three reactions with positive control plasmid, and inducer if required. The mean and standard deviation was calculated for each sample.

3.4.3 Statistical analysis

The mean of three replicate reactions for each clinical sample tested was calculated and samples were grouped by *Pseudomonas* infection status in mixed populations, or by the concentration of 3-oxo-C12-HSL according to LC-MS/MS. Unpaired t-test was performed using the software GraphPad Prism 7.0.

3.5 In vivo characterisation

3.5.1 Growth and fluorescence measurements

E. coli BL21-Gold (DE3) cells were transformed with plasmids and plated on LB agar with 100 µg/ml ampicillin. 5 ml M9 medium with 100 µg/ml ampicillin was inoculated with a single colony and incubated overnight at 37°C with 220 rpm shaking. Overnight cultures were then diluted into 5 ml pre-warmed M9 medium with 100 µg/ml ampicillin at 100X dilution and incubated at 37°C with 220 rpm shaking for 3-4 hours. The O.D.₆₀₀ of the cultures was measured using a spectrophotometer, diluted into 1 ml of pre-warmed M9 medium to a final O.D.₆₀₀ of 0.07, and incubated again at 37°C with 220 rpm shaking for 1 hour.

Three aliquots of 196 µl culture were transferred to a black clear-bottom 96-well plate (Greiner Bio-One). Cultures were induced with 4 µl AHL stock solutions to give final concentrations of 1, 100, and 10000 nM. To un-induced control cultures, 4 µl DMSO was added instead of AHL solution. Plates were incubated at 37°C with 200 rpm continuous shaking in the POLARstar Omega plate reader (BMG Labtech), and GFP fluorescence (485 nm emission, 520 nm excitation) and absorbance at 600 nm was measured every 15 min for 8 hours.

3.5.2 Data analysis

The background absorbance of M9 medium was subtracted from the absorbance of each culture. The background fluorescence of *E. coli* BL21 Gold(DE3) with the pSB1A2:I13504 plasmid was subtracted from each culture. The corrected fluorescence was divided by the corrected absorbance to calculate a normalised FI/OD₆₀₀ value for each sample at each timepoint. FI/OD₆₀₀ at 4 hours was taken when the majority of GFP expression had taken place. Mean and standard deviation of three repeats was calculated.

3.6 AHL extraction from CF clinical samples

The basic method for extraction of AHLs from clinical samples is described here, and modifications for method optimisation are described in greater detail in Results (Section 4.2). Briefly, BAL or sputum samples (approximately 1 ml) were added to 1 ml PBS. An equal volume – approximately 2 ml – of dichloromethane (acidified, 0.1% formic acid) or ethyl acetate (acidified, 0.1% acetic acid) was added and the mixture was gently shaken for 5 minutes. Samples were centrifuged at 4000 rpm for 1 min to help the separation of organic and aqueous phase, and then the organic phase was removed and collected in a glass vial. This was repeated three times in total. The collected solvent of each sample was divided into four aliquots of equal volume and dried under N₂ gas in a sample concentrator (Techne). One aliquot was resuspended in 25 µl methanol (acidified, 0.1% formic acid) for LC-MS/MS analysis. For the cell-free biosensor assays, one aliquot was re-suspended in 80 µl cell-free master mix and transferred to a 384-microwell plate in three reactions of 25 µl each. Fluorescence was monitored as described in section 3.4.1.

3.7 LC-MS/MS

LC-MS/MS analysis was conducted based on the protocol described in Struss et al. 2013 using the 1290 LC system and 6550 iFunnel Quadrupole-Time of Flight mass spectrometer with electrospray ionisation (Agilent). A Zorbax Extend C-18 LC column (Agilent), 2.1 x 50 m and 1.8 µm particle size, was used for LC. Protonated molecules [M+H]⁺ were subjected to collision induced disassociation (10 eV) and product ions monitored either throughout or based on retention time windows. Fragment ions (mass measured to 4 decimal places) were quantified by the summed peak area +/- 20 ppm. Calibration curves for quantitation were

generated by injecting increasing volumes of AHL standard solutions in methanol and plotting the summed peak area of fragment ions against loaded sample. The lower limit of quantitation was determined as the concentration of analyte with a signal-to-noise ratio of 10:1, while the lower limit of detection was calculated for a signal-to-noise ratio of 3:1.

Sputum sample extracts were reconstituted in 100 µl methanol (LC-MS CHROMASOLV[®]-grade, Sigma Aldrich) when whole, or 25 µl when aliquoted. 0.5 – 10 µl was injected. Each sample was analysed for the presence of eight AHLs. The concentration was calculated using the equation of the AHL standards calibration curves.

Table 3 AHLs analysed by LC-MS/MS

AHL	M+H+	MS/MS product ions					All fragment ions
	m/z	Protonated HSL ring	m/z	Acyl chain	m/z		
C4-HSL	172.09682	C4 H7 N O2	102.05495	C4 H7 O	71.04914		102.05495, 56.04948, 74.06004, 71.04914
C6-HSL	200.12812	C4 H7 N O2	102.05495	C6 H11 O	99.08044		102.05495, 71.0858, 74.06004, 99.08044
C7-HSL	214.14377	C4 H7 N O2	102.05495	C7 H13 O	113.09609		102.05495, 56.04948, 74.06004, 85.1012, 113.09609
C8-HSL	228.15942	C4 H7 N O2	102.05495	C8 H15 O	127.11174		102.05495, 57.0699, 74.06004, 127.11174
3-oxo-C8-HSL	242.13868	C4 H7 N O2	102.05495	C8 H13 O2	141.09101		102.05495, 56.04948, 74.06004, 141.09101
C10-HSL	256.19072	C4 H7 N O2	102.05495	C10 H19 O	155.14304		102.05495, 57.0699, 71.0858, 74.06004, 95.0855, 155.14304
3-oxo-C10-HSL	270.16998	C4 H7 N O2	102.05495	C10 H17 O2	169.12231		102.05495, 56.04948, 74.06004, 127.1117, 169.12231
3-oxo-C12-HSL	298.20128	C4 H7 N O2	102.05495	C12 H21 O2	197.15361		102.05495, 56.04948, 74.06004, 155.14304, 197.15361, 240.19581
[¹³C]₄-3-oxo-C12-HSL	302.2147	[¹³ C] ₄ H7 N O2	106.06837	C12 H21 O2	197.15361		106.0684, 59.05907, 77.0701, 155.14304, 197.15361, 242.20252

Table 3, supplementary information

AHL	Retention time (min)	LLOD (nM)*	LLOQ (nM)*
C4-HSL	1.2	44	150
C6-HSL	4.4	11	30
C7-HSL	6.0	11	30
C8-HSL	7.3	4	12
3-oxo-C8-HSL	5.5	4	12
C10-HSL	9.3	4	12
3-oxo-C10-HSL	7.9	4	12
3-oxo-C12-HSL	9.8	3	10
[¹³C]₄-3-oxo-C12-HSL	9.7	3	10

* Detection concentration calculated for 2.5 µl injection volume

4 Results

4.1 AHL biosensors development and characterisation

4.1.1 Aim & Introduction

While *P. aeruginosa* is the main pathogen in adult CF lung infections, there are numerous other bacterial species that can inhabit and colonise the CF lung (see Section 2.4.2). It would be desirable to be able to distinguish between species by monitoring the QS molecules that are produced by different bacteria. This would expand the functionality of the biosensor platform beyond the two genetic circuits previously developed for detecting *P. aeruginosa*, as reviewed in Section 2.5.1 (Chappell, 2013). The *Burkholderia cepacia* complex (Bcc) was chosen as a new target, both for its clinical relevance and the similarity of its QS mechanisms to the *P. aeruginosa* AHL system. These topics will be briefly summarised here.

The Bcc is a group of at least 20 different species that are closely related (Depoorter et al., 2016; Vicenzi et al., 2016), and were originally thought to belong to the *Pseudomonas* genus (Laraya-Cuasay, Lipstein, & Huang, 1977). Infections by Bcc in the CF lung can cause rapid and fatal ‘cepacia syndrome’ (Isles et al., 1984), although in some cases long-term survival with chronic Bcc infections has also been recorded (Drevinek & Mahenthiralingam, 2010; Vandamme et al., 1997). Infections by Bcc pathogens are particularly dangerous because they are transmittable between CF patients and have multiple antibiotic resistance mechanisms. The vast majority (85-97%) of CF infections by the Bcc are caused by two species, *Burkholderia cenocepacia* and *Burkholderia multivorans* (Drevinek & Mahenthiralingam, 2010).

The QS system in Bcc species was initially identified in *B. cenocepacia* (then known as *B. cepacia*) to be a LuxI/R-type QS system consisting of an AHL synthase, CepI, and an AHL-responsive regulator, CepR (Lewenza, Conway, Greenberg, & Sokol, 1999). It was subsequently found to be highly conserved amongst all species of the Bcc (Sokol et al 2007). CepI primarily synthesises N-octanoyl-HSL (C8-HSL). Additional QS regulation has also been identified in particular species of the Bcc, including the CciIR system in certain strains of *B. cenocepacia* (Baldwin, Sokol, Parkhill, & Mahenthiralingam, 2004; Malott, Baldwin, Mahenthiralingam, & Sokol, 2005), which uses N-hexanoyl-HSL (C6-HSL), and the BviIR pair in *Burkholderia vietnamiensis* (Conway & Greenberg, 2002; Malott & Sokol, 2007),

which uses N-decanoyl-HSL (C10-HSL). These could potentially enable species differentiation by detection of C6-HSL or C10-HSL, whereas presence of C8-HSL would indicate infection by any of the Bcc species. Since C8-HSL is an AHL like 3-oxo-C12-HSL and C4-HSL, it is straightforward to adapt the existing AHL-sensitive biosensors towards detection of C8-HSL. In contrast, the Gram-positive species which are also prevalent in CF, such as *S. aureus*, have small peptide-based QS mechanisms and would require a different biosensor design.

In this chapter, it will be discussed how a biosensor was designed and constructed for the detection of C8-HSL from the Bcc. This panel of three biosensors were then fully characterised by comparing their behaviour *in vivo* and *in vitro* when induced by their cognate inducer and closely related AHLs.

4.1.2 Design and construction of a C8-HSL biosensor

The CepIR QS system was selected for the construction of a C8-HSL biosensor that would be able to detect members of the Bcc. For the detection module, the CepR gene was placed under the control of the BBa_J23101 constitutive promoter and the BBa_B0034 medium-strength RBS from the BioBricks Part Registry (<http://parts.igem.org>).

A Cep-based sensor had been constructed before to study QS in mixed biofilms of *P. aeruginosa* and *B. cepacia* (Riedel et al., 2001). This was made up of a CepR gene and pCepI:GFP translational fusion. As this was found to be effective at responding to C8-HSL, the same promoter was selected for the responsive element of this biosensor design. The pCepI promoter, as defined in that work, is composed of a 344 bp sequence directly upstream of the CepI ORF, from the *B. cenocepacia* H111 genome. The entire CepR transcription unit and pCepI promoter were synthesised by GeneArt (ThermoFischer Scientific), flanked by BioBrick prefix and suffix sequences.

A full-length and a truncated promoter sequence were both tested to determine if the sequence directly upstream of the ORF could be replaced with a standard RBS without affecting promoter activity. Therefore, two versions of the C8-HSL biosensor were assembled using BioBrick restriction digest cloning and an In-Fusion DNA assembly kit. In the first version, the entire insert of the synthesised promoter was cut out with EcoRI and SpeI restriction enzymes. The destination plasmid pSB1A2:I13504 was linearised using the EcoRI and XbaI restriction enzymes, and the two parts were ligated together. This created a construct where the full-length 344bp pCepI promoter was upstream of the B0034 RBS and GFPmut3b gene, called pSB1A2:pCepI344 (Figure 4A).

In the second version, the pCepI sequence was truncated by removing the first 25bp upstream of the CepI ORF, making a promoter of 319 bp length (pCepI319). PCR primers were designed to amplify the CepR transcriptional unit and a truncated pCepI sequence using the synthesised promoter as template. A PCR fragment was then generated containing the plasmid backbone with the B0034 RBS and the GFPmut3b gene, using the pSB1A2:I13504 plasmid as template. The two fragments were assembled by the standard In-Fusion protocol to create the pSB1A2:pCepI319 plasmid (Figure 4A).

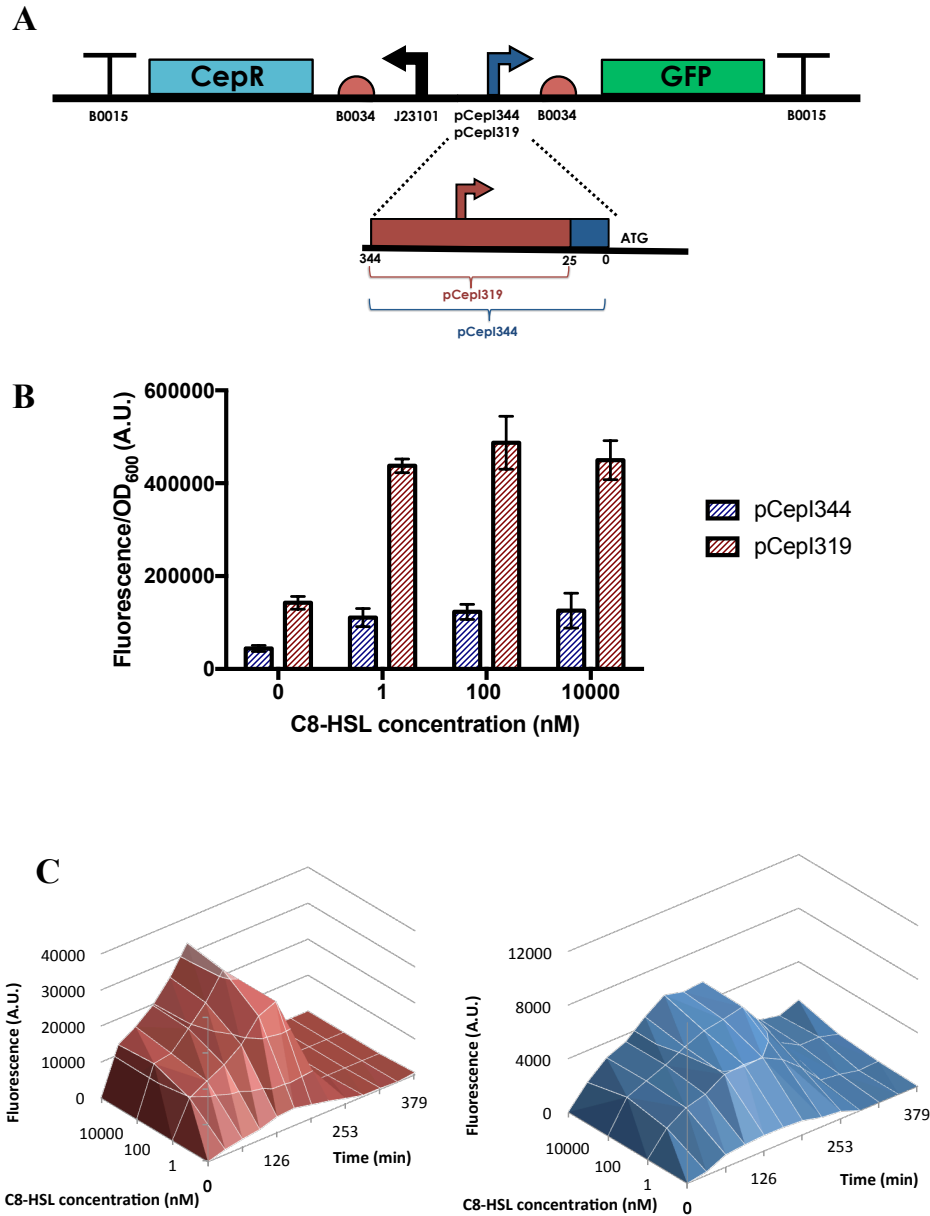


Figure 4 Characterisation of the C8-HSL biosensors *in vivo* (A) Design of C8-HSL biosensor constructs. Inset shows the structure of the pCepI344 and pCepI319 promoters, with numbers underneath representing the upstream distance in bp from the start codon of the native CepI ORF. (B) The GFP output of the biosensors following induction with varying concentration of C8-HSL. Fluorescence at 1h post-induction was normalised by OD₆₀₀ at same timepoint. Mean and standard deviation of three cultures is shown. (C) The rate of fluorescence increase, determined as change in fluorescence every 30 min. Left is the pSB1A2:pCepI319 plasmid and right is the pSB1A2:pCepI344 plasmid.

4.1.3 Characterisation of C8-HSL biosensor *in vivo* and *in vitro*

The biosensor constructs were first tested for their responsiveness to C8-HSL in a whole-cell context using *E. coli*. Since *E. coli* does not produce C8-HSL, the genetic circuit should be turned off in the absence of AHL and GFP expression would be induced with the addition of the AHL. *E. coli* BL21 Gold (DE3) cells were transformed with the pSB1A2:pCepI344 and pSB1A2:pCepI319 plasmids. Overnight cultures were diluted 1/100 in minimal M9 media, grown for 3 hours at 37°C, diluted to a standard OD₆₀₀ of 0.07, and grown for another hour. 200 µl aliquots induced with various concentrations of C8-HSL were transferred to a 96-well plate. Plates were incubated in a plate reader at 37°C with continuous shaking for 8 hours. *E. coli* harbouring the plasmid pSB1A2:I13504, which has the GFP gene without a promoter, was measured in parallel and the background fluorescence was subtracted from the final measurements. The background absorbance of M9 medium only was subtracted from absorbance of cell cultures. Fluorescence was divided by absorbance to normalise for cell growth.

For both pCepI variants, induction by C8-HSL led to GFP expression in the whole-cell biosensors (Figure 4B). There was no difference in the level of gene expression seen between the lowest and highest inducer concentration tested, 1 nM and 10 µM respectively, for either promoter. This suggests that the devices have a switch-like behaviour that is turned off in the absence of inducer and turned on at a concentration of 1 nM and higher. However, the level of gene expression did vary slightly between the two constructs, with pCepI319 having a 3-fold activation over uninduced versus 2.5-fold for pCepI344 at 1 nM induction with C8-HSL. The pCepI319 promoter also had a higher baseline expression of GFP when uninduced, meaning it is a more leaky promoter than pCepI344. Both constructs had increasing GFP expression during the first 4 hours after induction (Figure 4C), while the cells were in exponential growth phase (Appendix figure A1).

Next the two plasmids were tested within a cell-free context to determine if their behaviour was comparable *in vitro* to *in vivo*. Cell-free reactions were prepared with 1 µg plasmid DNA up to a total volume of 25 µl and incubated in a plate reader at 30°C. Reactions were induced with C8-HSL to final concentration between 1 and 10000 nM. Fluorescence was measured every 15 min for 8 hours.

In the cell-free systems, the dynamic range of the C8-HSL biosensors was greater than when tested in whole-cell context. Both constructs showed a dose-dependent response to the increasing concentrations of C8-HSL (Figure 5A). The dynamic range of pCepI344 and pCepI319 extended from 1 nM to their maximum activation at 1000 nM. Similarly as seen in the *in vivo* characterization, the promoter strength of pCepI344 was lower than that of pCepI319. At the lowest inducer concentration of 1 nM, the fold change of pCepI344 was 1.3 compared to 2.4 for pCepI319 at the same concentration. Comparing the signal output in terms of fluorescence, the pCepI344 promoter has 33% of the signal produced by pCepI319 at the maximum activation concentration of 1000 nM C8-HSL. Furthermore, in the absence of C8-HSL there was a higher basal expression of pCepI319 than pCepI344, which had also been observed *in vivo*. In terms of the dynamics of the genetic circuit, the highest rate of GFP expression was reached before the first hour, specifically between 30 and 45 min for pCepI319 (Figure 5B) and between 45 and 60 min for pCepI344 (Figure 5C).

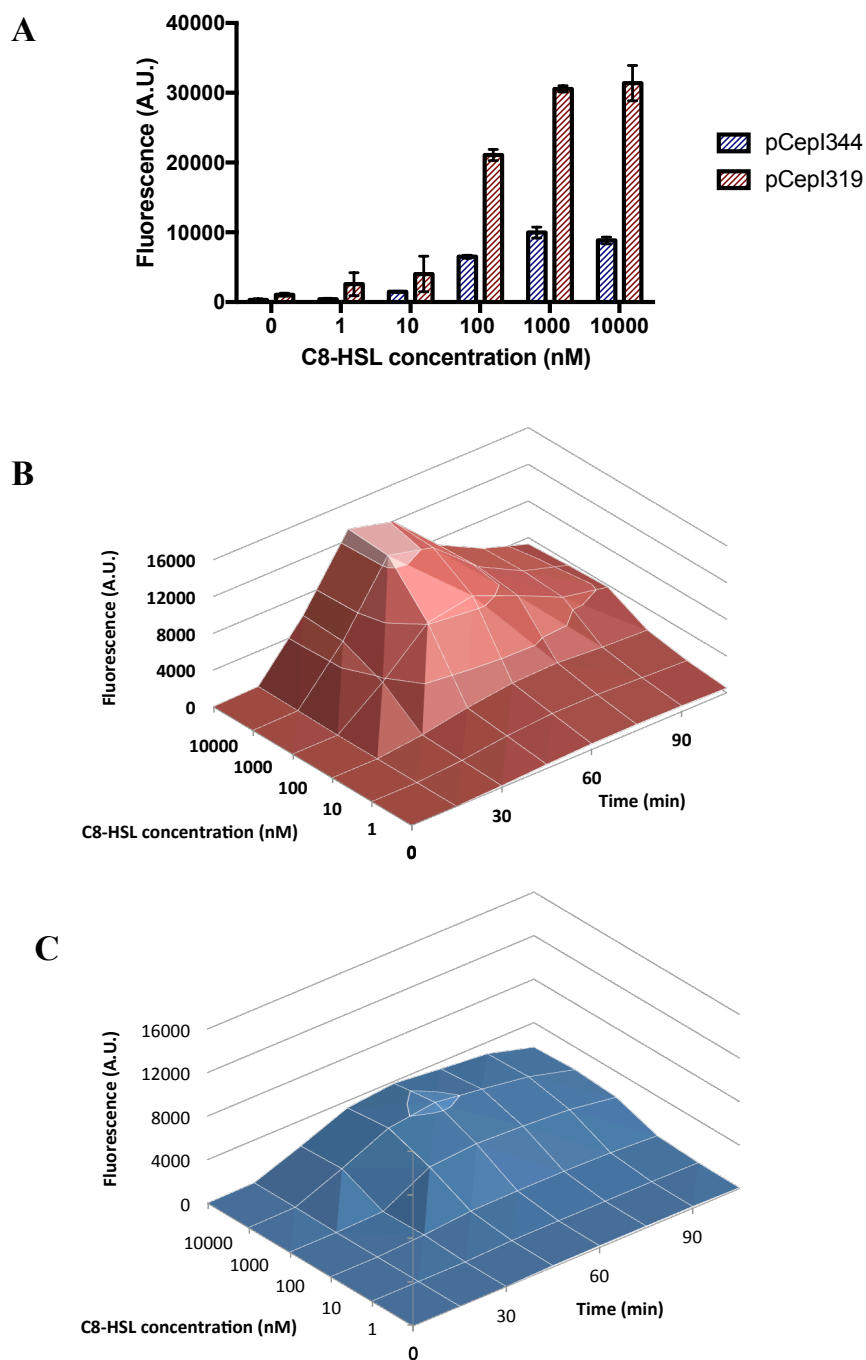


Figure 5 Characterisation of C8-HSL biosensors *in vitro*. (A) The GFP output of the biosensors following induction with varying concentration of C8-HSL. Fluorescence at 1h post-induction is shown with mean and standard deviation of three reactions. (B) The rate of fluorescence increase for pSB1A2:pCepI319, calculated as change in fluorescence every 15 min. (C) The rate of fluorescence increase for pSB1A2:pCepI344, calculated as change in fluorescence every 15 min.

The design and testing of these C8-HSL-detecting biosensors showed that the CepR transcription factor was able to respond to added C8-HSL and cause upregulation of GFP through both versions of the pCepI promoter. The behaviour of the circuits *in vivo* and *in vitro* did vary, with the *in vivo* devices having a more binary ON-OFF activation state (at least at the concentration range tested), whereas *in vitro* a concentration-dependent response was observed. Furthermore, the total output of the sensors was higher *in vivo* than *in vitro*, although in both cases the pCepI319-based device had a higher output than the pCepI344 one. Based on these results, the pSB1A2:pCepI319 construct was chosen as the most suitable genetic circuit to use as a C8-HSL biosensing device. Although it has a small basal level of expression, the promoter strength is much greater than pCepI344 and would therefore be able to produce a stronger signal. The construct will be referred to as the pSB1A2:CepRI biosensor from here onwards.

4.1.4 Characterisation of the AHL biosensors panel

Including the pSB1A2:CepRI biosensor, three different constructs were now available that targeted AHLs of varying acyl chain lengths. The pSB1A2:LasRV biosensor and pSB1A2:RhlRA can detect 3-oxo-C12-HSL and C4-HSL respectively (Chappell, 2013), the two AHLs synthesised by *P. aeruginosa*. With the inclusion of the C8-HSL-specific biosensor, it was desirable to directly compare performance across the panel of three biosensors. In particular, the specificity of the detection module towards various AHLs needed to be determined, in order to find out if the biosensors could be cross-activated by the same AHLs. Previously the two *P. aeruginosa*-targeting biosensors had been characterised in terms of their specificity *in vivo* (Chappell, 2013). Since the aim is eventually to use cell-free systems for the application of biosensors in healthcare settings, it was necessary to also determine the specificity of the constructs when expressed in *in vitro* systems.

