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An investigation of the molecular interactions between statins and bacterial pathogens, and their combined impact on the human immune system

A Thesis presented to the National University of Ireland

for the Degree of Doctor of Philosophy by

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School of Microbiology

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Cork

Supervisor: Professor Fergal O'Gara

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January 2014

Dedicated to my Parents

"I was taught that the way of progress was neither swift nor easy."

Marie Curie

"*Not all those who wander are lost.*" J. R. R. Tolkien

Declaration

I, the undersigned Emma Hennessy, hereby declare that this work has not been submitted for another degree, either in University College Cork or elsewhere and that the results presented in this thesis are derived from experiments carried out by myself in University College Cork, with the exception of the following:

Chapter 3 Figure 5B: Miller assy was undertaken by Dr F. Jerry Reen;

Chapter 3 Figure 6: Microarray hybridisation was carried out in DNAVision, Belgium;

Chapter 3 Table 4: 2-D gel electrophoresis and nano-LC/MS were carried out in the laboratory of Dr Olivier Lesouhaitier in University of Rouen, France.

_____

Emma Hennessy

January 2014

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Abbreviations

2-DGE: 2-dimensional gel electrophoresis

$3OC_{12}HSL$: N