Eight AHLs were chosen with acyl chain lengths spanning the range of the three biosensors' target molecules, from C4 to C12. Table 4 summarises the AHLs and bacterial species that are known to synthesise them.

Table 4 AHLs used for characterising biosensor specificity

AHL	Organism	Reference
C4-HSL	<i>Aeromonas hydrophila</i> , <i>A. salmonicida</i> , <i>P. aeruginosa</i> , <i>Serratia liquefaciens</i>	Lynch et al. 2002; Swift et al. 1997; Winson et al. 1995; Eberl et al. 1996
C6-HSL	<i>Chromobacterium violaceum</i> , <i>P. aureofaciens</i> , <i>Yersinia enterocolitica</i>	Mcclean et al. 1997; Wood et al. 1997; Throup et al. 1995
C7-HSL	<i>Edwardsiella tarda</i> , <i>Rhizobium leguminosarum</i>	Morohoshi et al. 2004; Lithgow et al. 2000
C8-HSL	<i>B. cepacia</i> , <i>B. cenocepacia</i> , <i>Y. pseudotuberculosis</i> , <i>R. leguminosarum</i>	Lewenza et al. 1999; Weingart et al. 2005; Ortori et al. 2007; Lithgow et al. 2000
C10-HSL	<i>B. vietnamiensis</i>	Conway & Greenberg 2002

3-oxo-C10-HSL	<i>V. anguillarum</i> , <i>B. pseudomallei</i>	Milton et al. 1997; Ulrich et al. 2004
C12-HSL	<i>Brucella melitensis</i>	Taminiau et al. 2002
3-oxo-C12-HSL	<i>P. aeruginosa</i>	Pearson et al. 1994

Firstly, the specificity of the biosensors *in vivo* was determined. *E. coli* BL21-Gold (DE3) was transformed with each plasmid. Single colonies were picked and cultured overnight in minimal M9 medium. Cultures were diluted 1/100 into pre-warmed M9 medium with ampicillin and grown for 3-4 hours at 37°C, then diluted to OD₆₀₀ 0.07 and grown for 1 hour. 196 µl culture was transferred to each well in a 96-well plate and 4 µl AHL solution in DMSO was added as inducer. The plate was incubated in a plate reader with continuous shaking at 200 rpm and 37°C. GFP and absorbance measurements were taken every 15 min for eight hours. *E. coli* cells containing empty plasmid pSB1A2:I13504 were measured in parallel and the GFP background fluorescence was subtracted from the final measurements. The background absorbance of blank M9 medium was likewise subtracted from absorbance of cell cultures. Fluorescence was divided by absorbance to normalise for cell growth. The fluorescence/absorbance (FI/OD₆₀₀) was taken at 4 hours when the majority of GFP production was complete.

The results of the *in vivo* characterisation clearly showed that the biosensors did not all have the same level of specificity towards their cognate inducer, and that this specificity was concentration-dependent (Figure 6). The pSB1A2:LasRV biosensor which targets 3-oxo-C12-HSL was only activated by its target and by 3-oxo-C10-HSL to a lesser extent at 1 nM (Figure 6A). All other AHLs at this concentration induced a fluorescence output comparable to the uninduced culture with no AHL added. When the concentration increased to 100 nM, 3-oxo-C8- and C10-HSL both induced a GFP-expressing response, but the highest signal was still seen with 3-oxo-C10- and 3-oxo-C12-HSL. At the highest concentration of 10000 nM, the maximum activation was equally induced by 3-oxo-C8-, C10-, 3-oxo-C10, and 3-oxo-C12-HSL. There were also lower responses seen with C8- and C6-HSL. Therefore in general the LasR receptor appears to be mostly activated by long-chain AHLs greater than C8, and becomes less specific the higher the concentration of AHLs present.

pSB1A2:RhIRa is the biosensor plasmid targeting C4-HSL, and it was found that while *in vivo* the construct will be preferentially activated by short-chain AHLs, it does not have

particular specificity for C4-HSL (Figure 6B). At 1 nM there was no measurable response greater than the uninduced control with any of the AHLs. At 100 nM, the AHLs in the range C4 to 3-oxo-C8-HSL all induced a small response. The highest concentration, 10000 nM, is within the μ M detection range that was previously defined for this biosensor (Chappell, 2013). At this concentration, all the tested AHLs activated the biosensor to varying degrees. The short-chain AHLs C4-, C6-, and C7-HSL induced the highest GFP output, followed by 3-oxo-C8-, 3-oxo-C10-, C8- and 3-oxo-C12-HSL respectively.

The C8-HSL-targeting pSB1A2:CepRI biosensor showed a concentration-dependent specificity similar to pSB1A2:LasRV (Figure 6C). At 1 nM the highest signal was measured in response to C8-HSL, followed by C10-HSL. At 100 nM the AHLs with chain length of C7 and higher all induced comparable output, while at 10000 nM the only AHL that did not activate the biosensor was C4-HSL. One difference with the Cep-based biosensor compared to the others was that the dynamic range of response was small; the lowest concentration of 1 nM C8-HSL induced a GFP output similar to the maximum output observed at 10000 nM.

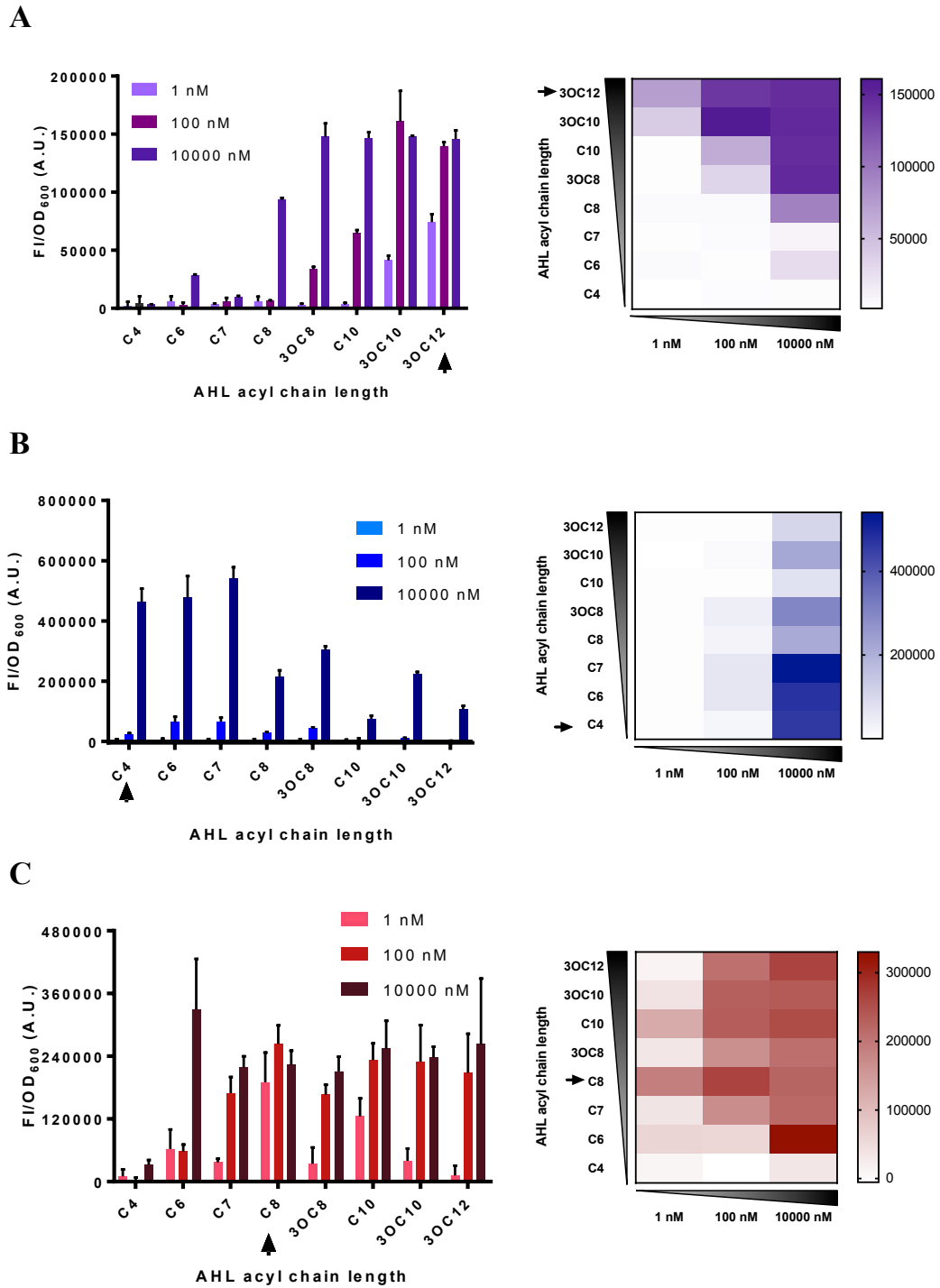


Figure 6 Characterisation of biosensor specificity *in vivo*. The GFP output of the biosensors following induction with varying concentration of AHLs: **(A)** pSB1A2:LasRV; **(B)** pSB1A2:RhlRA; **(C)** pSB1A2:CepRI. Fluorescence at 4h post-induction was normalised by OD₆₀₀ at same timepoint. Left: mean and standard deviation of three cultures is shown. Right: mean of three cultures is shown. Arrow indicates the targeted AHL.

The biosensors were then characterised in a similar manner *in vitro*. Cell-free reactions were prepared with 1 µg plasmid DNA for pSB1A2:LasRV and pSB1A2:CepRI, and 2 µg for pSB1A2:RhIRa, up to a total volume of 25 µl. 0.5 µl of AHL stock solutions were added to each reaction to achieve a final concentration of 1, 10, and 100 nM for pSB1A2:LasRV and pSB1A2:CepRI, and 0.1, 10, 1000 µM for pSB1A2:RhIRa. Reactions were transferred to a 384-well plate and incubated in a plate reader at 30°C, and fluorescence was measured every 15 min for 8 hours. In each run, three control reactions of pSB1A2:LasRV induced with 10 µM 3-oxo-C12-HSL were included as positive control. The fluorescence between 30 min, the lowest point of fluorescence, and 240 min, when fluorescence plateaued, was calculated. The background fluorescence of the uninduced control was subtracted, and a relative output was calculated by dividing fluorescence with the mean of the positive control reactions.

The pSB1A2:LasRV plasmid was overall more specifically activated by its cognate AHL *in vitro* than *in vivo* (Figure 7A). The target 3-oxo-C12-HSL produced the highest response at 1 nM, followed by 3-oxo-C8- and 3-oxo-C10-HSL which induced approximately half the output. Similarly, at 100 nM 3-oxo-C12-HSL, followed by 3-oxo-C10-HSL, induced the highest level of activation. A number of the other non-cognate AHLs did trigger activation at the maximum concentration tested, 10000 nM, including 3-oxo-C8-, C6-, C4- and C10-HSL. However, the AHLs that induced the highest response at this concentration were still 3-oxo-C12- and 3-oxo-C10-HSL. Compared to *in vivo*, this shows that the pSB1A2:LasRV biosensor has a greater level of specificity and is preferentially activated by its target 3-oxo-C12-HSL.

The pSB1A2:RhIRa plasmid was tested next. Due to the low level of expression observed, the concentrations of activator tested were 100-fold higher. At 0.1 µM, there was no GFP signal that was higher than the uninduced device (Figure 7B). At 10 µM, the target AHL C4-HSL as well as C6-HSL induced a low level of GFP expression. There was also a minimal positive induction observed by C7-HSL and 3-oxo-C12-HSL. This is very different to the *in vivo* measurements, where at this same concentration all the AHLs induced GFP expression to some degree (Figure 6B). In cell-free this behaviour is seen at 100 µM AHL induction. All of the AHLs except for C10-HSL produce a measurable GFP output, with the highest levels of activation achieved by C6-HSL and C4-HSL. Therefore, *in vitro* the pSB1A2:RhIRa

biosensor does not demonstrate a particular specificity for the cognate C4-HSL, but it is broadly activated more by short-chain AHLs than long-chain at higher concentrations.

The pSB1A2:CepRI plasmid has the strongest response of the three devices, as seen by the output value which is calculated relative to the pSB1A2:LasRV device at maximum activation (10 μ M 3-oxo-C12-HSL). The pSB1A2:CepRI output is 20-fold higher than pSB1A2:LasRV when induced with the same concentration of AHL, 10 μ M (Figure 7C). As seen before in the characterisation of pSB1A2:CepRI alone (section 4.1.3), the dynamic range of the device is markedly different between *in vivo* and *in vitro*. While there is a comparable level of activation between the lowest and highest concentrations tested in *E. coli*, in cell-free there is a broad range of activation between a relative output of 0.2 at 1 nM C8-HSL to 20 at 10000 nM. In terms of specificity, at the lowest concentration tested the cognate C8-HSL induces the highest output, followed by C10- and 3-oxo-C12-HSL. At 100 nM both C8- and C10-HSL induce the highest GFP expression, but all the other AHLs except C4- and C6-HSL also produce a strong signal. When the construct is induced with high concentrations of AHL at 10000 nM, there is almost no distinction between the level of response between all the AHLs from C7- to 3-oxo-C12-HSL, and there is also a lower output from C6- and C4-HSL. This shows that although the pSB1A2:CepRI device produces the highest level of reporter expression it also has a high degree of non-specific activation from AHLs other than its target molecule. As a biosensor therefore it would be most useful for detecting a broad range of AHLs rather than the specific C8-HSL molecule.

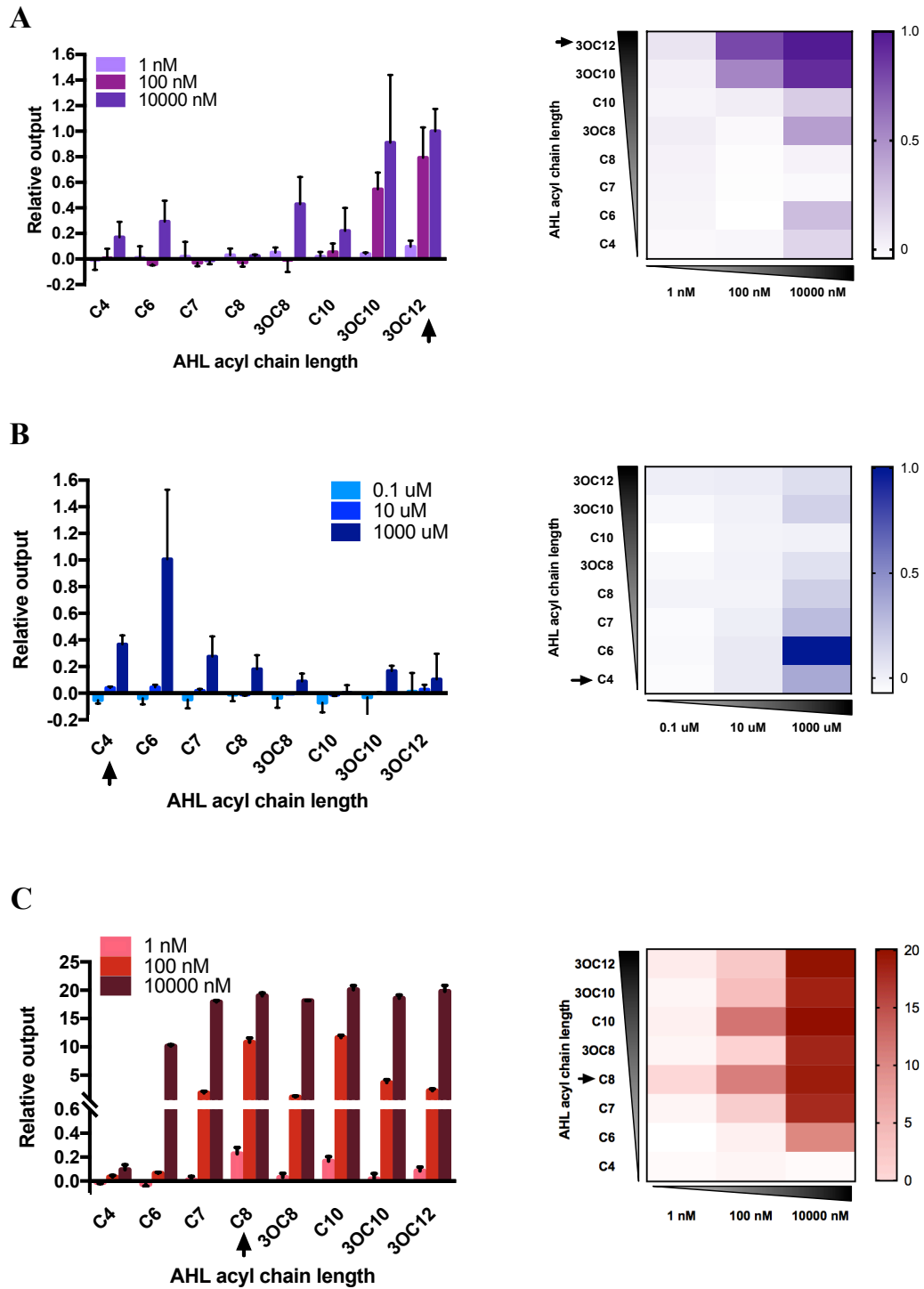


Figure 7 Characterisation of biosensor specificity *in vitro* (A) pSB1A2:LasRV (B) pSB1A2:RhlRA (C) pSB1A2:CepRI. Cell-free reactions with biosensor plasmid were induced with various concentrations of AHL. Fluorescence between 30 min and 4 hours was taken and normalised to the output of pSB1A2:LasRV induced with 10 μ M 3-oxo-C12-HSL to calculate a relative output value. Left: Mean and SD of three reactions is shown. Right: mean of three reactions is shown. Arrow indicates the targeted AHL.

In summary, the biosensor devices did not have the same specificity *in vivo* and *in vitro* when induced by a variety of AHLs. They appeared to be more specific towards their target molecules when implemented in cell-free than in whole cells. Of the three constructs, the pSB1A2:LasRV plasmid had the most specific response towards its cognate inducer with preferential activation by 3-oxo-C12-HSL at all levels of concentration tested, and minimal activation by the other AHLs except at 10000 nM. The pSB1A2:CepRI biosensor had the greatest output strength of the three but was strongly non-specifically activated. The pSB1A2:RhIRa plasmid had the lowest output and was not demonstrably specific towards C4-HSL at the concentrations tested.

4.1.5 Summary of results

The C8-HSL biosensor pSB1A2:CepRI was constructed based on the native CepRI system of *B. cepacia*. It was found to be responsive to C8-HSL with a minimum detection limit of 1 nM *in vivo* and *in vitro*, and with a dynamic range between 1 and 1000 nM *in vitro*. After characterizing the specificity of its response towards its target, it was found that it could be non-specifically activated by many other AHLs, with C10-HSL inducing an almost identical response and other AHLs (except for C4-HSL) also inducing GFP expression to a lesser degree. Therefore it would not be very effective as a species-specific biosensor. However, as an *in vitro* biosensor with an output that is 20-fold higher than that of the 3-oxo-C12-HSL-detecting pSB1A2:LasRV biosensor, it could potentially be a useful device for the general detection of QS molecules.

In terms of the two *P. aeruginosa*-specific biosensors, they were broadly more specific to AHLs that had similar acyl chain lengths to their target molecule. The pSB1A2:LasRV biosensor at all concentrations tested *in vitro* produced the strongest response when induced by its cognate AHL 3-oxo-C12-HSL. *In vivo* the biosensor had more non-specific activation; 3-oxo-C12-HSL was the strongest inducer at 1 nM, but at higher concentrations other long-chain AHLs were also able to induce equal activation as 3-oxo-C12-HSL. The pSB1A2:RhIRa biosensor for C4-HSL is a weak biosensor with the lowest promoter strength and the highest detection limit of the three. It was preferentially activated by short-chain AHLs instead of long-chain, but C4-HSL did not induce the highest response either *in vivo* or *in vitro*.

Determining the performance of the biosensors towards a range of AHLs at different concentrations was used to inform the choice of which biosensors to use for testing samples with unknown quantities of AHL. The analysis of such clinical samples will be discussed in the following section.

4.2 Detecting AHLs in cystic fibrosis patient samples

4.2.1 Aim & Introduction

The presence of QS molecules in clinical samples for CF is by now well-documented. In 2000, it was first observed that C4-HSL and 3-oxo-C12-HSL synthesis could occur in the sputum of CF patients, using a radiometric labeling method (P K Singh et al., 2000). This was interpreted by the authors as evidence that *P. aeruginosa* existed as a biofilm in the CF lung, and it was further hypothesised that AHL production could be used as a biomarker for monitoring the growth of biofilms.

Later studies sought to measure the presence of AHLs directly in CF patient samples. Using whole-cell *E. coli* biosensors based on the *luxR* and *lasR* genes, Middleton et al. identified the presence of AHLs in sputum samples from *P. aeruginosa* and *B. cepacia*-infected patients, but not in those infected by *S. aureus* (a non-AHL producing species) (Middleton et al., 2002). A similar study was performed using both *lasR*-based *E. coli* and *rhlR*-based *P. aeruginosa* biosensors to distinguish between 3-oxo-C12-HSL and C4-HSL, respectively, from patient sputum samples (Erickson et al., 2002).

Lung tissue collected from two CF patients was also found to contain AHLs, and using this type of patient sample enabled the authors to confirm that AHLs were detected in both bronchial and peripheral lung tissue (Favre-Bonté et al., 2002). Bronchoalveolar lavage (BAL) fluid is another type of commonly collected sample for monitoring infection. These were also found to contain AHLs, even from patients who had undergone lung transplants and had no clinical or microbiological evidence of infection (Ward et al., 2003). A third type of sample that was shown to contain AHLs was mucopurulent respiratory secretions collected from CF lung transplant recipients (Chambers et al., 2005).

While the above studies all relied on various types of whole-cell bacterial biosensors for the detection of AHLs, more recent work has focused on the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Barr et al., 2015; Struss et al., 2013). This technique is better suited to identifying specific AHLs, whereas whole-cell biosensors are generally only sensitive to either long- or short-chain AHLs – although this can be mitigated with an initial fractionation technique such as reverse-phase FPLC (Chambers et al., 2005).

Using LC-MS/MS, Struss et al. (2013) were able to measure and quantify levels of 3-oxo-C12-HSL, as well as a breakdown product called C12-tetrameric acid, in CF sputa. They showed that 3-oxo-C12-HSL was present in over 95% of samples tested, and in 10% of them it was measured at micromolar concentrations. Most recently, three types of clinical samples (sputum, plasma, and urine) from CF patients were analysed by LC-MS/MS for a diverse range of QS molecules, and the AHLs 3-oxo-C12-HSL and C4-HSL were identified only in the sputum samples (Barr et al., 2015). A strong positive correlation was also noted between the levels of C4-HSL and *P. aeruginosa* bacterial cell density ($p < 0.0001$), and a weaker correlation found for 3-oxo-C12-HSL ($p < 0.05$).

This body of literature provides strong evidence that AHLs are present in CF patient samples, and demonstrates that there is a continued interest in understanding the relationship between the presence of QS molecules and the clinical status of infected CF patients. However, neither whole-cell biosensors nor LC-MS/MS methods are amenable to high-throughput screening in clinical settings.

Determining that the cell-free biosensors can detect QS molecules in clinical samples is therefore required to show that they can be used as a diagnostic or research tool to further elucidate the relationship between QS and infection. The aim of this chapter is to measure QS molecules from infected patient samples using the cell-free AHL biosensors, and to identify the key parameters, such as limit of detection, which would be required for the optimal functioning of such tests.

4.2.2 Assessing extraction method with first sputum samples

The standard method developed for AHL extraction from bacterial cultures is liquid-liquid solvent extraction, followed by evaporation of the collected solvent to concentrate the extracted molecules (Camara, Daykin, & Chabra, 1998; Makemson, Eberhard, & Mathee, 2006; Shaw et al., 1997). CF sputum is a complex mixture of molecules such as extracellular DNA, lipids, and proteoglycans (Voynow & Menger, 2009), and in this context extraction is required to remove these polymers from interfering with downstream applications. A further advantage of preparing samples this way is to concentrate the extracted AHLs, which are likely to be present at low concentrations. The variations of the extraction method used on clinical samples are summarised in Table 5.

Table 5 Overview of methods used for AHL extractions from clinical samples

Type of sample	Solvent used	Method notes	Reference
Sputum	Ethyl acetate, acidified	• Evaporation under N₂ gas	Singh et al. 2000
Sputum	Dichloromethane	• Evaporation with rotary evaporator	Erickson et al. 2002
Sputum	Dichloromethane	• Diluted in 10ml PBS • Evaporation with rotary evaporator	Middleton et al. 2002
Lung tissue	Ethyl acetate, acidified	• Overnight extraction at -20°C • Evaporation under N₂ gas	Favre-Bonté et al. 2002
BAL	Dichloromethane	• Evaporation overnight	Ward et al. 2003
Mucopurulent respiratory secretions	Dichloromethane	• Evaporation, not specified	Chambers et al. 2005
Sputum	Dichloromethane, acidified	• Diluted in 15 ml PBS • Sonicated 1min15s • Evaporation under pressure	Struss et al. 2013
Sputum	Ethyl acetate, acidified	• Dried under vacuum	Barr et al. 2015
Plasma, urine	Solid-phase extraction	• Dried under vacuum	Barr et al. 2015

As the first aim was to replicate previous studies and demonstrate that the cell-free biosensors are capable of detecting AHLs from clinical samples, an extraction protocol with ethyl acetate was chosen for the initial testing. Three sputum samples from CF patients at Nottingham University Hospital were kindly provided by our collaborator, Dr. Alain Filloux, from a previous project where they were surplus to requirement. Since they were directly available but limited in number, they were an ideal case study with which test the extraction protocol for AHLs. It was decided to use the pSB1A2:LasRV biosensor for initial testing, because it has a higher signal output than the pSB1A2:RhIRa plasmid. Furthermore, at the time of testing the pSB1A2:CepRI plasmid had not yet been constructed.

Briefly, the sample was mixed with equal volume of acidified ethyl acetate (0.1% glacial acetic acid), spun down at low speed, and the organic phase collected. This was repeated three times in total, and the solvent was then removed by evaporation overnight before re-suspension in 50 μ l solvent. The extracted sample was added to cell-free reactions containing the pSB1A2:LasRV biosensor plasmid (Figure 8A). In the same assay, control reactions were included at known concentrations of 0, 1, 10, and 100 nM 3-oxo-C12-HSL to create a standard curve that could be used to calculate the concentration of AHL from fluorescence values of the clinical samples. Reactions were transferred to a 384-well plate and incubated in the plate reader at 30°C for at least 4 hours. Gain of fluorescence between 30 min (lowest level of fluorescence) and 240 min (maximum GFP expression) was calculated. Each sample was added to three independent cell-free reactions.

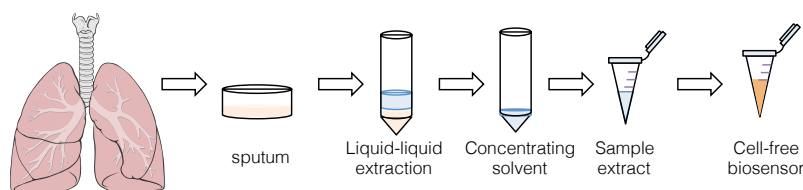
The first sample (PA25) was re-suspended in 50 μ l DMSO after the solvent was removed by evaporation, and 0.5 μ l was added to each cell-free reaction. There was an increased signal detected versus the uninduced control (the fluorescence of a cell-free reaction with the pSB1A2:LasRV plasmid and blank DMSO), but below the 1 nM standard (Figure 8B). To test if the solvent type had an influence on the outcome, the remaining two samples, PA55 and PA65, were extracted and the dried-down solvent was re-suspended in 50 μ l acidified ethyl acetate instead. Again 0.5 μ l of the concentrated extract was added to three cell-free reactions. The results showed a strong fluorescent output for both samples, between 1 nM and 10 nM on the standard curve (Figure 8C). The AHL solutions used for the standard curve were always in the same solvent as the tested patient sample – i.e. in ethyl acetate when

the sample was re-suspended in ethyl acetate – to take into account the potential effect of the solvent on the biosensor output.

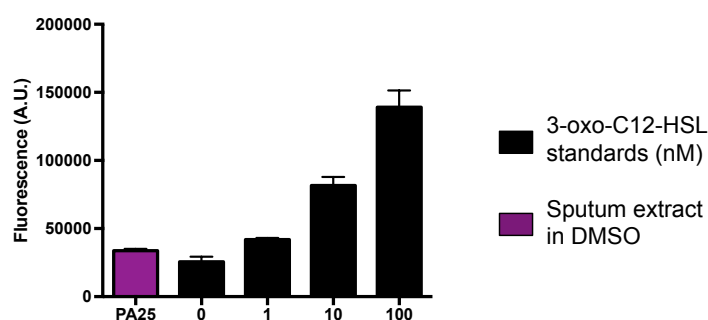
Since ethyl acetate can be removed by evaporation, it was possible to concentrate this extract again and re-suspend in DMSO before repeating the assay, thus providing a direct comparison of the two solvent types. This showed a much lower fluorescent signal than the previous results in ethyl acetate (Figure 8D). This suggests that the signal from the first patient sample PA25 might have been higher if it were re-suspended in ethyl acetate originally, and that ethyl acetate is a better solvent for re-suspension of dried AHLs than DMSO.

As these three clinical samples were not directly provided to us from the clinical collaborators, there was no further information about the medical condition of the patients, most importantly whether they were infected with *P. aeruginosa* or other species. Therefore no conclusions could be made from this small sample as to the correlation of AHLs with infection state. However it provided preliminary data that the extraction protocol was working as expected and was compatible with the downstream cell-free assay.

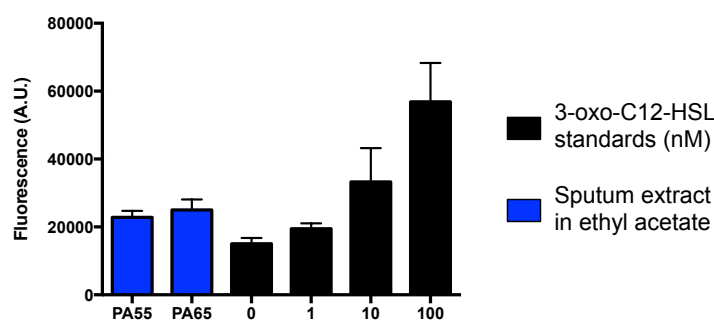
A



B



C



D

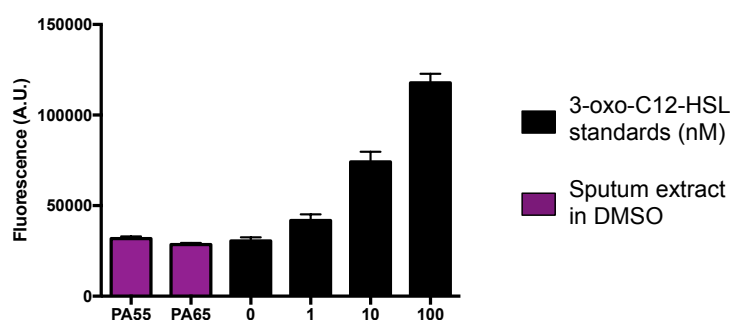


Figure 8 First CF sputum samples in a cell-free assay with the pSB1A2:LasRV biosensor (A) Extraction protocol for extracting AHLs from sputum and analysing with the cell-free biosensor **(B)** Fluorescence gain of cell-free reactions 4 hours post-induction with sputum sample extract is shown. The sample extract PA25 was re-suspended in DMSO after extraction. **(C)** The sample extracts PA55 and PA65 were re-suspended in ethyl acetate after extraction. **(D)** The sample extracts PA55 and PA65 in ethyl acetate from (C) were concentrated to dryness and re-suspended in DMSO before being added to the cell-free reactions. Standard reactions were prepared and induced with 3-oxo-C12-HSL stock solutions to 1, 10, and 100 nM final concentration. Mean and standard deviation of three reactions is displayed for each sample.

4.2.3 Assessing suitability of BAL sample type

The majority of previous research has focused on sputum samples from CF patients, but other types of patient material such as BAL are also routinely collected. While there has only been one previous publication about the presence of AHLs in BAL samples (Table 5, p.68), there are potential advantages to them over sputum samples. Firstly, they can be collected from a wider range of patients, including infants and young children for whom it is difficult to expectorate sputum (Ratjen et al., 2015). This could be important since CF patients tend to acquire *P. aeruginosa* infections as they enter adulthood, and the QS behaviour of bacteria is likely to change during the different stages of colonisation, establishment, and chronic infections. Also, because the samples are collected via bronchoscope from the lower lung area they are a more direct representation of the infection site, with less likelihood of contamination from the upper lung area and nasopharyngeal tract (Brennan, Gangell, Wainwright, & Sly, 2008). Furthermore, since BAL samples are mostly made up of the saline wash, they are a less complex sample type compared to sputum and therefore have less components that could inhibit or interfere with the cell-free reactions.

The aim of this experiment was therefore to test BAL samples using the same method as sputum to determine its suitability as a sample type. Ten BAL samples were obtained from the Biobank collection of patient samples at Royal Brompton Hospital, provided by our collaborator Prof. Jane Davies. The volume of each sample was approximately 1 ml. The experiments were carried out blind with regards to the patients' infection diagnosis to prevent bias in analysis of the results. The extraction procedure as described in Section 4.2.2 (p. 68) was carried out with the BAL samples, using ethyl acetate as the mixing solvent and re-suspending in 50 µl ethyl acetate after extracts were concentrated to dryness. The cell-free assay was again conducted as described in Section 4.2.2.

After the assay was completed, the infection status of each patient was disclosed and assigned to the results to investigate the correlation between infection and biosensor output. None of the samples produced a GFP signal that was significantly higher than the background signal ($p < 0.05$, unpaired t-test) (Figure 9A). The highest output that was recorded, sample A, came from a patient who did not have a diagnosed *P. aeruginosa* infection. The remaining nine samples all had fluorescence gains that were even lower than the uninduced control reaction.

When the results are grouped according to infection status (Figure 9B), the mean of the non-infected group was higher than the infected group, but this difference was not significant.

Based on these results, there was no evidence that measuring a positive biosensor output with the BAL samples was possible. The sample size is too small to be representative of the larger population of CF patients, however they were chosen at random from the clinical sample collection and contain a mixture from non-infected and infected patients. The volume of sample is likely to be a factor in this outcome: BAL samples are much more dilute than sputum, but the total volume from a single collection was not available and only 1 ml from each patient was used for testing. Although a limited number of sputum samples were tested in the initial method trial, two out of three did produce a positive signal. Furthermore there is a greater body of literature that suggests sputum samples rather than BAL samples contain AHLs. For these reasons it was decided that for further testing of clinical samples, sputum would be a preferable choice to BAL.

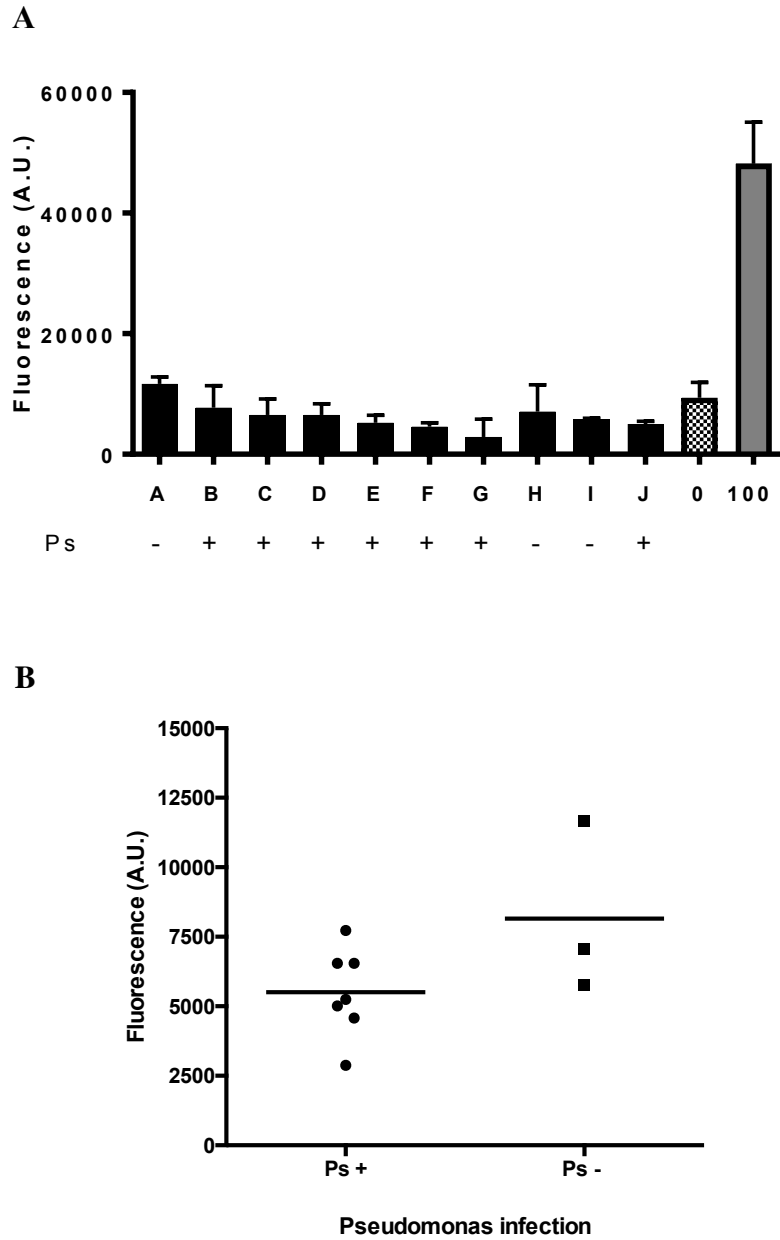


Figure 9 BAL samples in a cell-free assay with the pSB1A2:LasRV biosensor. (A) BAL samples were named A-J to remove patient ID numbers. Fluorescence gain of cell-free reactions 4 hours post-induction with BAL sample extract is shown. Mean and standard deviation of three reactions is displayed for each BAL sample. “0” is the background fluorescence of uninduced pSB1A2:LasRV, “100” is induced with 100 nM 3-oxo-C12-HSL. Ps – and + underneath the sample indicates if the patient was previously diagnosed with *P. aeruginosa* infections by bacterial culture. **(B)** BAL samples grouped by infection state. Dots represent individual BAL samples from *P. aeruginosa*-infected patients (n=7); squares represent individual BAL samples from non-infected patients (n=3). Solid line indicates the mean of each group. p= 0.10, unpaired t-test.

4.2.4 Expanding testing of sputum samples

A set of sputum samples from CF patients was collected from the Royal Brompton stock collection to further trial the extraction and detection of AHLs using the pSB1A2:LasRV biosensor in cell-free. 19 samples were obtained from both *P. aeruginosa* infected and non-infected patients, and as before with the BAL samples (4.2.3), the biosensor testing was carried out blind to the infection status of the patients. Samples were extracted with ethyl acetate and re-suspended in 50 μ l ethyl acetate. The sample extracts were added to cell-free reactions as described in section 4.2.2. The cell-free assay was carried out in two sets of samples, with ten sample extracts in the first assay and nine in the second. To normalise the fluorescence values from both sets so that they can be compared to each other, the fluorescence gain of each sample between 30 min and 4 hours was converted into a scaled output value between 0 and 1, where 0 was the fluorescence of the uninduced pSB1A2:LasRV reactions and 1 was the fluorescence induced at 100 nM.

The patient infection data revealed that the number of samples from infected or non-infected patients was 15 and 4, respectively. In the first assay, two samples (II and VI) out of ten induced GFP expression higher than the uninduced control reactions, and in the second assay eight out of nine samples were above background fluorescence (Figure 10A). However, the increases observed were not very high compared to maximum activation of pSB1A2:LasRV at 100 nM, with the highest signal producing an output of 0.2 compared to the expected 1.0 at 100 nM induction (Figure 10B). Furthermore, the results did not appear to be correlated with the known *Pseudomonas* infection diagnosis of the patients. When grouped together according to the infection status, there was no significant difference between the two groups (Figure 10B).

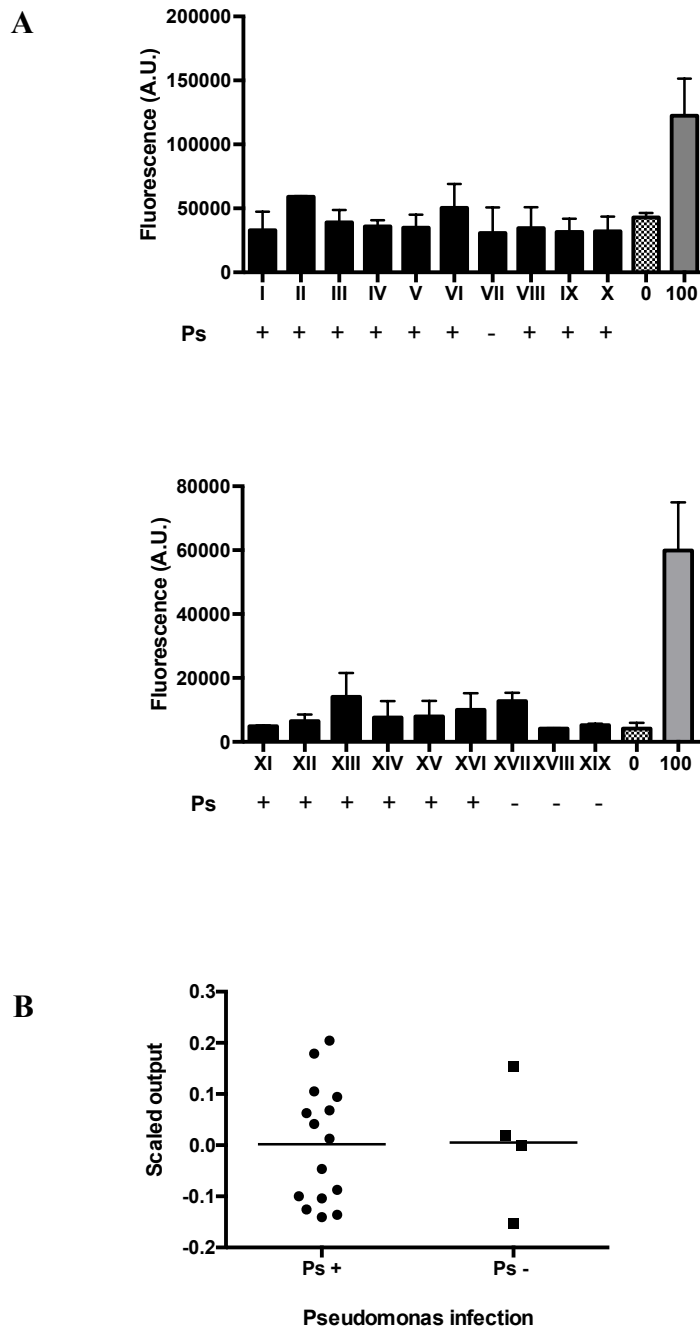


Figure 10 Sputum samples in a cell-free assay with the pSB1A2:LasRV biosensor. (A) Fluorescence gain of cell-free reactions 4 hours post-induction with sputum sample extracts I-XIX is shown. Mean and standard deviation of three reactions is displayed for each sample. “0” is the background fluorescence of 3 uninduced pSB1A2:LasRV, “100” is induced with 100 nM 3-oxo-C12-HSL. Ps – and + underneath the sample indicates if the patient was previously diagnosed with *P. aeruginosa* infections by bacterial culture. Stars indicate results that are significantly higher than the uninduced (0 nM) control ($p < 0.05$, unpaired t-test). **(B)** Sputum samples grouped by infection state. Dots represent individual samples from *P. aeruginosa*-infected patients ($n=15$); squares represent individual BAL samples from non-infected patients ($n=4$). Solid line indicates the mean of each group. $p = 0.96$, unpaired t-test.

The cell-free biosensor produced a positive response for 10/18 samples tested, which was a lower proportion than expected compared to previous studies (Erickson et al. 2002; Struss et al. 2013). This could either be due to differences between the sample sets used in this work and those previously reported, as a result of the different patient populations sampled, or it could have been an issue with the cell-free assay itself. The assay could be too insensitive to detect the low levels of AHL present in the samples. In order to be able to determine the cause of these differences, it was necessary to validate the cell-free results with a second AHL detection method, and confirm whether or not the positive outputs recorded by the biosensor corresponded to AHL presence in the sample extract. It would also be useful if the method were able to distinguish between different AHL chain lengths, as this would establish if the positive results were the result of a non-specific response by the biosensor. Therefore, LC-MS/MS would be preferable to the whole-cell biosensors used by previous studies. By collecting this additional information, it could also be determined if the concentration of extracted AHLs was below the detection limit of the current biosensor, and therefore if the sensitivity of the system needed to be improved.

4.2.5 LC-MS/MS analysis of sputum extracts

LC-MS/MS was utilised to determine the AHL content of the sputum samples that were being tested. A number of the previous studies summarised in Table 5 (p. 68) had used this method to analyse clinical samples (Barr et al., 2015; Struss et al., 2013), and the protocol from Struss et al. was adapted to the available equipment. Briefly, samples analysed by LC-MS/MS are processed as follows (Figure 11A). The sample is first separated on a liquid chromatography column and injected into the mass spectrometer. In electrospray ionisation, the sample is forced through a narrow capillary that causes a fine aerosol of droplets to form. The droplets are charged by the application of high voltage at the tip of the capillary, and then dried with N₂ gas leaving only the charged ions. With the hybrid quadrupole-time-of-flight (Q-ToF) analyser that was used, the ions can be identified by their fragmentation pattern. First they are selected based on their mass-to-charge ratio (m/z) in the quadrupole analyser, and these selected precursor ions are then sent through a collision cell, where nitrogen gas is introduced to fragment the ions. In the second analyser (ToF), the m/z of the individual fragment ions are measured and the peak area is quantified. This method provides accurate detection of molecules based on three levels of selection – retention time, molecular weight of precursor ion, and molecular weight of fragment ions – which makes it a highly precise technique. The LC-MS/MS data collection and analysis was performed by Dr. David Bell.

The LC-MS/MS method developed showed good detection of the eight AHLs for which standard solutions were available. The retention times, precursor, and fragment ions were determined for each AHL (section 3.7). It was found that there was a typical fragmentation pattern where the two main ions produced were the homoserine lactone ring and acyl chain, as shown for 3-oxo-C12-HSL (Figure 11B). Using an injection volume of 2.5 μ l, the lower limit of detection (LLOD) was between 3 nM for 3-oxo-C12-HSL up to 44 nM for C4-HSL. A calibration curve for each AHL was created by injecting increasing volumes of standard solutions, and plotting the peak area of the fragment ions against the amount injected (Figure 11C). Calibration curves were produced for each analysis to account for machine variation.

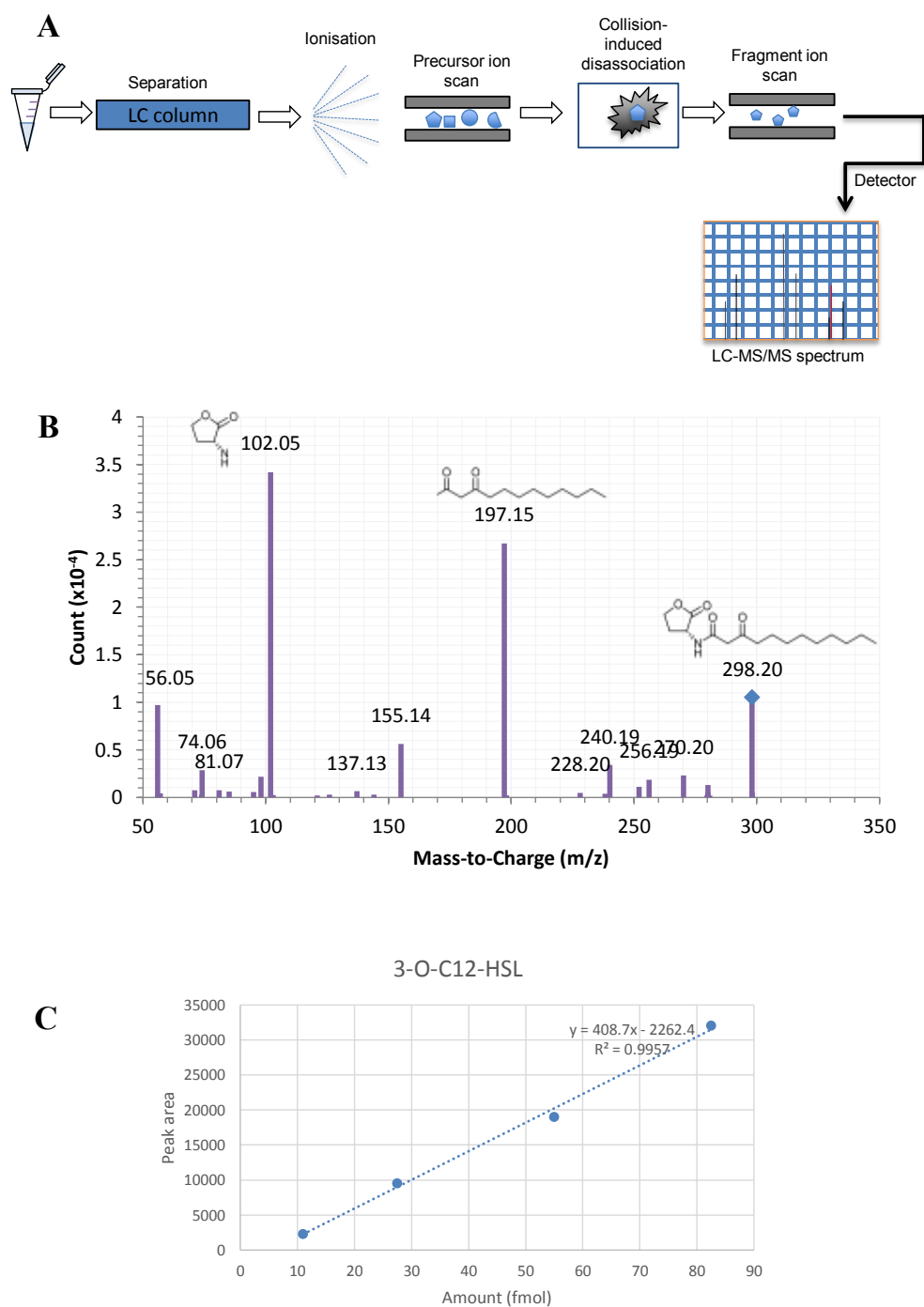


Figure 11 LC-MS/MS analysis of 3-oxo-C12-HSL (A) Scheme of the LC-MS/MS work flow. (B) The total ion spectrum from analysis of 3-oxo-C12-HSL. The m/z of the precursor ion (blue diamond) is 298.2013, and the two main fragments are the lactone ring and acyl chain with m/z of 102.05495 and 197.15361 respectively. (C) Example of calibration curve used to calculate AHL concentration. Increasing volumes between 0.1 - 0.75 μl of 110 nM 3-oxo-C12-HSL standard solution are injected and monitored. The peak area of 6 fragment ions is plotted against femtomoles of 3-oxo-C12-HSL and linear trendline is plotted. A calibration curve for each AHL is created in every LC-MS/MS analysis.

The Struss method used dichloromethane (DCM) instead of ethyl acetate as the solvent for extraction. Test extractions of blank water samples spiked with 3-oxo-C12-HSL found good recovery of AHL with negligible loss when measured by LC-MS/MS (data not shown). Twenty sputum samples were extracted using DCM and re-suspended in 100 µl methanol. Table 6 shows the AHLs that were detected in these samples, calculated as the concentration of AHLs in the re-suspended sample extract (p. 81). After the testing was concluded, the patient infection status was cross-referenced and it was found that all the samples with detected AHLs came from *P. aeruginosa*-infected patients. Another interesting observation is that besides the two *P. aeruginosa* AHLs, only one other AHL was found: 3-oxo-C10-HSL. 3-oxo-C10-HSL has been identified as the QS molecule for marine *Vibrio* strains (Buch, Sigh, Nielsen, Larsen, & Gram, 2003; Mohamad et al., 2015; W. S. Tan et al., 2014), but this is unlikely to be the source as it is not found in CF infections. The tomato rhizosphere isolate *P. putida* is also a known producer of 3-oxo-C10-HSL (Fekete et al., 2010; Steidle et al., 2001), and has been isolated from CF patients before (Ward et al., 2003). However, since 3-oxo-C10-HSL was only detected in tandem with 3-oxo-C12-HSL, it is thought to be more likely that they originate from the same source. Previous studies have demonstrated that LasI can produce small amounts of 3-oxo-C10-HSL as well as 3-oxo-C12-HSL (Gould, Herman, Krank, Robert, & Churchill, 2006), and 3-oxo-C10-HSL has been found in *P. aeruginosa* biofilms (Charlton et al., 2000), as well as in CF clinical samples (Chambers et al., 2005).

The concentrations at which the AHLs were found were much lower than expected, especially compared to the range (20 nM – 6 µM 3-oxo-C12-HSL) that was found by the previous work (Struss et al., 2013). Since the minimum detection limit of the pSB1A2:LasRV biosensor is 1 nM 3-oxo-C12-HSL final concentration, the AHLs found in the sample extracts would be diluted below this limit and would not produce a positive output; in the cell-free protocol, only 0.5 µl of sample extract is added to a 25 µl reaction meaning a 50-fold dilution takes place. For the C4-HSL found, the concentrations were in the nM range whereas the biosensor pSB1A2:RhIRa has a sensitivity in the µM range. Therefore, it appears that AHLs were present in sputum samples, but not at concentrations that were within the detection range of the cell-free biosensors.

Table 6 AHLs found in CF sputum samples as determined by LC-MS/MS

Sample ID	Concentration (nM)		
	3OC12-HSL	C4-HSL	3OC10-HSL
SpA	-	-	-
SpB	2.91	-	-
SpC	-	-	-
SpD	1.12	-	-
SpE	-	-	-
SpF	-	-	-
SpG	17.21	30.95	5.50
SpH	-	-	-
SpI	-	-	-
SpJ	-	-	-
SpK	-	-	-
SpL	-	-	-
SpM	-	-	-
SpN	-	-	-
SpO	17.34	11.23	5.53
SpP	-	-	-
SpQ	-	-	-
SpR	-	-	-
SpS	3.82	-	0.85
SpT	trace	6.63	-

4.2.6 Determining extraction efficiency

Since the levels of AHL detected in 20 sputum samples were both lower than expected and only detected in a minority of the samples, it was possible that AHLs were lost during the extraction process. To determine if this was the case, the following control experiments were devised and performed.

A calculation of extraction efficiency can be determined by adding a known amount of the molecule of interest to the aqueous matrix, performing the extraction protocol, and then measuring the amount of molecule that has been extracted. In this case, it was not possible to obtain a blank matrix (i.e. a sputum sample) that was known to be free of AHLs without undergoing extraction. No sputum samples were available from healthy patients, and CF patients may harbour infectious bacteria even if they have not yet been diagnosed with *P. aeruginosa* by microbiological culture. Instead of starting with a blank matrix, a target molecule that is similar to the molecule of interest could be added to matrix samples, and extracted instead. $^{13}\text{C}_4$ -labeled 3-oxo-C12-HSL was used as an internal standard in Struss et al. 2013, and is suitable because it has the same chemical structure but a greater molecular mass. This means that the partition coefficient of the two molecules will be similar and will not affect the calculation of the extraction efficiency, while the difference in mass will allow it to be distinguished from 3-oxo-C12-HSL in LC-MS/MS. A standard solution of $^{13}\text{C}_4$ -labeled 3-oxo-C12-HSL was kindly provided by Dr. Yonek Hleba.

Four sputum samples were spiked with 20 μl 500 nM $^{13}\text{C}_4$ -3-oxo-C12-HSL and then extracted as described in section 4.2.5 (Figure 12A). Sample extracts were re-suspended in 100 μl MeOH, reaching a final expected concentration of 100 nM, and then analysed by LC-MS/MS targeting the product ions of $^{13}\text{C}_4$ -3-oxo-C12-HSL. Extraction efficiency was calculated as a percentage of $^{13}\text{C}_4$ -3-oxo-C12-HSL standard solution. The presence of the full panel of eight AHLs from C4- to 3-oxo-C12-HSL was also measured.

Due to the similar structure of the internal standard and native 3-oxo-C12-HSL, the extracted samples were analysed by LC-MS/MS only and not in parallel by the pSB1A2:LasRV biosensor. To confirm that the biosensor is unable to distinguish between 3-oxo-C12-HSL and the labelled internal standard, cell-free reactions with the biosensor plasmid were prepared and induced with either 100 nM 3-oxo-C12-HSL or the labelled internal standard. It

was shown that the labelled standard would induce GFP expression at a similar level to the 3-oxo-C12-HSL target molecule (Figure 12B).

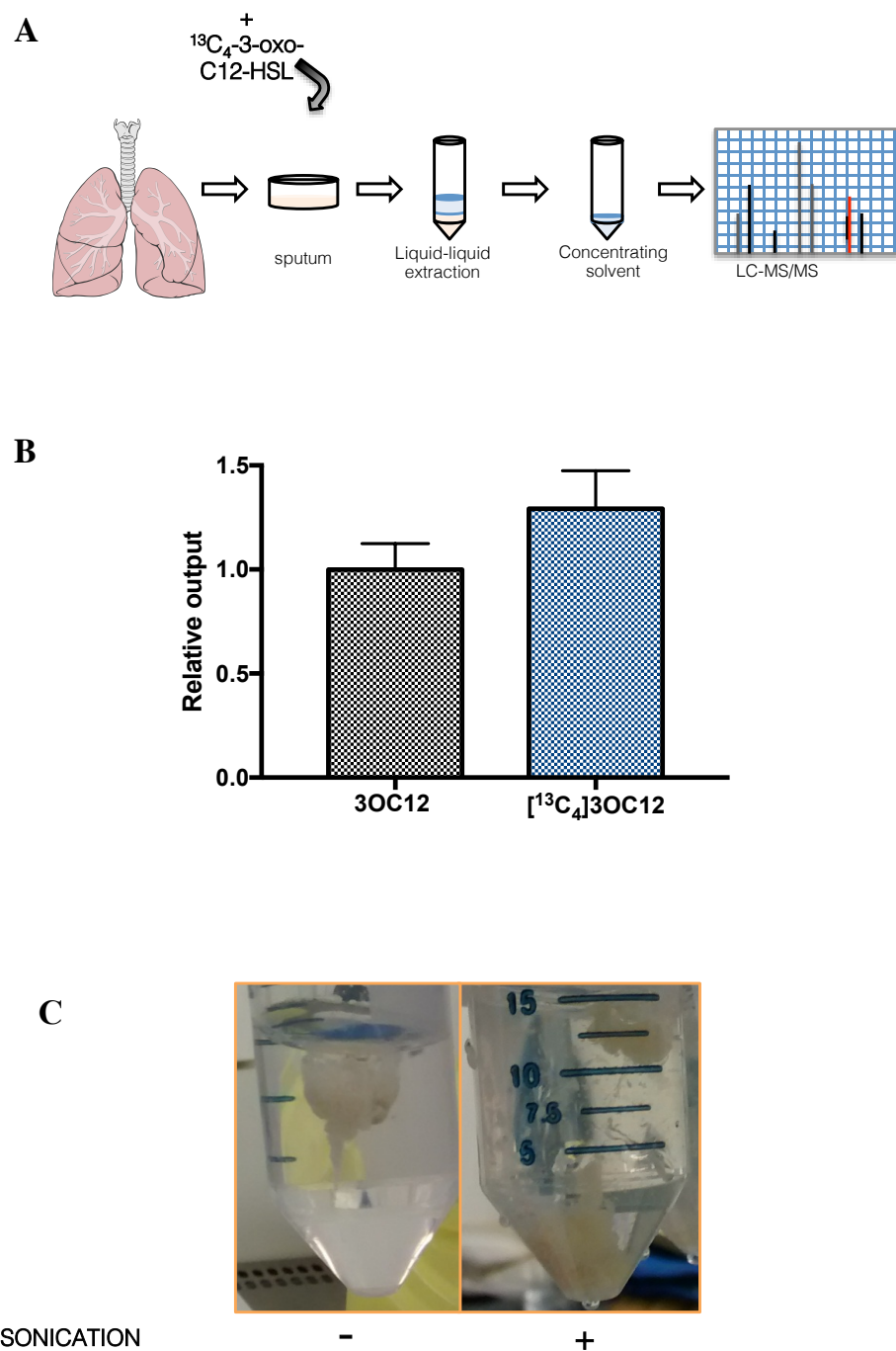


Figure 12 Calculating extraction efficiency of AHLs from CF sputum (A) Protocol for determining extraction efficiency. The internal standard $^{13}\text{C}_4\text{-3-oxo-C12-HSL}$ is added to sputum, then extracted and measured by LC-MS/MS. **(B)** Output of cell-free pSB1A2:LasRV biosensor when induced by either 100 nM 3-oxo-C12-HSL or $^{13}\text{C}_4\text{-3-oxo-C12-HSL}$. Fluorescence between 30 min and 240 min is taken and relative output is calculated by normalising to the mean of pSB1A2:LasRV-biosensor induced with 100 nM oxo-C12-HSL. Mean and standard deviation of three reactions is shown. **(C)** Effect of sonication on sputum sample. Sputum is added to 15 ml PBS and sonicated for 5 min (1 min off, 1 min on) in an ultrasonic bath.

The calculated extraction efficiency from the 4 sputum samples had a mean of 40.5% ($\pm$ 12.4%, 1 S.D.) (Table 7, p.86). It was observed that the variation in the viscosity of the sputum samples appeared to correspond with extraction efficiency, with thicker samples causing poorer extraction. To try and improve this, a sonication step to break up the sputum before extraction was included as reported in Struss et al. 2013. Five sputum samples were spiked with the same volume of $^{13}\text{C}_4$ -3-oxo-C12-HSL, added to 15 ml PBS and placed in an ultrasonic bath for 5 min total sonication (1 min on, 1 min off) (Figure 12C). The extraction protocol was then performed as described with the volume of DCM adjusted to 15 ml to maintain a 1:1 ratio of solvent: aqueous solution. The extraction efficiency of sonicated samples had a mean of 40.0 % ($\pm$ 8.2%, 1 S.D.). This showed that the sonication did not improve efficiency, although it did reduce the variation in the results. It was decided that the sonication step could be omitted for future experiments.

An advantage of using $^{13}\text{C}_4$ -3-oxo-C12-HSL is that the AHLs present in the sputum can still be measured by LC-MS/MS in parallel. It was found that in these eight samples, the ones from the *P. aeruginosa*-infected patients were the only three to have measurable AHLs. 3-oxo-C12-HSL was found in two samples, while C8-HSL was found for the first time in sample 11S. As discussed in section 4.1.1, C8-HSL is synthesised by the *B. cepacia* complex, a group of closely related species that are known CF pathogens, although this patient had not been diagnosed with *B. cepacia* infection before. This sample also contained 3-oxo-C10-HSL, which in this case was not found together with 3-oxo-C12-HSL as seen before (section 4.2.5). All three samples with measured AHLs were confirmed to be from *P. aeruginosa*-infected patients.

Determining the extraction efficiency of AHLs had shown that approximately 40% of an added AHL standard could be recovered from sputum using a dichloromethane extraction method, regardless of sonication. This showed that there was a loss of the target molecule that would mean an expected 2.5-fold decrease in signal of extracted AHLs. Although improving this efficiency would be beneficial, having 2.5x higher concentration of AHL in most samples where 3-oxo-C12-HSL were found would still not lead to a positive signal output from the biosensor. For this to be possible, the detection limit of the cell-free assay would need to be improved.

Table 7 Extraction efficiency of $^{13}\text{C}_4$ -3-oxo-C12-HSL from sputum using dichloromethane

Sonication	Sample ID	3-oxo-C12-HSL (nM)	C8-HSL (nM)	3-oxo-C10-HSL (nM)	$^{13}\text{C}_4$-3-oxo-C12-HSL extraction efficiency (%)	Mean extraction efficiency (S.D.)
-	5S	-			51	40.5% (12.4)
	6S	-			25	
	7S	-			50	
	8S	3.32			37	
+	9S	-	-	-	42	40.0% (8.2)
	10S	-	-	-	49	
	11S	-	31.25	41.86	45	
	12S	6.60	-	-	29	
	13S	-	-	-	35	

4.2.7 Lowering the limit of detection for cell-free assay

The process of extraction and cell-free biosensor assay was re-examined to identify how to optimise it for low concentrations of AHL. It was noted that the addition of the sample extract to the cell-free reaction had the largest effect, as there was a 50-fold dilution of sample extract into the final reaction. The volume of sample extract added could not be increased, because a higher percentage of inorganic solvent would have an adverse effect on the functioning of the cell-free system. Instead, the final step of the extraction protocol was modified to avoid the need for adding solvents to the cell-free reaction; after collecting the dichloromethane, the pooled solvent would be divided into four aliquots of equal volume. Each one of these was concentrated to dryness individually and could be used for separate purposes. One was re-suspended in methanol for LC-MS/MS analysis, and each of the three remaining aliquots could be re-suspended directly into the cell-free reaction containing biosensor plasmids (Figure 13A).

Since the analysis to date of sputum samples by LC-MS/MS had shown that the samples with detectable AHLs were all from patients positive for *P. aeruginosa*, it was decided to collect samples only from *P. aeruginosa*-infected patients rather than a mixture of non-infected and infected for the following experiment. Ten samples were analysed using the modified extraction protocol described above. The previous method required a concentration of 50 nM in the sample extract to be measured at the 1 nM limit in the cell-free (accounting for the 0.5 µl volume of sample extract added to 25 µl cell free). With the new method, to achieve a final concentration of 1 nM in the cell-free extract would be equivalent to a sample extract with 12.8 nM. The extract is divided into 4 aliquots but there is no dilution which means the limit would be 4 nM. However each aliquot is re-suspended into 80 µl cell-free volume (instead of 25 µl) to produce three reactions, therefore the effective limit would be 12.8 nM in a sample extract that was prepared as before. This would allow us to see the higher concentrations that were found in samples during the LC-MS/MS screen (Table 6).

The cell-free assay with pSB1A2:LasRV showed 5/10 samples with output greater than the uninduced control (Figure 13B). The concentration of 3-oxo-C12-HSL that was found in the samples by LC-MS/MS is shown below the biosensor results. It has been adjusted to show the expected final concentration that would be in the cell-free reactions. In these samples no other AHLs were detected. Comparing the cell-free biosensor and LC-MS/MS results it was

clear that the concentration of 3-oxo-C12-HSL found did not correspond to the output of the biosensor. Samples N1 and N3 had high concentrations of 3-oxo-C12-HSL, yet produced a similar GFP response to sample N2 which had 7-8 times less AHL. Furthermore N4 and N5 had no detected AHL and yet produced a response above background. The samples were grouped according to whether or not a positive biosensor response would be expected (concentration greater than the detection limit of 1 nM) and analysed (Figure 13C). It was found that although the mean of the expected positive response group was higher, this difference was not significant ($p=0.0506$).

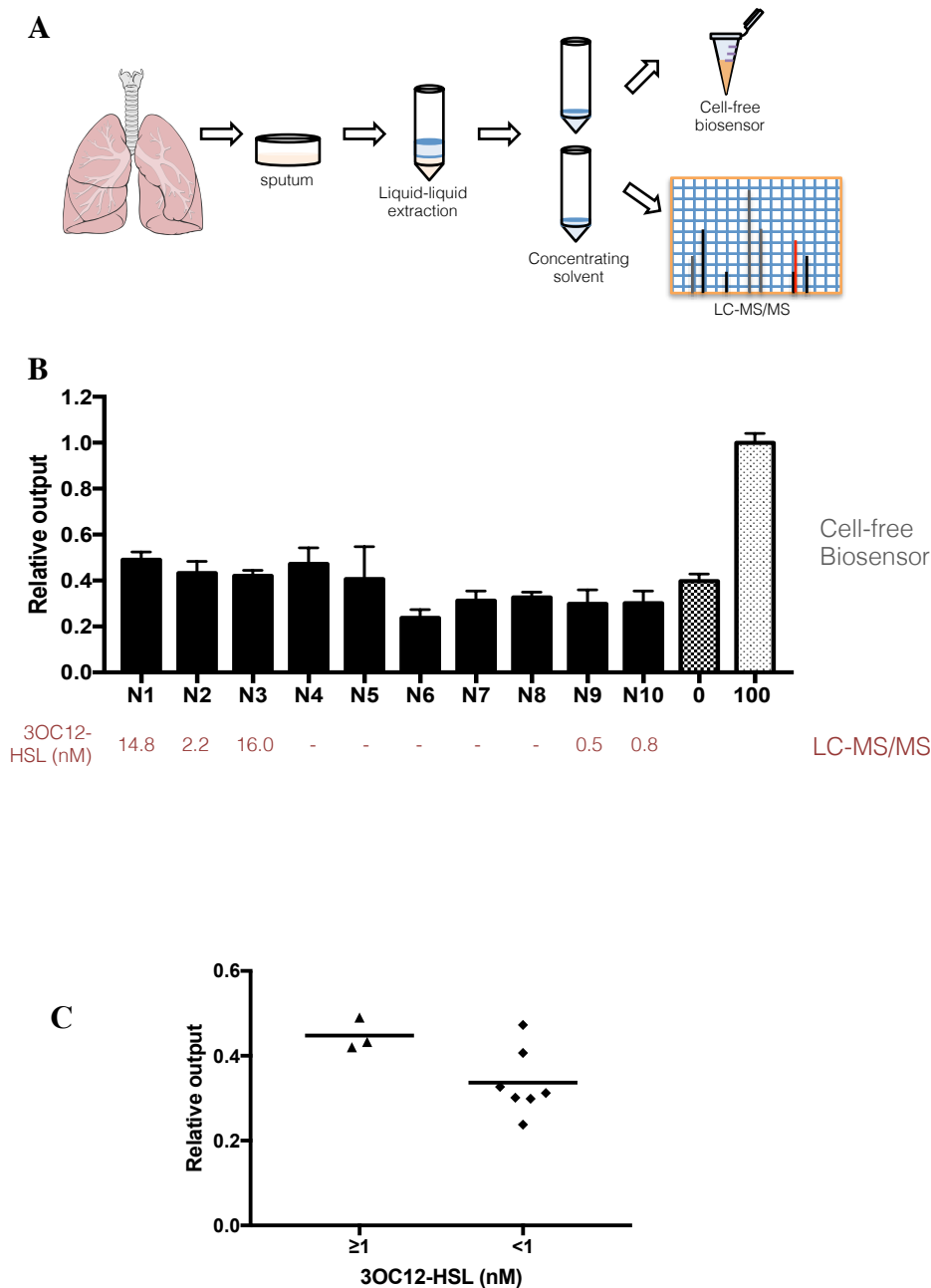


Figure 13 Sputum extracts analysed by the pSB1A2:LasRV cell-free biosensor and LC-MS/MS
(A) Extraction protocol optimised to lower the limit of detection in cell-free reactions. At the final step sample extract is resuspended directly into cell-free. **(B)** Above signal output of the pSB1A2:LasRV cell-free biosensor induced with sputum samples N1 to N10. Samples were extracted using dichloromethane. Normalised output value was calculated relative to biosensor induced with 100 nM 3-oxo-C12-HSL for fluorescence at 4 hours post-induction. “0” is the background fluorescence of uninduced biosensor and “100” is output induced with 100 nM 3-oxo-C12-HSL Mean and standard deviation of three reactions is shown. Below: expected concentration of 3-oxo-C12-HSL as determined by LC-MS/MS analysis. **(C)** Sputum samples grouped by LC-MS/MS detected AHL concentration. Samples equal or greater than 1 nM (n=3) are expected to produce a positive output in the biosensor, while samples below 1 nM (n=7) would not be detected. $p=0.0506$, unpaired t-test.

From this experiment it was concluded that the biosensor was not able to consistently detect 3-oxo-C12-HSL from sputum sample extracts. As the LC-MS/MS and cell-free assay analysis were conducted in parallel, it was considered unlikely that there would be differences in the AHL levels between aliquots that could account for this inconsistency. In particular, it was concerning the two samples with relatively high concentrations of 3-oxo-C12-HSL (N1 and N3) did not produce a correspondingly high output. It was therefore necessary to examine whether the extracts from sputum were affecting the output of the biosensor in the cell-free assay.

4.2.8 Determining the effect of sputum extract on cell-free assay

As discussed earlier in Section 4.2.6 (p.82), a challenge of working with sputum samples is the lack of true negatives – samples that are known to not contain AHLs before they are analysed. Samples from patients that were not diagnosed with *P. aeruginosa* infections could still produce a positive output with the biosensor (Section 4.2.4, p.75). Furthermore, once samples had been used for extraction they were broken down through prolonged contact with the solvent and could not be reused. With the modified extraction protocol (Figure 14A), however, four aliquots of extract had been produced per sample. Since two were used for the pSB1A2:LasRV cell-free biosensor assay and LC-MS/MS, there were two aliquots per sample remaining. The samples that had been confirmed to have no or low levels of AHL could serve as background-only controls to determine what the effects of sputum sample extracts were on the output produced by biosensors in cell-free.

Cell-free reactions were prepared containing the plasmid pSB1A2:LasRV. The sample extracts N7 to N10 had been prepared most recently and were therefore used for this experiment to minimise any possible effects of long-term storage. Although samples N9 and N10 had 3-oxo-C12-HSL levels detectable by LC-MS/MS, the concentration (< 1 nM) was considered low enough to be negligible. Sample extracts were re-suspended in 80 μ l cell-free master mix, and then 3-oxo-C12-HSL was added to a final concentration of 100 nM. Reactions were transferred to a 384-well plate in three aliquots of 25 μ l each, and GFP expression was measured. Fluorescence values were divided by the fluorescence of the positive control, cell-free reactions containing pSB1A2:LasRV and 100 nM 3-oxo-C12-HSL, to calculate a normalised relative output.

By adding a known concentration of 3-oxo-C12-HSL to the cell-free reactions, it was possible to see how the presence of the sample extracts would affect the biosensor's ability to detect the AHL and express GFP. The results showed that the effect of the sputum extract on the cell-free activity varied significantly between samples (Figure 14A). None of the reactions containing sputum extract were able to recover the level of GFP expression seen in the positive control. Sample N8 had the highest expression, with a relative output of 0.72 compared to the expected 1.0 of the positive control, while samples N7, N9, and N10 had fluorescence levels comparable to the uninduced pSB1A2:LasRV (0 nM). This showed that

there was a severe impact on the cell-free functioning with the addition of sample extract, which in most cases even completely suppressed the expected signal.

To identify if this effect was due to interference in the biosensor's ability to detect and respond to AHL, or if it was acting on the cell-free mechanism of protein expression, a second experiment was conducted with extracts of the same samples. Cell-free reactions were prepared containing the plasmid pSB1A2:GFP, which has the GFP gene under the control of the constitutive promoter J23101. Sample extracts were re-suspended in 80 µl cell-free master mix, transferred to a 384-well plate in three aliquots of 25 µl, and GFP expression was measured. Fluorescence values were divided by the fluorescence of the positive control, cell-free reactions containing only the pSB1A2:GFP plasmid, to calculate a normalised relative output.

The results observed with the pSB1A2:GFP plasmid were similar to the biosensor plasmid with added 3-oxo-C12-HSL (Figure 14B). Sample N8 again had the highest GFP expression (0.69 relative output), while samples N7 and N9 had output levels comparable to the negative control (cell-free reactions containing no plasmid). The one difference compared to the previous results was sample N10, which did have some GFP expression over background (0.39 relative output) rather than complete loss of signal as observed with the biosensor. These results showed that the suppression effect observed originally with the biosensor plasmid did not involve the AHL detection function specifically, but that there is a general global dampening of output signal when sputum extracts were added to cell-free systems. Furthermore, this effect varies considerably between samples.

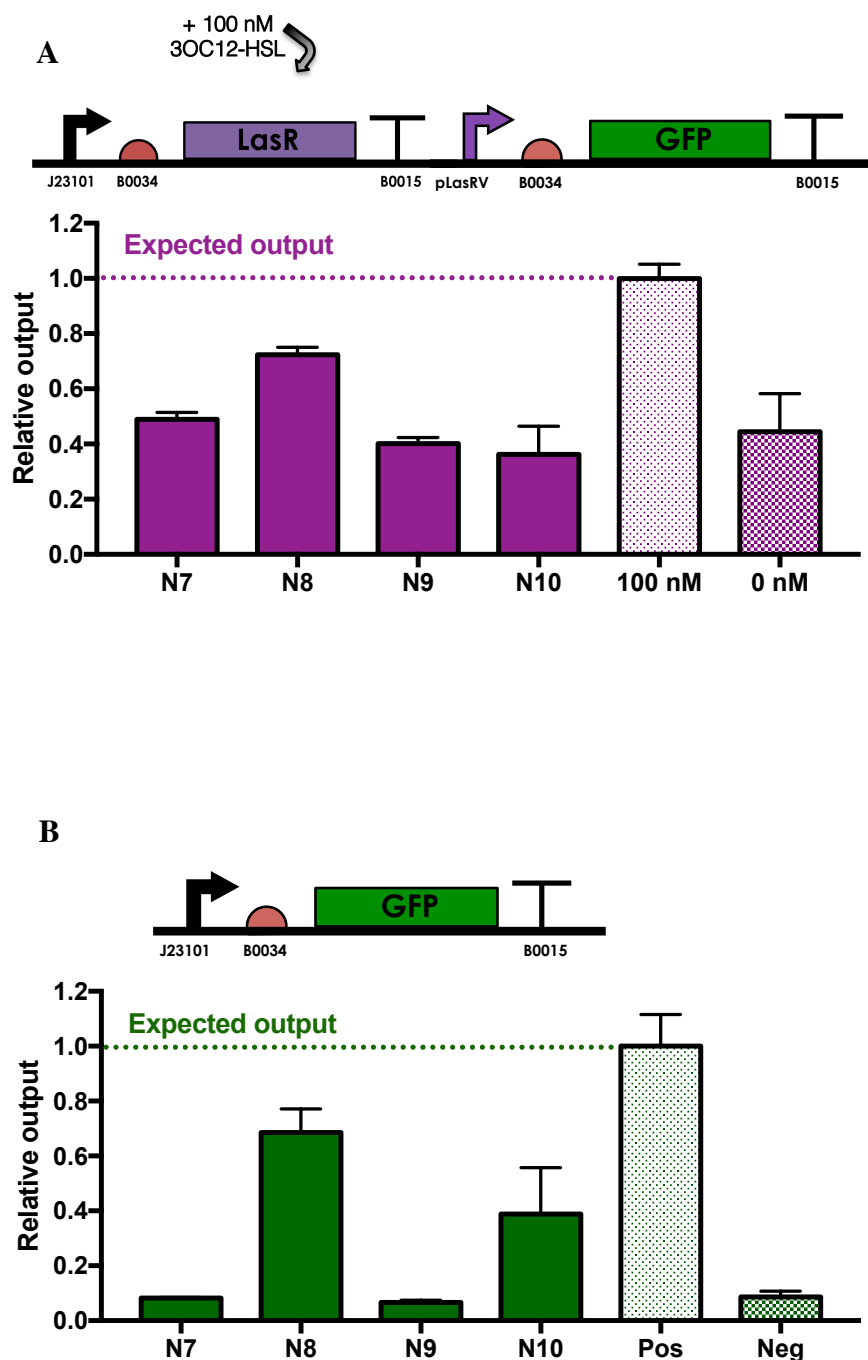


Figure 14 Effect of sputum extract by dichloromethane extraction on cell-free activity (A) GFP output of the pSB1A2:LasRV biosensor cell-free assays containing sputum extracts N7-N10. Sputum samples were extracted using dichloromethane, then extracts were re-suspended in cell-free reactions and spiked with 100 nM 3-oxo-C12-HSL to recover activity. Relative output compared to control reaction containing 100 nM 3-oxo-C12-HSL only was calculated. Mean and standard deviation of three replicates is shown. (B) GFP output of pSB1A2:GFP cell-free assays containing sputum extracts N7-N10. Sputum samples were extracted using dichloromethane, then extracts were re-suspended in cell-free reactions. Relative output compared to control reaction containing pSB1A2:GFP only (Pos) was calculated. Blank cell-free reactions with no DNA (Neg) were used to measure background fluorescence. Mean and standard deviation of three replicates is shown.

4.2.9 Optimised extraction with ethyl acetate

DCM had been used as the solvent for extraction in sections 4.2.5 to 4.2.8, following the Struss et al (2013) protocol. The development of LC-MS/MS validation methods made it possible to explore if the initial solvent used (ethyl acetate) could be a better choice for these assays. The testing of extraction efficiency and effect of sputum extract on cell-free were the two control experiments performed to compare the suitability of ethyl acetate as an extraction solvent versus DCM. Following these, a set of eighteen sputum samples were analysed by cell-free biosensor and LC-MS/MS using this method.

Extraction efficiency was determined as described in section 4.2.6 (p.82). Four sputum samples were spiked with $^{13}\text{C}_4$ -3-oxo-C12-HSL, processed by liquid-liquid extraction using ethyl acetate, and the final concentration of spiked analyte in sample extracts was determined by LC-MS/MS. In Table 8 the calculated extraction efficiency for the four samples is shown. The mean efficiency was 71% ($\pm 17\%$, 1 S.D.), which was an improvement over the 40% extraction efficiency that was achieved when using DCM (Table 7, p.86).

Table 8 Extraction efficiency of $^{13}\text{C}_4$ -3-oxo-C12-HSL from sputum using ethyl acetate

Sample ID	3-oxo-C12-HSL (nM)	$^{13}\text{C}_4$ -3-oxo-C12-HSL extraction efficiency (%)	Mean extraction efficiency (S.D)
2335		46	71% (17)
1958	19.58	74	
2346		87	
1960		78	

The effect of sputum extract on the cell-free assay when ethyl acetate was used instead of dichloromethane was also tested. Four sputum samples were extracted using ethyl acetate and concentrated to dryness. Sample extracts were re-suspended in cell-free reaction master mix containing the pSB1A2:LasRV biosensor plasmid. 3-oxo-C12-HSL was added to a final concentration of 100 nM. Reactions were analysed as described in section 4.2.8.

As seen before when dichloromethane was used, the samples affected the activity of the cell-free reactions to varying degrees (Figure 15A). In the case of L1, the level of output signal was comparable to the uninduced biosensor, showing that the addition of the sample extract completely suppressed any GFP signal. The three other samples L2-L4 had relative outputs of 0.72 – 0.87, and were therefore closer to recovering the level of output seen by the positive control but did still have some suppression of signal. The mean of the biosensor activity for four sputum samples was calculated for both the dichloromethane and ethyl acetate extractions (Figure 15B). Although the variation among samples was high, overall the recovery of activity following ethyl acetate extraction was slightly improved compared to the dichloromethane-treated samples. Taken together with the results showing extraction efficiency with ethyl acetate (Table 8) was better than with dichloromethane (Table 7), this data suggested that ethyl acetate was a preferable solvent for the extraction of AHLs from sputum samples.

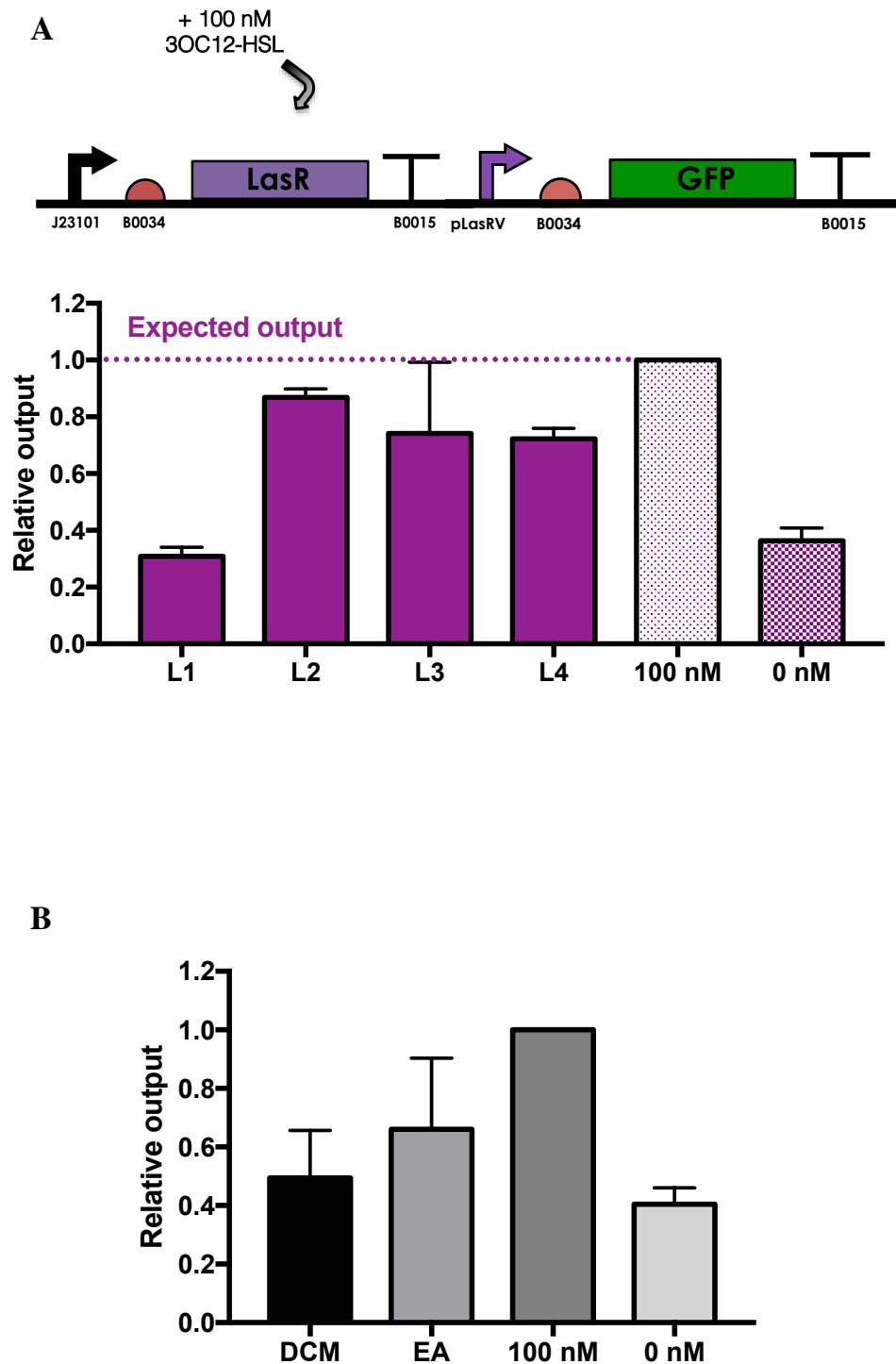


Figure 15 Effect of sputum extract by ethyl acetate extraction on cell-free activity (A) GFP output of the pSB1A2:LasRV biosensor cell-free assays containing sputum extracts L1-L4. Sputum samples were extracted using ethyl acetate, then extracts were re-suspended in cell-free reactions and spiked with 100 nM 3-oxo-C12-HSL to recover activity. Relative output compared to control reaction containing 100 nM 3-oxo-C12-HSL only was calculated. Mean and standard deviation of two repeats is shown. **(B)** Comparison of the effect on cell-free activity by sputum extracts from dichloromethane (DCM) and ethyl acetate (EA) extractions. Mean and standard deviation of four sputum samples per solvent is shown.

Eighteen sputum samples from *P. aeruginosa*-positive patients were analysed by the pSB1A2:LasRV cell-free biosensor and LC-MS/MS in parallel. The extraction method was performed as described in Section 4.2.7, using ethyl acetate instead of DCM. Additional positive and negative controls were included for the extraction process. The negative control was 2 ml water and the positive control was 2 ml water spiked with 3-oxo-C12-HSL to achieve an expected concentration of 100 nM in the LC-MS/MS analysis. These samples were processed in parallel with the sputum samples, which provided an additional control to monitor the extraction protocol. The positive and negative control extracts were also analysed in the cell-free assay and the positive control output was used to normalise the output of the sputum samples. To do this, the fluorescence gain of the cell-free reactions containing sputum extract was divided by the equivalent value of the positive extraction control. The relative output value produced would therefore take into account potential differences in extraction efficiency between experiments, since the sputum samples were processed in sets of four each time. The background signal was taken as the output of the negative control extract.

The LC-MS/MS analysis showed that eight of the eighteen samples had detectable levels of 3-oxo-C12-HSL (Figure 16B). No other AHLs were found. The two samples with the highest concentration were P2 and P7. In the pSB1A2:LasRV cell-free biosensor assay, P2 and P7 were also the samples that produced the highest output signal (Figure 16A). The strength of the biosensor output did not correspond with the concentrations calculated by LC-MS/MS, however, as the concentration found in P2 was nearly two-fold that of P7, but the relative output with the biosensor was higher for P7 than P2. As it was shown previously that the sputum extracts could have varying signal suppression effects on the biosensor, this would explain why the output would no longer correspond directly with an expected concentration. Similarly, the sample P9 had a concentration of 1 nM that should be detectable by the biosensor but produced no positive response. This can be explained by the suppression effect described above (and Figure 15); dampening of the biosensor output by sample extract would mean the effective detection limit of the biosensor is higher than 1 nM. When the samples are grouped based on the 1 nM of 3-oxo-C12-HSL benchmark, (Figure 16C), the difference between the two groups is not statistically significant ($p=0.09$, unpaired t-test). However if sample P9 is not expected to produce a positive output and therefore excluded, the distinction between the two groups would be significant ($p=0.01$). These data confirm that while the true

LLOD for the biosensor is close to 1 nM, if sample suppression effects are taken into account, the effective LLOD for the biosensor is higher. A much larger number of samples would need to be tested experimentally to determine exactly what this LLOD is.

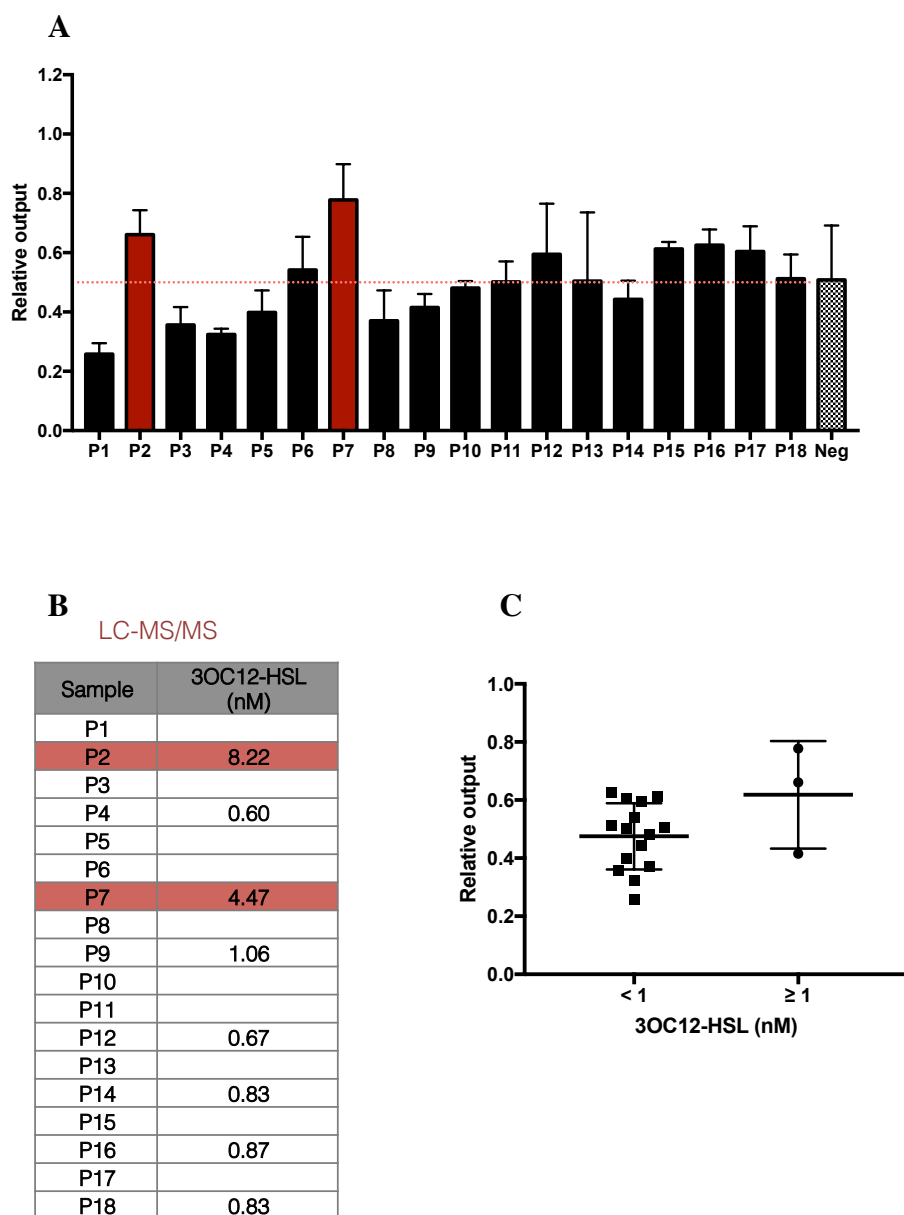


Figure 16 Sputum extracts analysed by the pSB1A2:LasRV cell-free biosensor and LC-MS/MS (A) Signal output of the pSB1A2:LasRV cell-free biosensor induced with sputum samples P1 to P18. P1 to P18 were extracted using ethyl acetate. Normalised output value was calculated relative to a positive control extract sample with added 3-oxo-C12-HSL. Mean and standard deviation of three reactions is displayed for each sample. ‘Neg’ is the background fluorescence of a negative control extract sample with no added AHL. (B) Concentration of expected 3-oxo-C12-HSL in cell-free reactions according to LC-MS/MS analysis. (C) Sputum samples grouped by LC-MS/MS detected AHL concentration. Samples equal or greater than 1 nM (n=3) are expected to produce a positive output in the biosensor, while samples below 1 nM (n=15) would not be detected. p=0.09, unpaired t-test.

4.2.10 Summary of results

In this section, the efforts towards developing a cell-free biosensor assay for the detection of AHLs in CF clinical samples were described. Two types of samples were tested, sputum and BAL, and sputum was deemed the more promising candidate for containing measurable levels of AHL. A secondary AHL quantification method, LC-MS/MS, was established to validate the results of the pSB1A2:LasRV biosensor assay. The extraction protocol for isolating AHLs from sputum was improved through a series of optimisation steps to achieve a protocol that was compatible with the downstream analysis methods. It was determined that addition of the sputum samples extracts to cell-free reactions did result in a lower signal output, and that this effect was not consistent between samples. Therefore AHL extracted from sputum samples could not be accurately quantified in terms of concentration. However in some samples it was shown that the presence of 3-oxo-C12-HSL could still be detected by the assay. With the final extraction method used, the presence of 3-oxo-C12-HSL was detected by both the pSB1A2:LasRV biosensor and LC-MS/MS in two sputum samples with concentrations of 4.5 nM or higher.

The LC-MS/MS analysis showed that 3-oxo-C12-HSL was the most commonly detected AHL in sputum samples. 3-oxo-C10-HSL was the next most common, followed by C4-HSL and C8-HSL. This suggests that the pSB1A2:LasRV biosensor is the most useful of the three AHL biosensors for analysis of CF samples, and also that there is unlikely to be cross-activation by a non-specific AHL producing a false positive result. The concentration of C4-HSL, when found, was below the detection limit of the pSB1A2:RhIRa biosensor. In the one case where C8-HSL was detected, the sample had been spiked with ¹³C-labeled 3-oxo-C12-HSL which would have masked the signal induced by the C8-HSL. The pSB1A2:CepRI and pSB1A2:RhIRa biosensors were therefore not tested to analyse any clinical samples.

4.3 Expanding the biosensor platform with a c-di-GMP biosensor

4.3.1 Aim & Introduction

C-di-GMP has emerged as a key regulator of biofilm growth (as reviewed in section 2.3.3, p. 27) and is therefore a potentially useful marker to monitor with a targeted biosensor. In order to design a biosensor for c-di-GMP, a literature review was first conducted to find examples of gene expression that are directly regulated by c-di-GMP levels. Examples of both transcriptional- and translational-level regulation were identified from a variety of bacterial species, as shown in Figure 17A. This included five transcription factors and two types of riboswitches. Since the time of review, two further transcription factors have been identified as c-di-GMP-regulated effectors: BldD from *Streptomyces*, which binds to tetrameric c-di-GMP (Tschowri et al., 2014), and LtmA from *Mycobacterium smegmatis* (W. Li & He, 2012). The cellular concentration of c-di-GMP is estimated as “<50 nM to a few μ M” (Hengge, 2009), which corresponds with the binding affinities determined for known protein effectors (Figure 17A). Interestingly, however, the riboswitches have the highest binding affinity by a significant margin, in the picomolar to nanomolar range. It is not yet clear why the riboswitch affinity for c-di-GMP is orders of magnitude lower than the estimated basal concentration (Romling, Galperin, & Gomelsky, 2013), but one possibility may be that they regulate certain functions when there is high activity by c-di-GMP-degrading enzymes and subsequently a low level of c-di-GMP (Hengge, 2009).

The identified transcription factors have different mechanisms of action, namely de-repression, upregulation, and downregulation of gene expression. Biosensor circuits can be designed with each of these mechanisms, but the most straightforward approach is to use an upregulating or activating mechanism. This enables a positive output to be measured in the presence of the compound of interest. The *vpsT* gene from *V. cholerae* was selected as a promising candidate for a c-di-GMP biosensor circuit, as it was the most well-characterised of all the known c-di-GMP-regulated transcription factors. Its protein structure was determined in 2010, and it was found to have a similar mechanism as the 3-oxo-C12-HSL-regulating transcription factor LasR (Krasteva et al., 2010). One molecule of c-di-GMP binds to a single VpsT monomer, which then forms a protein dimer that can bind to DNA. The binding affinity of VpsT for c-di-GMP was previously calculated as 3.2 μ M (Krasteva et al., 2010).

4.3.2 Design & construction of the biosensor circuit

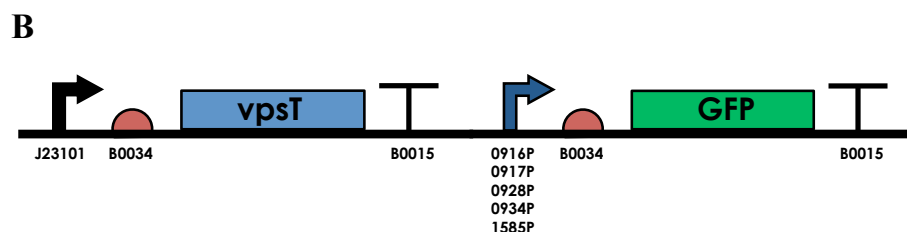
In the biosensor circuit design, the VpsT gene was placed under the control of a strong constitutive promoter, BBa_J23101 from the Anderson promoter library and a medium strength RBS, BBa_B0034 (<http://parts.igem.org/>). This combination of promoter and RBS was previously used in the same cell-free system and is known to produce strong level of protein expression (Chappell et al., 2013).

The VpsT gene sequence (VCA0952) was identified from the genome sequence of *Vibrio cholerae*. To avoid the restriction digest sites for the BioBrick assembly method (EcoRI, XbaI, SpeI and PstI), a degenerate codon was chosen for residue 62 to remove an XbaI restriction site. The gene was synthesised by IDT with J23101 promoter and B0034 RBS sequences upstream, flanked by the BioBrick prefix and suffix regions.

As the exact DNA-binding domains of VpsT had yet to be determined (Krasteva et al., 2010), the next step was to identify and test promoters that were recognised to be under the regulation of VpsT. 31 genes were upregulated (≥ 2 -fold) when VpsT was introduced into a $\Delta vpsT$ mutant strain (Krasteva et al., 2010), suggesting that the promoter regions of these ORFs could be used to upregulate the expression of a reporter gene in the presence of the VpsT transcription factor and c-di-GMP. The 31 genes were organised into 19 ORFs in total.

The promoter regions of the 19 ORFs were taken from the intergenic region directly upstream of the ORFs, up to a cut-off length of approximately 400 bp. Five candidates were selected for initial promoter screening by rejecting sequences with high GC content or potential problematic DNA secondary structures. These were the promoter regions of the ORFs VC0916, VC0917, VC0928, VC0934 and VC1585. The native RBS of these promoters was replaced with the RBS B0034 by removing the first 25 bp upstream of the start codon and inserting the B0034 RBS flanked by scar sites, which is also 25 bp long. This was done to standardise the effect of RBS on expression levels and to isolate the promoter region. The five promoters were synthesised by IDT with flanking BioBrick prefix and suffix sequences for assembly. To assemble the plasmids, BioBrick cloning was used to insert the synthesised DNA sequences into a pSB1A2 plasmid. The final plasmid design is shown in Figure 17B.

A	VpsT	FleQ	mrkH	Bcam1349	CLP	Riboswitch class I & II
Species of origin	<i>Vibrio cholerae</i>	<i>Pseudomonas aeruginosa</i>	<i>Klebsellia pneumoniae</i>	<i>Burkholderia cenocepacia</i>	<i>Xanthomonas campestris</i>	Multiple species
Protein size (kDa)	25.8	55.3	26	28.7	27.6	N.A.
Mechanism of gene regulation	Transcription activation	Release of repression	Transcription activation	Transcription activation	Transcription inactivation	Transcriptional and translational control
Binding affinity for c-di-GMP	3.2 μM ¹	15-25 μM ²	107 nM ³	10 μM ⁴	3.5 μM ⁵	Class I: 10 pM ⁶ Class II: 2.2 nM ⁷



C

ORF	Promoter length (bp)	Gene/Operons	Proteins expressed
VC0934	375	<i>vpsL-P</i>	Vibrio polysaccharides, cluster II
VC0917	287	<i>vpsA-K</i>	Vibrio polysaccharides, cluster I
VC0916	236	<i>vpsU</i>	Vibrio polysaccharides, cluster I
VC0928	170	<i>rbmA</i>	Secreted matrix protein RbmA
VC1585	148	<i>katB</i>	Catalase enzyme KatB

Figure 17 Design of c-di-GMP biosensor. (A) Candidates for effectors of gene expression regulation by c-di-GMP. Sources for binding affinity are provided in footnotes. **(B)** Design of c-di-GMP biosensor plasmid **(C)** Promoter features of VpsT-regulated genes identified for screening.

¹Krasteva et al. 2010; ²Hickman and Harwood 2008; ³Schumacher and Zeng 2016; ⁴Fazli et al. 2011; ⁵Chin et al. 2010; ⁶Kathryn D Smith et al. 2009; ⁷Shanahan et al. 2011

4.3.3 Screening of VpsT-regulated promoters

The five selected promoters were assembled upstream of GFP together with the constitutively expressed VpsT module to form the pSB1A2:VpsT plasmids. In order to measure c-di-GMP-regulated expression, the constructs needed to be tested in cell-free assays. They could not be tested *in vivo* because c-di-GMP is synthesised by *E. coli* and the biosensors could therefore be in a permanently switched-on state.

Cell-free reactions were prepared with 1 µg plasmid DNA and incubated at 30°C in a plate reader and GFP fluorescence was measured for 6 hours. 10 µM c-di-GMP was added at the start of the reaction to induce GFP expression. The AHL biosensor plasmid pSB1A2:LasRV, induced with 10 µM 3-oxo-C12-HSL, was used as a positive control for GFP expression. The empty vector pSB1A2:I13504, which contains the B0034 RBS and GFP gene but no promoter, was used as a negative control for background fluorescence.

None of the five promoters were found to up-regulate GFP expression in the presence of c-di-GMP. The positive control plasmid pSB1A2:LasRV did have strong expression of GFP when induced by 10 µM 3-oxo-C12-HSL, proving that the cell-free platform was functional as expected (Figure 18A). However, all of the reactions containing the pSB1A2:VpsT series of plasmids had fluorescence values similar to the negative control plasmid pSB1A2:I13504 (Figure 18B), showing that there was no positive induction by c-di-GMP at 10 µM, and also that there was no measurable leakiness of the selected promoters.

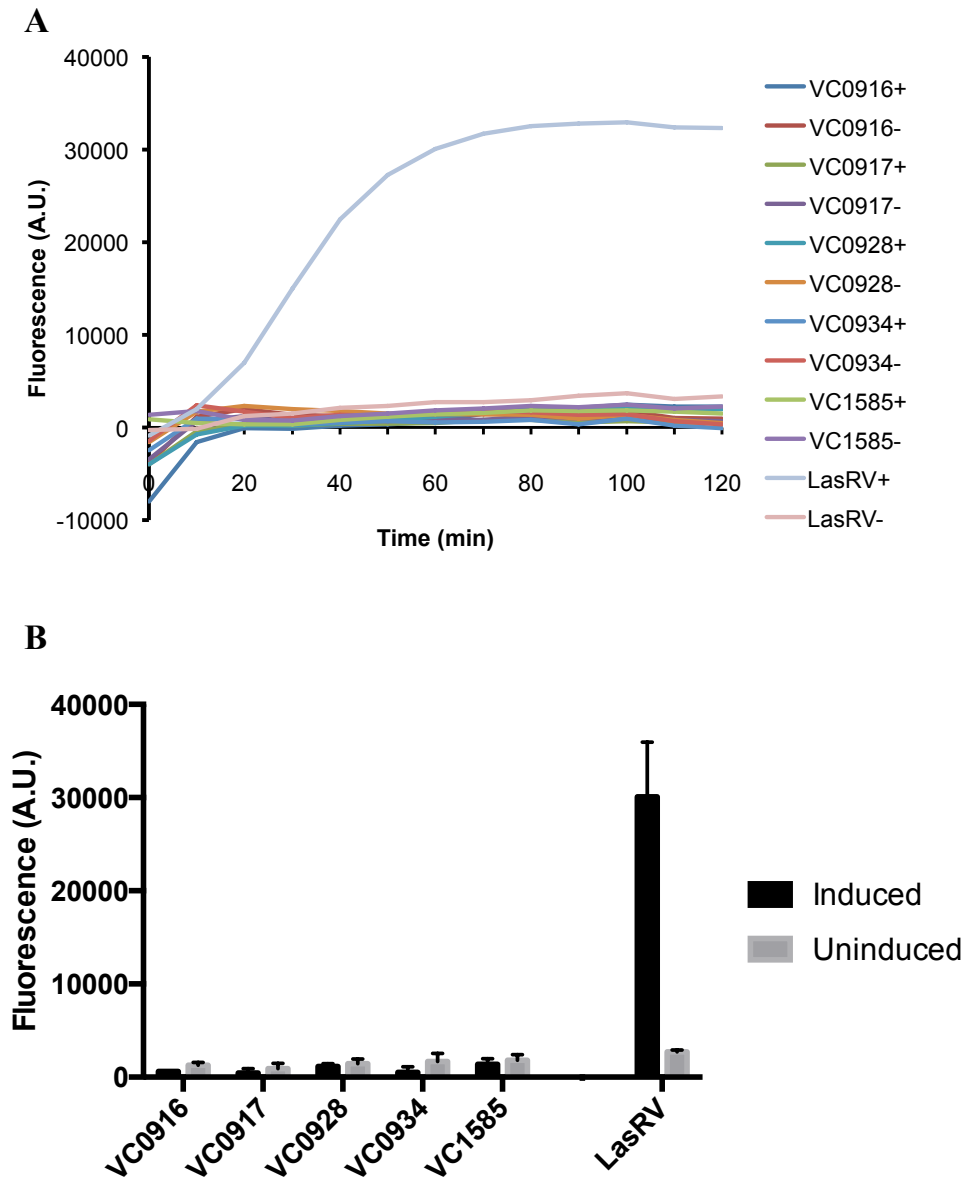


Figure 18 Screening of five VpsT-regulated promoters (A) GFP fluorescence of cell-free reactions with pSB1A2:VpsT constructs. Reactions were incubated for 6 hours at 30°C. Reactions labeled ‘+’ were induced with 10 μ M c-di-GMP, or 10 μ M 3-oxo-C12-HSL in the case of the pSB1A2:LasRV plasmid. Mean of three reactions is shown. (B) Fluorescence at 60 min when GFP production has plateaued for the pSB1A2:LasRV control plasmid. Mean of 3 reactions is shown, and error bars are + S.D.

4.3.4 Modified promoter design

The initial design of c-di-GMP biosensors did not produce any circuits that would upregulate gene expression in the presence of c-di-GMP. Since the promoters chosen were selected from an expression study in the native *V. cholerae* organism, there could be further regulation beyond the VpsT transcription factor required for these ORFs to be activated, which would be absent when the VpsT system was isolated and used in another context. However, it had been shown in previous literature that for at least one of the five tested promoters this regulation did work when taken out of the native context. The promoter for the *vps-II* operon containing genes VpsL-P, named VC0934 in this work, had been used to create a plasmid for monitoring c-di-GMP levels (Shikuma & Yildiz, 2009). In this work, the entire upstream region had been fused to the reporter ORF, whereas in the pSB1A2:VpsT₀₉₃₄ construct the sequence was cut off at 400 bp upstream of the start codon. Furthermore, the native RBS regions, which had been replaced with the B0034 RBS in pSB1A2:VpsT₀₉₃₄, was maintained in this version.

A new pSB1A2:VpsTL plasmid was therefore assembled where the VC0934 promoter was swapped with the full-length promoter – named pVpsL – that retained these features (Figure 19A). The same cell-free assay was performed to measure upregulation of GFP expression when induced by c-di-GMP. The results showed that pSB1A2:VpsTL could be activated by induction with at least 1 μ M, which was the lowest concentration tested. The maximum activation concentration is approximately 100 μ M (Figure 19B).

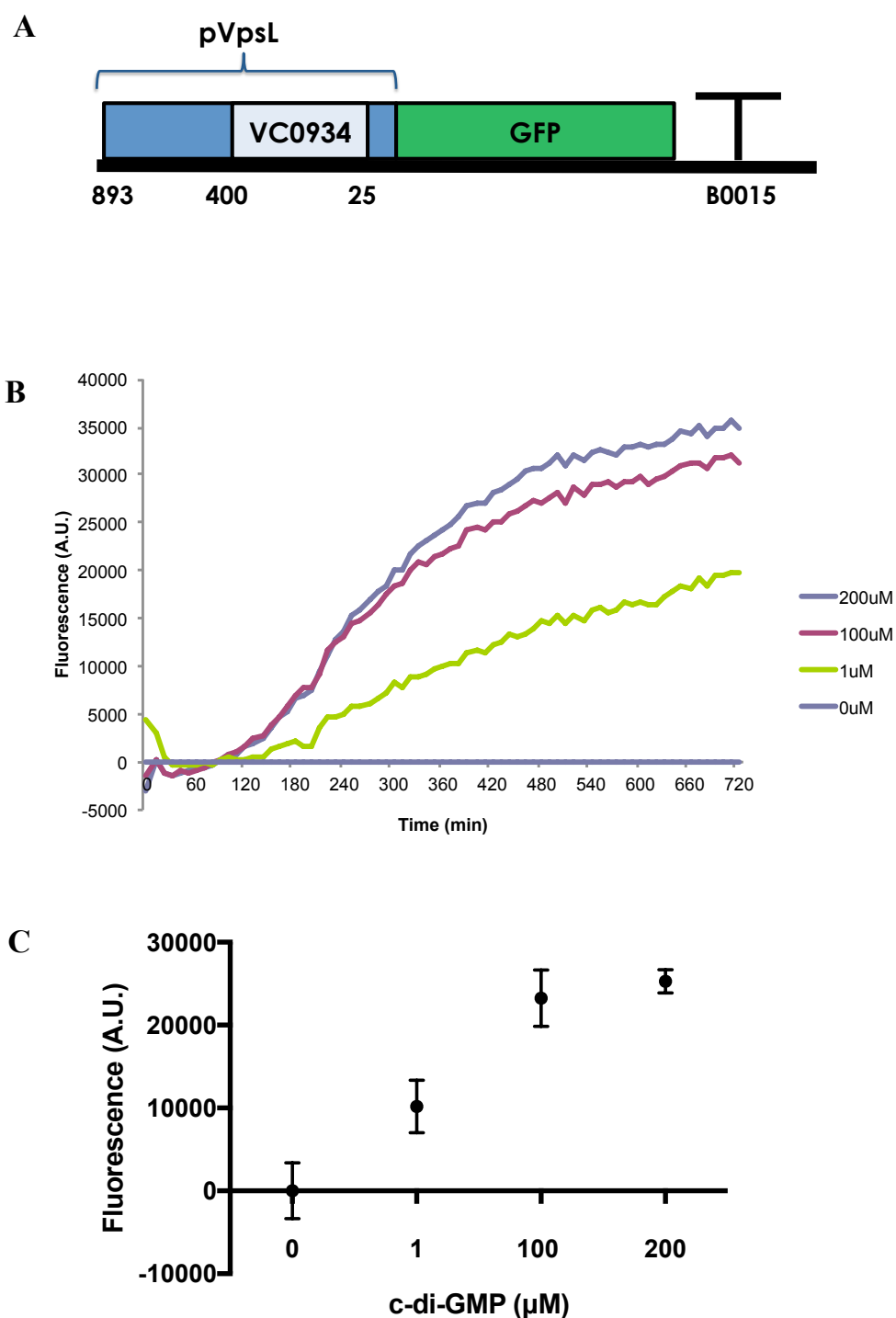


Figure 19 Activity of VpsT-regulated promoter pVpsL. (A) A comparison of the original VC0934P and the modified pVpsL promoter sequences. Numbers underneath represent the position upstream to the translational start site of the VC0934 ORF. (B) GFP fluorescence of cell-free reactions containing the pSB1A2:VpsTL plasmid induced with increasing concentrations of c-di-GMP. Reaction were incubated at 30°C for 12 hours and fluorescence was normalised to the background of pSB1A2:VpsTL with no inducer. (C) Fluorescence output of pSB1A2:VpsTL in response to a range of c-di-GMP concentration. The fluorescence increase between 30 and 360 min was taken. The mean $\pm$ standard deviation of three independent reactions is shown.

4.3.5 Summary of results

Following the design and testing of c-di-GMP biosensors, one promoter was identified that was responsive to c-di-GMP in the biosensor standard structure and when implemented in a cell-free platform. The VpsT transcription factor from *V. cholerae* was selected as the detection module for c-di-GMP. Five putative VpsT-regulated promoters were identified and a minimal sequence of these were cloned upstream of the reporter gene GFP. The initial constructs were not active in response to c-di-GMP. A full-length version of the VC0934 promoter, pVpsL, was then tested in the same context and found to be responsive to c-di-GMP induction. The minimal detected concentration with this promoter was 1 μM c-di-GMP, and the maximal output was measured at 100 μM .

There were two differences in the sequence between the truncated and full-length versions of the VpsL promoter: a) 25bp directly upstream of the start codon, assumed to contain the native RBS, was replaced with a standard BioBrick RBS BBa_B0034; b) an additional 493 bp at the start of the promoter was absent in the minimal sequence. Future work could test variants of this promoter sequence to identify whether either the native RBS or the complete length of the promoter is the feature necessary for regulation of the promoter by VpsT. The biosensor plasmid also functioned differently than the available 3-oxo-C12-HSL biosensor pSB1A2:LasRV, with a delayed response time and longer overall expression of GFP. Future work could investigate the reasons behind this difference, such as potentially slower expression and/or folding of the VpsT protein, or dynamics of c-di-GMP binding to VpsT.

5 Discussion

5.1 AHL biosensors development and characterisation

To expand the detection range of the available biosensors, a genetic circuit was designed and constructed to sense C8-HSL synthesised by the *B. cepacia* complex species. The biosensor was validated as being able to detect C8-HSL in both *E. coli* whole cell and cell-free chassis. The specificity of this circuit as well as the existing *P. aeruginosa*-specific biosensors towards their cognate autoinducers were characterised in both chassis as well. The 3-oxo-C12-HSL-specific pSB1A2:LasRV biosensor was determined to be the most specific towards its target molecule.

5.1.1 Comparing the promoter strength of pCepI319 and pCepI344

When constructing the C8-HSL biosensor, two different promoter variants were selected. pCepI344 contained the entire upstream sequence of the CepI ORF from the *B. cepacia* genome and pCepI319 was the same sequence minus the first 25 bp upstream of the start codon. It was shown that expression from the pCepI319 promoter was ~4-fold higher than pCepI344 when induced at maximum activation *in vivo*, and ~3-fold higher *in vitro*. Comparing the sequences directly provides a probable reason for this difference in the architecture of the promoter (Figure 20). A *cep* box consensus sequence where the CepR transcription factor binds has previously been determined as N-t-g-N-N-A-N-A-N-T-t-G-N-N-a-G-N-T; the bases in lower case were found in more than 50% of the aligned promoters, and bases in upper case were present in more than 70% (Chambers, Lutter, Visser, Law, & Sokol, 2006). In the pCepI promoters, the *cep* box, -10 binding site, and transcription start site has been maintained in both versions and the CepR-C8-HSL complex would therefore be able to bind and initiate transcription. This was evidenced by the fact that positive regulation by C8-HSL was observed for both promoters. However, the pCepI344 promoter sequence also has the native RBS for the CepI ORF contained in it, which would be removed in the pCepI319 promoter with truncated 25 bp. Since they were cloned into the same location on the backbone plasmid, the pSB1A2:pCepRI344 construct therefore has two consecutive RBS sequences: the native CepI RBS and the B0034 RBS, upstream of the GFP gene. Ribosomes can presumably bind to both, however if they are bound to the CepI RBS, there is no AUG start codon at the appropriate distance and therefore no protein would be produced. Therefore, it is possible that while the same number of mRNA transcripts could be

synthesised from pSB1A2:pCepRI319 as from pSB1A2:pCepRI344 plasmid, on the pCepRI344 transcripts unfavourable ribosome-ribosome interactions would occur when there is binding to the ‘incorrect’ upstream RBS. This could explain the significant difference between expression levels from the pCepI319 and pCepI344 promoter that was observed.

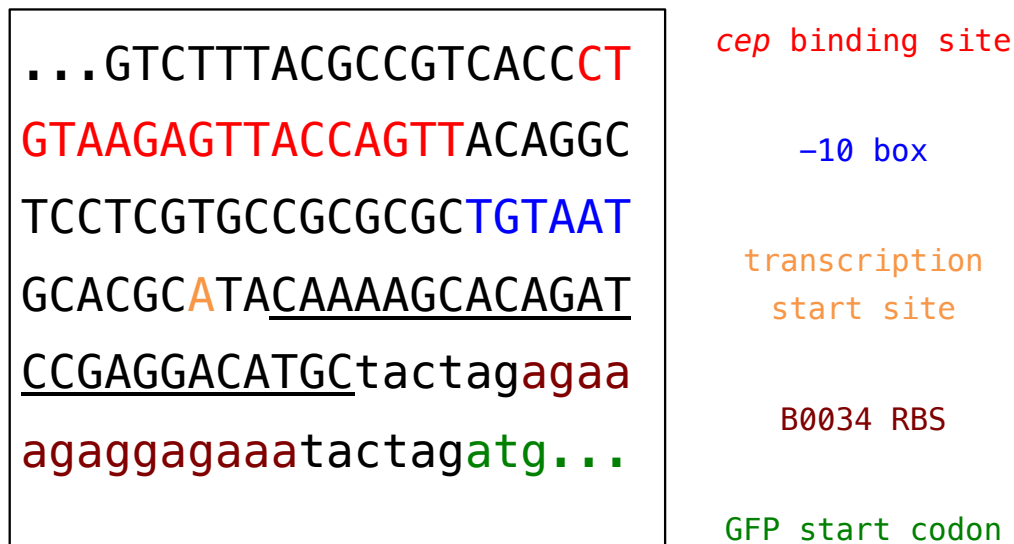


Figure 20 Architecture of pCepI344 promoter. Part of the promoter sequence of pCepI344, with downstream B0034 RBS (maroon) and start codon of GFP (green). Underlined sequence is the 25 bp which is absent in the pCepI319 promoter, containing the presumed native RBS sequence of the CepI ORF. Position and sequence of the *cep* binding site (red), -10 box (blue), transcription start site (orange) were annotated as described in Chambers et al. 2006.

5.1.2 Dynamic range of C8-HSL biosensors *in vivo* and *in vitro*

When the two C8-HSL biosensor plasmids were characterised in cell-free reactions, a high level of output signal was observed after induction by C8-HSL. One noticeable difference between the response observed *in vivo* and *in vitro* is that the dynamic range was much greater *in vitro* at the tested concentration range of inducer (Figure 5 and Figure 6). This is unlike the 3-oxo-C12-HSL-targeted pSB1A2:LasRV biosensor where the dynamic range is similar between *in vivo* and *in vitro* measurements. The pCepRI promoter has a considerably higher promoter strength than pLasRV, which could explain the difference observed. In whole cells, the protein expression capacity is much greater than in cell-free; it is estimated that the concentration of transcription and translation machinery proteins is 25-30 fold lower in *E. coli* cell-free than in cells (Takahashi et al., 2015). The rate of mRNA and protein synthesis is correspondingly 1-2 orders of magnitude slower when measured in the TX-TL cell-free system. This could explain why the expression of GFP when induced by 1 nM C8-HSL is somewhat lower *in vitro* than *in vivo*, with 2.4-fold expression over uninduced *in vitro*, compared to 3-fold *in vivo*.

On the other hand, the protein expression machinery of the cell-free is clearly not being used at maximum capacity when induced at 1 nM, since the same reaction (with the same amount of DNA template) is able to produce a much higher GFP output when induced by higher concentrations of C8-HSL. Therefore it could be that in the whole cell system there is a limit on expression from the biosensor plasmid template. Research on heterologous circuits in whole cell hosts has shown that cells can limit protein yield when the burden of additional gene expression is imposed on the finite resources of the cell (Borkowski, Ceroni, Stan, & Ellis, 2016). It was not observed that the growth rate of the C8-HSL biosensor *in vivo* was decreased when induced with AHL (Appendi Figure A1), which would be expected if a significant burden were imposed by the expression of GFP from the pCepI promoter. However, a study using a “capacity monitor” in the form of an integrated GFP expression cassette to measure burden on cells did show that the capacity of the cell for heterologous gene expression can be greatly reduced even while the impact on growth rate is not as pronounced (Ceroni, Algar, Stan, & Ellis, 2015). Therefore it could be that with the high strength of the pCepI promoter, induction at higher concentrations (100 nM and greater) would not result in an increase in GFP expression because of the limit placed on protein expression by the cell. There are many factors that can influence this behaviour, and the

expression of CepR transcription factor from the plasmid will of course also have an impact on the dynamics of the system. Creating a mathematical model for this genetic circuit would be extremely helpful in understanding how the many different stages in transcription and translation contribute to this observed difference between *in vivo* and *in vitro* behaviour. It would also be useful to test the pSB1A2:CepRI circuit in *E. coli* with lower concentrations of C8-HSL inducer to find out if the dynamic range is greater at a lower range of inducer.

5.1.3 The specificity of the biosensors towards AHL inducers

All three biosensor plasmids were induced with a range of AHLs to determine their specificity towards the intended target molecules, and each genetic circuit displayed a different degree of specificity. The pSB1A2:LasRV biosensor shows the most selective activation by its target 3-oxo-C12-HSL, while pSB1A2:RhlRA and pSB1A2:CepRI had greater non-selective activation.

Comparing all three biosensors as a whole *in vivo* versus *in vitro*, there is less non-specific activation in cell-free than in whole cells (Figure 6 and Figure 7). For example, at 100 nM *in vitro* the pSB1A2:CepRI plasmid has the strongest output for its target C8-HSL as well as C10-HSL, while at the same concentration *in vivo* an additional two AHLs (the long-chain 3-oxo-C10- and 3-oxo-C12-HSL) induced a similar level of response. As discussed above, the protein expression capacity of cell-free is lower compared to a whole cell chassis. The slower rate of transcription and translation may be contributing to this difference; in the whole cell systems the rapid protein synthesis results in measurable signal output from the non-specific AHLs, whereas *in vitro* there is less likelihood of a measurable signal being produced before the reaction becomes inactive. Therefore, although a whole cell biosensor may be preferable for certain applications because it is capable of producing a higher signal output, here it is demonstrated that the biosensors in cell-free may be more suitable where specificity is a key requirement for applications.

It is interesting to consider why the C4-HSL-targeted pSB1A2:RhlRA biosensor does not appear to be preferentially activated by its intended target. One major difference between this biosensor and the 3-oxo-C12- and C8-HSL biosensors is that activation is observed at the micromolar range rather than nanomolar. Other QS circuits such as the 3-oxo-C6-HSL-

induced LuxR have also been shown to have nanomolar sensitivity (Canton et al., 2008). If the observed sensitivities of these circuits are a reflection of the concentrations required to induce expression in the original quorum-sensing systems, it could be that the RhlR transcription factor did not need to have evolved such targeted specificity towards C4-HSL since it is unlikely to encounter equally high concentrations of other AHLs. It was previously shown that the pSB1A2:RhlRA biosensor produced a positive response when induced with *P. aeruginosa* biofilm culture supernatant, suggesting that at least micromolar concentrations of C4-HSL were produced (Chappell, 2013). The position of the RhlR/I system in the *P. aeruginosa* QS hierarchy, as the second stage of regulation after LasR/I activation, could be the reason that C4-HSL is functional at higher concentrations than other QS molecules. It is also important to note that for the *in vitro* measurements, although the C6-HSL has a higher mean output than C4-HSL at 1000 μ M, the promoter strength of pRhIA is the lowest of all three biosensors and the fluorescence measurements are therefore susceptible to noise. This can be seen by the high standard deviation of the reactions.

The C8-HSL-targeted pSB1A2:CepRI also shows a significant degree of activation by non-cognate AHLs. It is possible that the CepR/I regulatory system is the opposite of RhlR/I, in terms of being activated at a much lower concentration than other systems. This could imply either that the *B. cepacia* complex (Bcc) species switch on their quorum-sensing regulated behaviours of biofilm formation and virulence at a lower cell density than *P. aeruginosa*, or that they regulate these behaviours as a response to detection of other species. In mixed *B. cepacia* and *P. aeruginosa* biofilms, it was shown that unidirectional communication occurred, with *B. cepacia* being able to detect AHLs produced by *P. aeruginosa*, but not vice versa (Riedel et al., 2001). It is interesting to consider this possibility in light of how Bcc infections in CF occur. They are often found as co-cultures with *P. aeruginosa*, and colonise after *P. aeruginosa* has already established chronic infections (Govan & Deretic, 1996). The sudden and rapid decline in pulmonary function occurs with patients that get the so-called cepacia syndrome (Isles et al., 1984). It is possible that the *B. cepacia* species use QS to sense other bacteria and out-compete them. A study using a *Caenorhabditis elegans* infection model found that the *cep* system was necessary for invasion but not colonisation of the host, and *cep* mutant strains, unlike the wild-type, did not cause *C. elegans* killing (Kothe et al., 2003). In terms of the specificity assays shown here, it appears likely that the concentration

range that was tested is at the maximum activation response of the system *in vivo*, and at a lower concentration the observed specificity towards C8-HSL may be greater.

5.1.4 Optimising the biosensor genetic circuits

The current three biosensors were constructed based on sequences from existing QS regulation networks. In order to take a more engineering-based approach, the genetic circuits could be re-designed in a number of ways. Firstly, a library of promoters could be synthesised by maintaining the known binding sites of the transcription factors and randomly varying the remaining bases. In the case of pSB1A2:LasRV, it was one of three *P. aeruginosa* promoters that were previously identified to be upregulated when induced by 3-oxo-C12-HSL, and these three did display differences in their dynamic range and response levels (Chappell et al., 2013). Therefore it is probable that a library of promoters would show a range of different output responses to AHL induction. A second method of optimising the biosensor function could be directed evolution of the transcription factors to improve their specificity. This has been demonstrated with EsaR from *Pantoea stewartii*, where a 70-fold increase in sensitivity towards its cognate 3-oxo-C6-HSL inducer was achieved by screening a library of EsaR variants (Shong, Huang, Bystroff, & Collins, 2013), as well as with LuxR from *V. fischeri*, which was mutated to increase its sensitivity towards C8-HSL (Collins, Arnold, & Leadbetter, 2005). Finally, another approach could be to exploit the modularity of the transcription factors in terms of their structure. The N-terminal domain confers the specificity towards the AHLs, while the C-terminal domain enables DNA binding (Z. Li & Nair, 2012). According to crystal structure studies of a number of LuxR-type transcription factors, there is a highly conserved pocket in which the homoserine lactone binds, and then the acyl chain either extends outwards in the case of short-chain AHLs, or curls back towards the interior in the case of long-chain AHLs. It would be interesting to investigate whether the AHL recognition and DNA binding functions of the protein could be de-coupled to create hybrid transcription factors. These could combine the desirable characteristics of each one and avoid the weaker features; for example, the specificity of LasR could be coupled with the high activation strength of CepR. Whether this is possible has yet to be investigated. Ultimately the current biosensors can be said to have been through one round of the design-build-test cycle, and further iterations will be informed by the specifications of the particular application.

5.2 Detecting AHLs in CF patient samples

5.2.1 Sample preparation with liquid-liquid extraction

The liquid-liquid solvent extraction method was a required sample preparation process to isolate the AHLs from clinical samples and prepare them for the cell-free assay. An extraction process was developed that was compatible with both downstream analysis methods of cell-free biosensors and LC-MS/MS.

Two solvents were tested during the extraction optimisation process, ethyl acetate and dichloromethane (DCM). In previously described extraction methods, both these solvents were used as well as solid-phase extraction. The latter method was used for aqueous plasma and urine samples, and would not be compatible with the viscous condition of the sputum. Ethyl acetate was initially selected for extraction as the safer choice to avoid the carcinogenic effects of DCM exposure. When the LC-MS/MS analysis was introduced, DCM was tested to fully reproduce the method described by a similar study (Struss et al., 2013). Of the two solvents, ethyl acetate was shown to have a better extraction efficiency, as measured with the internal standard $^{13}\text{C}_4$ -3-oxo-C12-HSL, and to produce sample extract that was more compatible with the cell-free expression system (Figure 15B). The final extraction efficiency of 71% was in line with the reported figure of “up to 60%” for a similar method (Struss et al., 2013).

The sample preparation step is critical for medical diagnostic tests and can be the most challenging aspect of producing reliable and reproducible results. In the current experimental method, it takes ~ 2h for extraction of samples, ~ 0.5 hours to concentrate the collected solvent, and ~ 4 hours to incubate the cell-free reaction to completion. Including the time taken to set up the experiments, the testing of up to ten samples can be completed in one day. Of this total time, however, the extraction protocol is the most labour-intensive step because it requires the samples to be manually mixed and separated, whereas the following two steps do not need constant attention. Furthermore, it was shown here that there is significant sample variation that affected both the extraction process and the downstream assay. This sample preparation step can therefore be further developed to improve the testing of CF patient samples. One potential improvement would be to use automated liquid-handling for the solvent extraction. The volumes involved (at the millilitre scale) and the individual steps

are compatible with commercial liquid-handling robots such as the Cy-Bi FeliX (Analytik Jena). This could reduce variability between samples and would remove the need for manual extraction. The cell-free assay has also been shown to be compatible with automation at a smaller scale, with the use of the Echo (Labcyte) acoustic liquid-handler in our group (data not shown). Developing this method would allow more rapid and high-throughput testing of samples, which would enable the high-volume collection of data that is required to study the clinical relevance of AHLs in CF patient samples.

5.2.2 Choosing the right kind of patient sample

Two types of CF patient samples, BAL and sputum, were analysed with the pSB1A2:LasRV cell-free biosensor in this work. These two types were chosen for testing because of the existing evidence that they contain AHLs from infectious bacteria (summarised in Table 5, p.68). Although it was a small sample size, only one out of the ten tested BAL samples had a greater signal output than background level, which was not significant. The conclusion, therefore, was that BAL samples were a less promising sample type than sputum for the detection of AHLs. It was not possible to verify that these samples were free of AHLs, because during the time of BAL testing the LC-MS/MS validation method had not yet been developed. However, with the addition of this verification method and the improvements to the sensitivity of the extraction protocol, BAL samples could be re-tested to confirm their suitability as a sample type. In a previous study where AHLs in BAL samples were analysed, the patients were lung transplant recipients, and those who tested positive for AHL were negative for *P. aeruginosa* infections and vice versa (Ward et al., 2003). This supports the possibility that QS behaviour is upregulated during initial colonisation, and down regulated after establishing chronic infection. Since BAL samples are more practical to collect from young CF patients than sputum, it is worth investigating this sample type further to explore this hypothesis.

Sputum samples are the most commonly studied CF clinical samples in terms of the presence of QS molecules, and the work presented in this thesis supports the existing evidence that they contain measurable concentrations of AHL. There can be a number of drawbacks to using this sample type, however. Firstly the high viscosity and complexity of the matrix requires a time-consuming and labour-intensive sample preparation method, as described

above. Secondly the variation between individual samples can lead to measurement bias, which was seen in the major differences observed in extraction efficiency (Table 8) and in the impact on the cell-free activity (Figure 15) between samples. There is also the risk of sampling bias, since each sample will not be a perfect representation of the lung state and could therefore contain variable amounts of AHL depending on the sample used. This is particularly pertinent because research has found that even within the same lung there are significant regional differences in bacterial strain genotype (Jorth et al., 2015).

In a recent publication, it was demonstrated for the first time that QS signalling molecules can be measured in plasma and urine samples as well (Barr et al., 2015). Although AHLs were only found in sputum, members of the quinolone signalling family including PQS were found in plasma and urine. This study was particularly significant because it provided evidence that there was a correlation between certain QS markers and the clinical state of the patient; for example, the PQS-related 2-nonyl-4-quinolone (NHQ) molecule was measured at elevated levels in plasma before a pulmonary exacerbation. This highlights the potential for these alternative clinical sample types to be used for diagnostic purposes, with the added advantages that they are easier to collect and handle than sputum. The further expansion of the biosensor platform to target quinolone signalling molecules would be needed facilitate this development.

5.2.3 Measuring extracted AHLs with LC-MS/MS

The LC-MS/MS analysis method was developed to validate the biosensor detection of AHLs in sputum samples. It provided a way of categorically identifying AHLs by their mass, so that it could be determined if the output of the biosensor was producing false positive or negative results. Furthermore, because LC-MS/MS is not only qualitative but also quantitative, it enabled us to determine the biosensor sensitivity for AHLs extracted from sputum samples. The data acquired by LC-MS/MS confirmed that in the available sample group a number of them did have measurable AHLs, but the concentrations at which these AHLs were present was too low to trigger a positive biosensor signal. This knowledge enabled us to optimise the extraction procedure so that concentrations of AHL in the final sample extract would be within the detection range of the biosensor. The lowest concentration of 3-oxo-C12-HSL

from a sputum sample extract that was detected by the pSB1A2:LasRV cell-free biosensor was thus 4.5 nM (Figure 16).

When the LC-MS/MS method was first explored for this research, the work by Struss et al. (2013) was the most thorough study available that had quantitative measurements of AHLs in CF sputum samples. In that study they reported 45/47 samples had detectable 3-oxo-C12-HSL. This was a much higher proportion than the 6/20 AHL-positive samples that we initially identified using LC/MS, and the reported concentrations of 3-oxo-C12-HSL in their sample group was also higher than in the ones measured in this work (20-6833 nM compared to <1-17 nM, see Table 6). It should be noted that the concentrations are not directly comparable because Struss et al. applied a correction for the extraction efficiency, calculated by the recovery of spiked internal standard per sample. In addition their original sample volume ranged from 1-5 ml, compared to our consistent 1 ml samples. Even taking these factors into account, however, the reported values in their study would be higher than the concentrations of AHL detected in our samples. It is thus clear that there are major differences between the cohorts of patient samples analysed in these studies. A more recent study by Barr et al. (2015) detected 3-oxo-C12-HSL in 72/110 samples, with a median concentration of 4 nM. This further illustrates that variability between sample groups plays an important role in the concentrations of AHL found.

The sample group used for this work was acquired from the Biobank at Royal Brompton Hospital, which is a depository of clinical samples and strain isolates collected for research purposes. Since these samples are deposited over an extended period of time from a diverse group of patients, they are likely to be varied in terms of the clinical status of the patients at the time of collection. It would be beneficial to gather more information about the clinical status of the patients in our cohort, or to collect samples with more stringent requirements (such as only from early-stage infection). This would allow us to identify differences between our sample group and those reported in other studies, and may help elucidate the factors that are responsible for the varying AHL concentrations found. This is now being explored in our group with the collection of clinical samples from patients admitted for pulmonary exacerbations.

5.2.4 Measuring extracted AHLs with cell-free biosensors

The cell-free biosensor assay was applied towards the measurement of AHLs from CF clinical samples. Although three biosensor circuits were constructed with different AHL targets, ultimately only the 3-oxo-C12-HSL-targeted pSB1A2:LasRV biosensor was used for testing clinical samples. This was firstly because the C8-HSL pSB1A2:CepRI plasmid had not yet been designed when testing started, and secondly because it was hypothesised that the concentrations of AHL in clinical samples would be too low for the micromolar sensitivity range of the C4-HSL pSB1A2:RhlRA biosensor. After the LC-MS/MS validation method was developed, it was confirmed that 3-oxo-C12-HSL was the most prevalent AHL in CF sputum, and that the detected concentration of C4-HSL was over 1000-fold too low for the biosensor limit of detection. C8-HSL was also extremely rarely encountered in the tested samples, with only one sample found to contain measurable C8-HSL using LC-MS/MS (Table 7, p.86). Therefore the pSB1A2:LasRV biosensor was deemed the most suitable for the testing of this sample group. It would be interesting to acquire clinical samples specifically from Bcc-infected patients to validate the pSB1A2:CepRI biosensor as well.

The main challenge encountered with the cell-free biosensor was that the concentrations of AHL found in the tested sputum were at, or around, the lower limits of detection for pSB1A2:LasRV, making it difficult to reliably distinguish a positive signal from background expression. The dynamic range of this circuit is 1-100 nM, which meant that the majority of AHL-positive samples did not have a high enough concentration to induce a strong response. This was partially mitigated by re-suspending dried solvent extract directly into cell-free reactions so that there would be more AHL per reaction. It was also shown, however, that the addition of the sputum extract to cell-free reactions resulted in a dampening of the signal output (Figure 14 and Figure 15). The combination of the low native concentrations of AHL and the dampening effect of extract addition therefore led to a number of false-negative results, where 3-oxo-C12-HSL could be measured in samples by LC-MS/MS but could not be detected by the cell-free biosensor.

A key development for this assay would therefore be to improve either the sensitivity or the signal-to-noise ratio of the pSB1A2:LasRV biosensor, so that low concentrations of 3-oxo-C12-HSL would still produce a strong detectable output. The sensitivity could be improved by engineering the genetic circuit, which has been discussed above (section 5.1.4, p.114). The

signal-to-noise ratio of the existing circuit could also be boosted in a number of ways to produce a stronger output response that is clearly above background levels. One method could be to replace the GFP reporter gene with a more suitable output module. At the emission spectrum of GFP there is a significant level of background autofluorescence from cell-free extract (Pardee et al., 2014), which results in a high baseline signal that can obscure small increases in GFP expression. Other fluorescent proteins that have an emission spectrum with low background signal in cell-free would be a better alternative, such as mCherry. Alternatively, an enzymatic output such as LacZ or luciferase would be suitable because they are able to catalyse multiple reactions and thereby amplify the total signal output. Another, more sophisticated, approach to this could be to introduce additional DNA elements to produce a genetic cascade that would amplify the signal output. These have been demonstrated to be feasible in cell-free reactions, with different designs such as transcriptional cascades using RNA attenuator sequences (Takahashi et al., 2015) and endogenous sigma factors or viral polymerases (Noireaux et al., 2003; J Shin & Noireaux, 2012).

Another way of improving the signal output is to use cell-free systems with a higher protein expression capacity. A commercial *E. coli* S30 extract (Promega) was used for the experiments in this work, and provided a reliable and reproducible test platform for the cell-free assays. However, in recent years *E. coli* transcription-translation (TX-TL) systems have been developed that have vastly improved protein yields and are also much more cost-effective than commercial systems (Jonghyeon Shin & Noireaux, 2010; Sun et al., 2013). Initially, the use of such TX-TL extracts was explored by preparing cell-free systems with *E. coli* BL21-Gold (DE3), which is the same strain used to produce the commercial S30 system. It was not observed that the signal output was greater with this extract compared to the commercial version. Further work in this area has since been carried out by members of the group, however, and it has been shown that the *E. coli* Rosetta strain produces over 2-fold greater activity compared to the commercial S30 extract (Figure 21). Using this system, additional testing of sputum samples has found there is a marked improvement in the ability of the assay to detect AHLs (data not shown). It is therefore clear that the major bottleneck for the cell-free biosensor assay was the limited signal output of the platform, and developing a high-producing cell-free platform has been a major breakthrough for this technology.

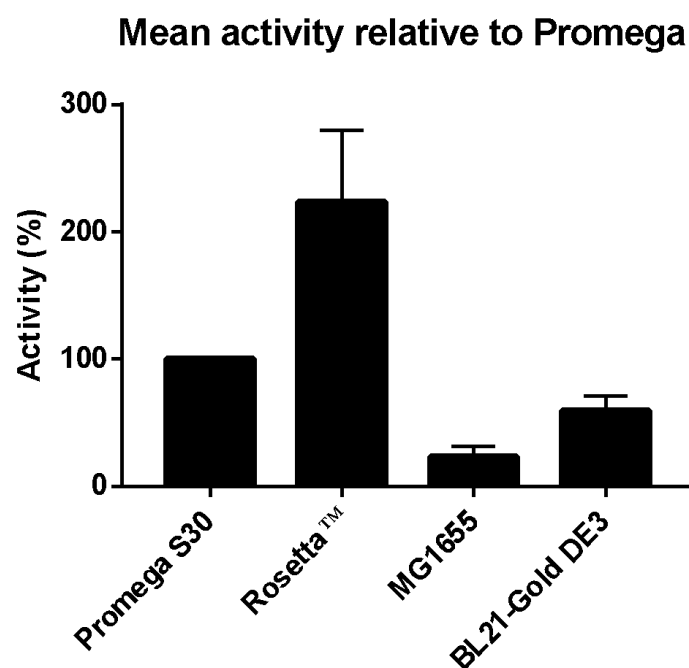


Figure 21 Comparison of commercial cell-free extract with TX-TL from three *E. coli* strains. Cell-free reactions were prepared with 1 μ g pSB1A2:LasRV plasmid DNA and induced with 110 nM 3-oxo-C12-HSL. The mean and standard deviation for three reactions per extract is shown. Data provided by Loren Cameron.

The cell-free expression system can provide a valuable alternative platform for the implementation of designed genetic circuits in the field. The assay in its current form, however, requires storage of the cell extract and buffers at -80°C before they are combined together directly before measurement. This would be impractical outside of a research setting, especially if the assay is to be used as a point-of-care diagnostic. To improve the usability of the cell-free system, it was demonstrated that reactions could be prepared in advance and freeze-dried. After storage at room temperature for 24 hours, they were re-constituted with water and upon induction with AHL were shown to have the same level of activity as newly prepared reactions (Figure 22). Although the freeze-dried extracts were only stored for 24 hours in our case, it has previously been reported to be stable for up to a year (Pardee et al., 2014). This method expands the potential for cell-free systems to be a suitable chassis in non-research settings, as it eliminates the need for cold-chain storage and requires minimal training to set up.

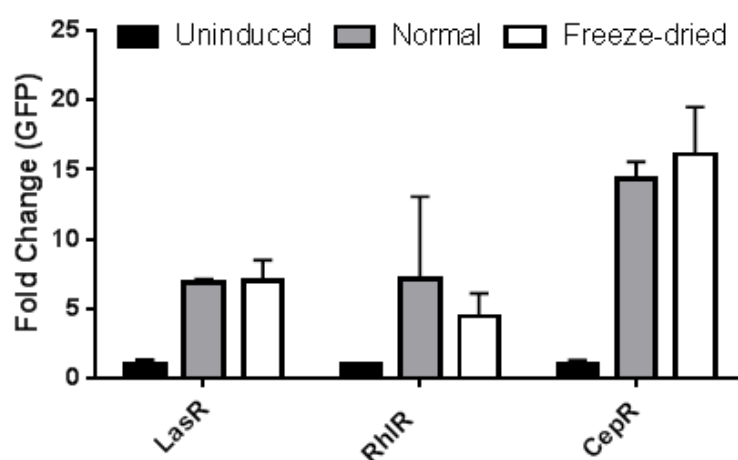


Figure 22 Freeze-dried cell-free biosensor assay. Cell-free reactions were prepared with biosensor plasmid DNA and freeze-dried, stored at room temperature for 24 hours, and reactivated by addition of water and AHL solution. Fluorescence gain was measured between 30 min and 240 min, and calculated as fold change compared to uninduced reactions containing the same biosensor DNA. Samples were activated at maximal induction concentration – LasR with 100 nM 3-oxo-C12-HSL, RhlR with 1 mM C4-HSL, and CepR with 100 nM C8-HSL. The mean and standard deviation of six reactions are shown for uninduced and freeze-dried, which is made up of two sets of three reactions freeze-dried separately. The mean and standard deviation of three reactions are shown for the normal cell-free conditions. Data provided by Richard Kelwick.

5.3 C-di-GMP as a marker for biofilm formation

The cell-free biosensor assay was expanded to include a non-QS signalling molecule, the intracellular secondary messenger c-di-GMP. Since this molecule has been widely implicated in the regulation of biofilm formation, it provides a potential marker for identifying biofilm-based infections regardless of bacterial species. A biosensor device based on the *V. cholerae* VpsT transcription factor and pVpsL regulated promoter was developed with micromolar sensitivity to c-di-GMP.

5.3.1 Comparing the VC0934 and pVpsL promoter activity

When the c-di-GMP biosensor was designed, the DNA binding sites for VpsT had not yet been experimentally determined (Krasteva et al. 2010). Five putative promoters were therefore screened for inducible expression by VpsT, but showed no measurable response in the presence of c-di-GMP. One of these promoter sequences, VC0934, was extended to include the full upstream region of the VC0934/*vpsL* gene (named pVpsL), and this did produce a positive output in response to c-di-GMP induction.

Recently Zamorano-Sánchez et al. (2015) studied the architecture of the *vpsL* promoter and identified the site of VpsT binding using DNase I footprinting. The sequence, named the T box, was found to consist of a 47 bp sequence that was 250 bp upstream of the start codon. The transcriptional start site, RBS, -10 and -35 sites were also annotated. Comparing these regions to the promoters screened in this work, it can be seen that all of the promoter elements, including the T box, were retained in the truncated VC0934 promoter as well as in pVpsL. In theory, the VC0934 sequence should therefore be a functional inducible promoter. Furthermore, a 22 bp palindromic motif within the T box was also conserved among other promoter regions, including the VC0917 and VC0928 ORFs that were tested in this work. The only difference between the native sequences in the *V. cholerae* genome and the tested promoters here is the RBS region, where the native RBS was replaced with the B0034 RBS (plus flanking BioBrick scar sites, making a total of 25 bp). Approximate estimates of the RBS strength using the RBS calculator tool (<https://salislab.net/software/>) estimates that the B0034 RBS has ~5-fold higher strength than the native RBS in the pVpsL sequence. Therefore it remains unclear why the truncated promoter sequences did not respond to c-di-GMP induction.

5.3.2 Implementing c-di-GMP as a biomarker for biofilm formation

The pSB1A2:VpsTL biosensor was shown to be responsive to chemically synthesised c-di-GMP inducer, but it has not yet been tested for the detection of endogenous c-di-GMP from bacterial cells. There are a few challenges that could be encountered if it were used for clinical sample testing. Firstly, since c-di-GMP is an intracellular signalling molecule, an initial cell lysis step would be required in order for it to be detectable by the biosensor. This could be achieved with a short heating step, as was shown to be possible for the detection of viral RNA in infected plasma samples (Pardee et al., 2016). Secondly, the sensitivity of the current biosensor is probably too low to measure c-di-GMP from endogenous sources. The cellular concentration of c-di-GMP has been estimated to be in the nanomolar to micromolar range (Hengge, 2009). An extremely high cell density would consequently be required to reach the micromolar limit of detection in the cell-free assay, which is unlikely to be achievable in clinical samples. Alternative detection modules that could provide greater sensitivity are the c-di-GMP-responsive riboswitches, which have been experimentally determined to have binding affinities of ~10 pM and 2 nM (Shanahan, Gaffney, Jones, & Strobel, 2011; Kathryn D Smith et al., 2009).

Beyond clinical testing, a c-di-GMP biosensor could be used as a semi-quantitative tool to further understand how c-di-GMP concentration is correlated with biofilm regulation. It is known that synthesis of c-di-GMP corresponds with the switch from motile to sessile phenotype (Boyd & O'Toole, 2012). However it has been hypothesised that c-di-GMP-regulated behaviours are effected through local concentrations of signalling molecule, which allows multiple pathways to be regulated in isolation (Hengge, 2009). If this is the case, the total cellular pool of c-di-GMP may not be measurably different even when synthesis of the molecule is upregulated by individual proteins. On the other hand, there is also evidence that c-di-GMP regulation can be mediated through its global concentration to which multiple synthesis proteins contribute (Shikuma, Fong, & Yildiz, 2012). Currently, methods for measuring cellular c-di-GMP concentrations include HPLC (Fazli et al., 2011) and mass spectrometry (Massie et al., 2012; H. Tan et al., 2014). The cell-free c-di-GMP biosensor could potentially provide a simple and low-cost alternative for the quantification of c-di-GMP levels, which would enable further research into the intracellular regulation by this widely conserved signalling molecule.

6 Conclusion

In this work, the development of cell-free biosensors for detection of clinically relevant biomarkers has been described. Although the field of synthetic biology has led to many promising new technologies being proposed, particularly in the healthcare sphere, there are still very few examples of genetic devices that have been successfully shown to function with real-world samples. The aim of this project was, therefore, to determine the potential and limitations of using cell-free biosensors for diagnostic purposes. The role of quorum sensing in bacterial infections is still being elucidated, and the biosensors that have been developed here for the measurement of QS molecules offer a rapid, cheap, and easy-to-use tool that can help to understand this process.

The number of QS molecule targets was expanded with the construction of a C8-HSL-sensitive device to monitor species from the *B. cepacia* complex. This increases the utility of the cell-free biosensor platform to detect other pathogens as well as *P. aeruginosa*, for which AHL biosensors had previously been constructed. In addition, a biosensor for the intracellular regulator molecule c-di-GMP was designed, built, and shown to be functional in cell-free systems. This has the potential to be a general biofilm detection tool that can be used to further analyse the phenotypic state of pathogens during infections.

The three AHL biosensors, targeted towards C4-, C8-, and 3-oxo-C12-HSL, were shown to have vastly different device characteristics, both in terms of their output strength and the specificity of response towards their target inducers. The biosensor with the greatest specificity towards its intended AHL was the pSB1A2:LasRV biosensor for 3-oxo-C12-HSL. This biosensor was then applied in a cell-free assay to the detection of AHLs in cystic fibrosis patient samples.

Sputum samples collected from cystic fibrosis patients were analysed for the presence of AHLs using both the cell-free biosensor and LC-MS/MS. The most commonly-detected AHL was 3-oxo-C12-HSL, with C4-, C8- and 3-oxo-C10-HSL being detected less frequently. The targets for which biosensors have been developed are therefore shown to be in agreement with the AHLs that are likely to be found in these clinical samples. It was shown that the concentration of AHLs present in samples are very low and require highly sensitive measurement tools. Through extensive optimisation of the sample preparation method

through solvent extraction and of the cell-free assay, the biosensor was demonstrated to be able to measure 3-oxo-C12-HSL in sputum samples. Increasing the output of the current cell-free system to produce a stronger output is the next logical step in order to be able to clearly distinguish between a positive response and background signal. This is currently in development and has produced highly promising results.

The initial hypothesis was that patients with diagnosed *P. aeruginosa* infections would have detectable levels of AHL in collected clinical samples. This was shown not to be the case, with many of the *Pseudomonas*-positive samples being negative for AHL presence. However, all of the samples with confirmed AHL presence were from *Pseudomonas*-infected patients, which raises further interesting questions about what distinguishes the production of QS molecules in some infections but not others. The time since initial colonisation, whether infections are in a stable chronic state or causing exacerbations, or the presence of co-infections by other species are all possible variables that can be explored in greater depth using cell-free biosensor devices.

7 Bibliography

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8 Appendices

8.1 Growth of pSB1A2:pCepI319 and pSB1A2:pCepI344 in *E. coli*

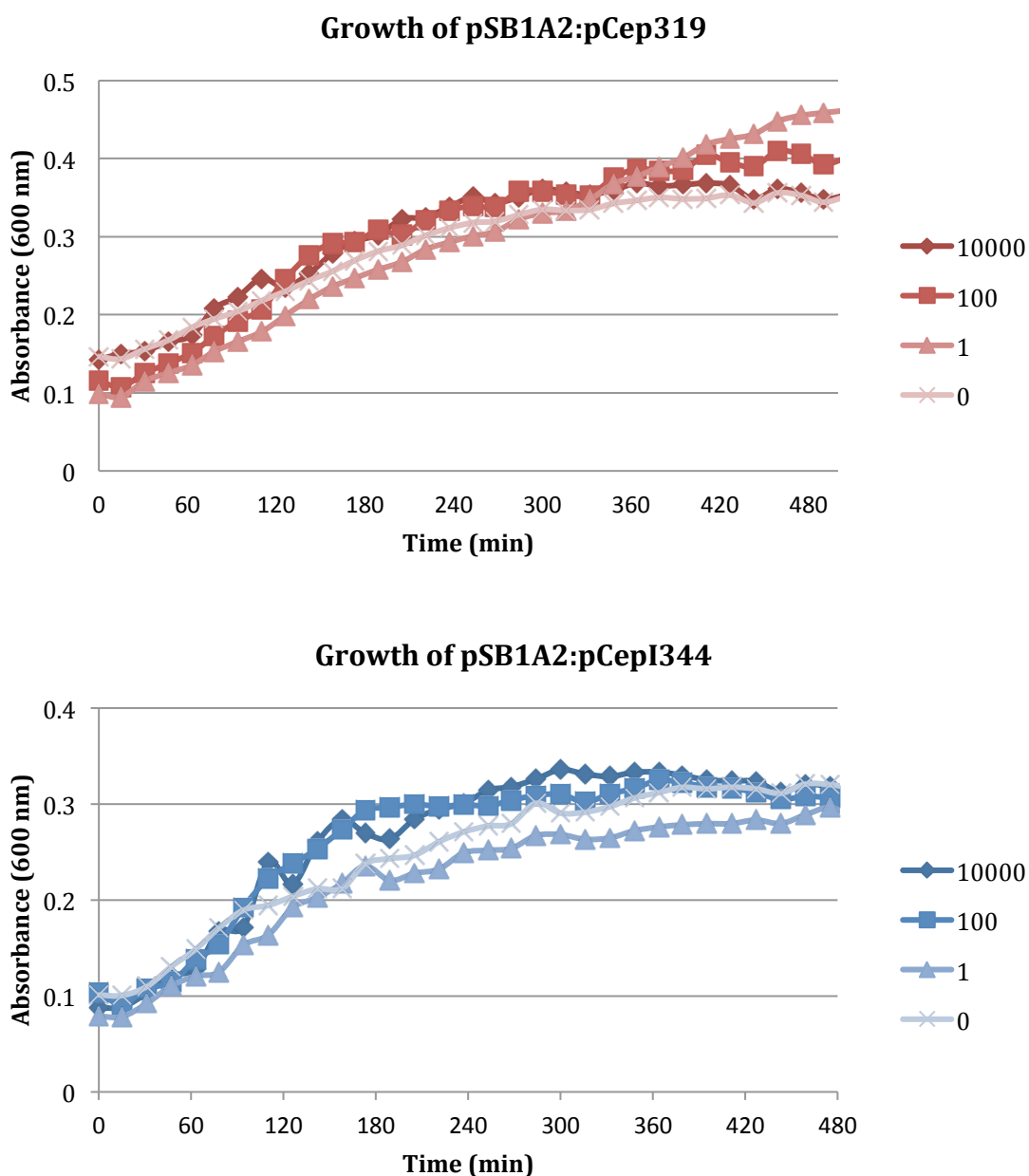


Figure A1: Growth of pSB1A2:pCepI319 and pSB1A2:pCepI344 in *E. coli*.

E. coli BL21 Gold (DE3) strains transformed with plasmid were cultured overnight in M9 media with ampicillin at 37°C. Cultures were diluted 100x into fresh media and grown for 3 hours, then diluted to an O.D.₆₀₀ of 0.07 and grown for 1 hour. After transferral to a 96-well plate and induction with C8-HSL, cultures were incubated in a plate reader and monitored for GFP fluorescence and absorbance every 15 minutes for 8 hours. Concentration of C8-HSL (nM) is shown in the key above. The mean of three cultures was taken at each concentration.

8.2 Inhaled medications prescribed to CF patients who provided samples N1-N10

Inhaled medication	<u>Function</u>	<u>Dosage</u>	<u>Mode of action</u>
DNase	Mucolytics	2.5 mg/day	Digests extracellular DNA
Mannitol	Mucolytics	400 mg BD	Increase osmolality of aqueous surface layer
Hypertonic Saline	Mucolytics	7% 4 ml/day or BD	Hydrate thick mucus.
Colomycin	Antibiotics	2MU BD	Cyclic polypeptides A and B. Acts like a detergent to solubilise membranes. Active against Gram-negative bacteria.
Tobramycin (usually as TOBI)	Antibiotics	300 mg BD	Aminoglycoside. Binds to 30S and 50S ribosome, thus stopping translation. Active against Gram-negative bacteria.
Aztreonam (as AZLI)	Antibiotics	75 mg TDS	Monocyclic Beta-lactam. Active against Gram-negative bacteria. Inhibits cell wall synthesis, blocking peptidoglycan crosslinking.
Symbicort	Inhaled Steroids	200/6 or 400/12 strength (200 µg budesonide/ 6 µg formoterol)	Budenoside is a glucocorticoid. Potential anti-inflammatory benefits to limit lung damage
Seretide	Inhaled Steroids	500 mg BD	Fluticasone (corticosteroid)/ salmeterol (prevents airway constriction).

8.3 Supplementary Materials

Table A2 Media for bacterial growth

LB medium	10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl
LB agar	10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15g/L agar
SOC medium	20 g/L Tryptone, 5 g/L Yeast Extract, 4.8 g/L MgSO ₄ , 3.6 g/L dextrose, 0.5 g/L NaCl, 0.186 g/L KCl
M9 medium, supplemented	1X M9 salts (6.78g/L Na ₂ HPO ₄ , 3g/L KH ₂ PO ₄ , 0.5g/L NaCl, 1g/L NH ₄ Cl), 0.4% (w/v) glycerol, 0.2% (w/v) casamino acids, 2 mM MGSO ₄ , 0.1 mM CaCl ₂ , 1 mM thiamine HCl

Table A3 Antibiotics for plasmid selection

Ampicillin	100 µg/ml
Kanamycin	50 µg/ml
Chloramphenicol	34 µg/ml

Table A4 Gel electrophoresis

TAE buffer	40 mM Tris-acetate, 1 mM EDTA, pH 8.3
Agarose gel	1% w/v agarose, 1X TAE buffer, 0.5X GelRed or 1X SYBR Safe
6X DNA loading buffer	50% glycerol, 0.1% bromophenol blue, 10 mM EDTA
SDS running buffer	25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3
4X protein loading buffer	20% SDS, 40% glycerol, 0.4% bromophenol blue
Tris-glycine gel	Novex 4-20% tris-glycine gel (Life Technologies)

Coomassie stain	0.05% Coomassie Blue R-250, 30% ethanol, 10% acetic acid
Destain solution	30% ethanol, 10% acetic acid

Table A5 Kits

	Supplier
QIAprep Spin miniprep plasmid purification	Qiagen
QIAfilter Maxiprep plasmid purification	Qiagen
QIAquick gel extraction	Qiagen
QIAquick PCR purification	Qiagen
In-Fusion cloning	Clontech
<i>E. coli</i> S30 extract system for circular DNA	Promega

Table A6 Oligonucleotides

13504_fwd	ATGCGTAAAGGAGAAGAAGCTTTTCAC
13504_rvrs	CTTTCTCTAGAAGCGGCCG
PVPSL_fwd	CGGCCGCTTCTAGAGAAAGTACGATCGCTTGAGCAAG
PVPSL_rvrs	CTTCTCCTTTACGCATTAGACGCTCCTAACCGATG
GFPseq_rvrs	GCCCATTAACATCACCATCTAA
J23101B34_F	CGA TGT AAC CCA CTC GTG C
J23101B34_R	CTAGTATTTCTCCTCTTTCTCTAGTAG
BSV_F	GTTTATATACTAGAGCCTTCCGAAACGAAACAAGTTG
BSV_R	CCTCTTTCTCTAGTACTCGGCGTTATGTCATGAAGAG
B34GFP_F	AAAGAGGAGAAATACTAGATGCGTAAAGG
Bbprefix_R	CTAGAAGCGGCCGCGAATTC
BBprefix_F	GAATTCGCGGCCGCTTCTAG
pCepI_R	GTATTTCTCCTCTTTCTCTAGTATATGCGTGCATTACAGCGC
VF2	TGCCACCTGACGTCTAAGAA
VR	ATTACCGCCTTTGAGTGAGC

Table A7 Acyl homoserine lactones

N-butyryl-DL-homoserine lactone (C4-HSL)	Sigma Aldrich (#09945)
N-hexanoyl-DL-homoserine lactone (C6-HSL)	Sigma Aldrich (#09926)
N-heptanoyl-DL-homoserine lactone (C7-HSL)	Sigma Aldrich (#10939)
N-octanoyl-DL-homoserine lactone (C8-HSL)	Sigma Aldrich (#10940)
N-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL)	Sigma Aldrich (#O1764)
N-decanoyl-DL-homoserine lactone (C10-HSL)	Sigma Aldrich (#17248)
N-3-oxo-decanoyl-L-homoserine lactone (3-oxo-C10-HSL)	Sigma Aldrich (#O9014)
3-oxo-C12-HSL lactone (3-oxo-C12-HSL)	Cayman Chemicals (#10007895)
[¹³ C] ₄ -3-oxo-C12-HSL	Kindly synthesised by Dr. Yonek Hleba

Table A8 DNA part sequences

Name	Part type	Sequence	Source
J23101	Promoter	TTTACAGCTAGCTCAGTCCTAGGTATTATGCTAG	Parts registry
B0034	RBS	AAAGAGGAGAAA	Parts registry
B0015	Double terminator	CCAGGCATCAAATAAACGAAAGGCTCAGTCGAAAGAC TGGGCTTTTCGTTTTATCTGTTGTTGTGTCGGTGAACGCTC TCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCTTT CTGCGTTTATA	Parts registry
LasR	Gene	ATGGCCTTGGTTGACGGTTTTCTTGAGCTGGAACGCTCAA GTGGAAAATTGGAGTGGAGCGCCATCCTCCAGAAGATGG CGAGCGACCTTGGATTCTCGAAGATCCTGTTCCGGCTGTT GCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGT CGGCAACTACCCGGCCGCTGGCGCGAGCATTACGACCG GGCTGGCTACGCGCGGGTCGACCCGACGGTCAGTCACTG TACCCAGAGCGTACTGCCGATTTCTGGAACCGTCCAT CTACCAGACGCGAAAGCAGCAGGATTCTTCGAGGAAGC CTCGGCCGCGGCTGGTGTATGGGCTGACCATGCCGCT GCATGGTGCTCGCGCGAACTCGGCGCGCTGAGCCTCAG CGTGGAAGCGGAAAACCGGGCCGAGGCCAACCGTTTCAT AGAGTCGGTCTGCCGACCTGTGGATGCTCAAGGACTA CGCACTGCAAAGCGGTGCCGACTGGCCTTCGAACATCC GGTCAGCAAACCGGTGGTTCTGACCAGCCGGGAGAAGG AAGTGTTGCAGTGGTGCGCCATCGGCAAGACCAGTTGGG AGATATCGGTTATCTGCAACTGCTCGGAAGCCAATGTGA ACTTCCATATGGGAAATATTTCGGCGGAAGTTCGGTGTGA CCTCCCGCGCTAGCGGCCATTATGGCCGTTAATTGG GTCTTATTACTCTCTAATAA	Parts registry
RhlR	Gene	ATGAGGAATGACGGAGGCTTTTTGCTGTGGTGGGACGGT TTGCGTAGCGAGATGCAGCCGATCCACGACAGCCAGGGC GTGTTTCGCCGTCTTGAAAAGGAAGTGCAGCGCGCTGGG TTCGATTACTACGCCTATGGCGTGCGCCACACGATTCCCT TCACCCGGCCGAAGACCGAGGTCCATGGCACCTATCCCA AGGCCTGGCTGGAGCGATACAGATGCAGAACTACGGG GCCGTGGATCCGCGATCCTCAACGGCCTGCGCTCCTCG GAAATGGTGGTCTGGAGCGACAGCCTGTTTCGACCAGAGC CGATGCTCTGGAACGAGGCTCGCGATTGGGGCCTCTGT GTCGGCGCGACCTTGCCGATCCGCGCGCCGAACAATTTG CTCAGCGTGCTTTCCGTGGCGCGGACAGCAGAACATC TCCAGCTTCGAGCGCGAGGAAATCCGCCTGCGGCTGCGT TGCATGATCGAGTTGCTGACCCAGAAGCTGACCGACCTG GAGCATCCGATGCTGATGTCCAACCCGGTCTGCCTGAGC CATCGCGAACGCGAGATCCTGCAATGGACCGCCGACGGC AAGAGTTCCGGGGAAATCGCCATCATCCTGAGCATCTCC GAGAGCACGGTGAACCTCCACCACAAGAATCCAGAA GAAGTTGACGCGCCGAACAAGACGCTGGCTGCCGCTA CGCCGCGGCGCTGGGTCTCATCTAATAA	Parts Registry
CepR	Gene	ATGGAATGCGCTGGCAGGATGCCTACCAACAATTACG GCCGCGGAGGACGAGCAGCAGCTCTTCCAGCGGATCGCC GCCTATTCCAAGCGGCTCGGATTGCAATACTGCTGTTAC GGCATTTCGCGTACCGCTGCCCGTTTCGAAGCCGGCCGTC GCCATCTTCGATACCTATCCGGACGGCTGGATGGCGCAC TACCAGGCGCAGAACTACATCGAGATCGATTTCGACGGTT CGTGACGGCGCGCTCAGCACCAACATGATCGTGTGGCCG GACGTCGACCGGATCGATCCGTGCCCGCTGTGGCAGGAC GCGCGCGATTTCGGATTGTGGTGGGCGTCCGCGCAGTCG AGCTGGGCGGCGCGCGCGGCTTCGGGCTGCTAAGCATC GCCC GCCATGCCGACCGGCTGACGCCGCGCGAGATCAAC ATGCTGACGCTGCAGACCAACTGGCTCGCAAACCTGTG CATTCGCTGATGAGCCGCTTCATGGTGCCGAAGCTGTG CCGGCGGCGGGCGTACGCTGACCGCGCGGAGCGCGA GGTGCTGTGCTGGACGGCCGAGGGCAAGACCGCGTGCG AAATCGGCCAGATCCTCAGCATCTCGGAGCGGACGGTCA ACTTCCACGTGAACAACATCCTCGAGAAGCTCGGCGCGA CCAACAAGGTCCAGGCGGTGCTCAAGGCGATCTCGGCCG GGCTCATCGAAGCACCTGA	<i>B. cenocepacia</i> H111 genome (Genbank accession no. HG938371.2)
VpsT	Gene	ATGAAAGATGAAAACAACTAAACGTTAGAATGCTTTCT GATGTTTGCATGCAATCCAGATTGTTGAAAGAGGCGTTA GAATCAAAACTTCCTTTGGCGCTGGAAATTACACCATTTT	<i>V. cholerae</i> O1

		CTGAGCTCTGGCTTGAAGAGAATAAACAGAAAGTCGCA GTATTCAGATGCTGGTGATTGATTATTCAAGAATTTCTGA TGATGTTTTGACCGATTACAGCTCGTTTAAAGCACATCAGT TGTCCTGATGCGAAAAGAGGTCATCAAACTGTCCGACAG GATATTGAGCATAAGCTGCTCTTTAAGTGAATAATTTG GCTGGAGTATTTATATTGATGATGATATGGATACCTGA TCAAAGGCATGAGTAAAAATTTGCAAGATGAAATGTGGT TAACGCGTAAACTGGCCCAAGAATACATTCTCCATTATC GTGCCGGTAACTCAGTCGTGACCTCACAATGTACGCAA AATTAACCAAAAAGAGAACACAGATTATCAAGTTACTTG GTAGTGGTGTCTCTAATATTGAAATTGCAGATAAACTCTT TGTGAGTGAATAACAGTAAAAACACATCTGCATAATGT CTTTAAGAAAAATTAATGCCAAAAATCGCTTGCAGGCACT GATTTGGGCGAAAAATAATATTGGAATTGAGGAAGTCAA TTCTTAA	biovar El Tor str. 16961 genome (Genbank accession no. NC_002505.1 and NC_002506.1)
GFPmut3b	Gene	ATGCGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCA ATTCTTGTGTAATTAGATGGTGATGTTAATGGGCACAAA TTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATAC GGAAACTTACCCTTAAATTTATTGCACTACTGGAAAA CTACCTGTTCATGGCCAACACTTGTCACTACTTTTCGGTT ATGGTGTTCAATGCTTTGCGAGATACCCAGATCATATGA AACAGCATGACTTTTCAAGAGTGGCATGCCCCGAAGGT ATGTACAGGAAAGAACTATATTTTCAAAGATGACGGGA ACTACAAGACACGTGCTGAAGTCAAGTTGAAGGTGATA CCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAA AGAAGATGGAAACATTCTTGGACACAAATTGGAATACAA CTATAACTCACACAATGTATACATCATGGCAGACAAACA AAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAA CATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCA ACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTACCA GACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAA GATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAG TTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAA CTATACAAATAATAA	Parts Registry
pLasV	Promoter	CCTTTCCGAAACGAAACAAGTTGGATTTTGCACCTACCA GAACTGGTAGTTCTGACCTGTGGCTATCTTCGAAGGCAT CGATATTATGCACATTGGAACCTTTCATGACATAACGCC GAG	Chappell 2013
pRhIA	Promoter	TCCTGTGAAATCTGGCAGTTACCGTTAGCTTTCGAATTGG CTAAAAAGTGTTTC	Chappell 2013
pCepI319	Promoter	GATCCGACATCGGCATGTTGCGCACGGCCGCGCGCTCG CCGCACGGCTCGCCGCCCTCGACTGGCCCGCCGCGGTG CGTCGGCCCTGCTCGGGCACAACGACGCCATCATGCCG GTCCGACGCTCCAGTGACAACCGGCCGCGCATTCCTCTG ACGATTTTGCAAGACTGGCGCTCTTTATAAGCAAAAAGCG GCGCGGTTTTCAATCCCGTTGATCAAGAAACCGTTACCA CGTCCCGAATGGCGTCTTTACGCCGTACCCCTGTAAGAG TTACCAGTTACAGGCTCCTCGTGCCGCGCGCTGTAATGC ACGCATA	This work
pCepI344	Promoter	GATCCGACATCGGCATGTTGCGCACGGCCGCGCGCTCG CCGCACGGCTCGCCGCCCTCGACTGGCCCGCCGCGGTG CGTCGGCCCTGCTCGGGCACAACGACGCCATCATGCCG GTCCGACGCTCCAGTGACAACCGGCCGCGCATTCCTCTG ACGATTTTGCAAGACTGGCGCTCTTTATAAGCAAAAAGCG GCGCGGTTTTCAATCCCGTTGATCAAGAAACCGTTACCA CGTCCCGAATGGCGTCTTTACGCCGTACCCCTGTAAGAG TTACCAGTTACAGGCTCCTCGTGCCGCGCGCTGTAATGC ACGCATACAAAAGCACAGATCCGAGGACATGC	This work
pVpsL	Promoter	TACGATCGCTTGAGCAAGCTCTTGTGGCGCACTTTCAATC GCGGCTTGTAACACATCGGCACGTTGTTCTTGAACGATA CTGCGCATGCGTCAACGGCGAGTTGTACTCGTTGGTCAT CCACAACATCACTAAAAAGCACGAGATAAATTTCTCGTG TtagTGACGGATTTAGGTTGCTCGCAAGGGCTGCGATTT GCACGGCATTCTCAGGACATGTCACCATGAGTGATGCCA CCGTTGTCAGTAGATCGGTAGTTTGATTTAAAGCGCTGTT CAACTGACTCTCTTGTGTACATAGAGCAGGATCAAATGA AGCGGTAGTGTTTATTTTCAAGATTCTGCGAATGCATTCATG CTAGGAAAAAATGCAATCAATAGCACTAGTGGAATGGT ACGGGTTTCATATTGTTCTGTTTTTCCTTTTCGATAAACGA TACATATTATTTCTTTATGGTATTTTAAATCACGGCTGGT CTATGTGGCTTGTTAAATAATTTGTTTTTACTTTTCTCAA AAATAATTCAATGTAAATCCAAAATGTAATACAAATCTT ATTTTTATTAAATTGGTCTTTTCGTTTTTATTATCTGTT	Shikuma & Yildiz 2009

		TTGATTAACCTATTAACCATCATAAAAGTAACTAAAGT TTATTTTCATGGTGGTTAATAAGTGAGTCTCAAGGTTTTA TTTGATTAAGTGTGTTTATATTTATTTTAAATTTAA TGCCAACACTAGATTATATGAACTTGGTTGTTTCAATT GGTTTCATCATGTTTTTATTTTATTTCAGTCTTAGAATTG ATGCAGATATTTCTATTGATAAAGATGTAGTCTTAAAA AAGAATTTAATACATTCCAATTATCTTCGGGAAATCTTA CATCGGTTAGGAGCGTCTA	
VC0916	Promoter	CTGCAACTTTAAGTCTTTTGGGCATTTAACGAAGATGTCA ACCTTCATTATTAGTAAATATGCCCTGTCGTATTATTGA TTCAAATCGATGGCTTTTAATACTAATACAATAAATAAA ACTACTTTTCATTTTACTGCTTTAGTTCTAATTAATATCCA GCTCAATTATGAGACAGACATTATTGGTAAGCTTGGGAT ACTCTGATAATGAGTAGATTGCGTATTACTTTAACAAT	This work
VC0917	Promoter	CTTAGTCGCTTTTGGGTGTGTCTAAAAGATAAGTTGGAAT GAAAAATAACTAAAGTTTATATCGCGCTTAACTATATG TTTATTTTCTGTTCCCTTAAGAAGGCCATAAATTTCAA GTAAATAAGATTGTTTATAACGACGAATTTAATAATAA GCTGACATACATGGGTAATCTCCCTCGTTGAGATTTAATA CAACAACACGAAATAATTTCAGTGTTTCGCTTATGTATATG TTCCTCATTTAAGGAGCAGTGGGACTTTATTGCTTGAATT TTGTGAAATT	This work
VC0928	Promoter	CCTTTGGGTGAAAAATAAACTTTGGTTTATTTTGGCTTT CTGGTTTTTATTTCGTTGAATCAAGTTTTTTAATATTATTA ATGCTTAGCCAATGCAATTGTCTTAGATTTTGATAAAGGT AGATCCTTTAATAAAAAAGCGGATGTATTTCTATAGTTAT GAATTAGCCT	This work
VC0934	Promoter	TTTGTTTTTACTTTTCTCAAAAAATAATTCAATGTAAATC CAAAATGTAATACAAATCTTATTTTATTTAAATTGGTTC TTTCGTTTTTATTTATCTGTTTGGATTAACTATTAACCAT CATAAAAGTAACTAAAGTTTATTTTCATGGTGGTTAAT AAGTGAGTCTCAAGTTTTTATTGATTAAGTGTTGTTTT ATATTTATTTTTTAATTTAATGCCAACACTAGATTATAT GAACTTGGTTGTTTTCAATTGGTTTCATCATGTTTTTAT TTTTATTTCAGTCTTAGAATTGATGCAGATATTTCTATTGA TAAAGATGTAGTCTTTAAAAAAGAATTTAATACATTCCA ATTATTCTTCGGGAA	This work
VC1585	Promoter	GAGGTTGAAAAATTCATTCACGCTTTTATATCGTATTTGG ACATGAGTTATAACCAAGTTTAAAAAACAGCCGGCATGCT TAAAAAACGCCGAGAATAGAGAGCTAATTTAACCATCT CTAATAAATAATTCTGTGAACCAATATCAC	This work

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Fig 1 The QS network in *P. aeruginosa*

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Fig 2 Classes of CFTR mutations according to their effect on function

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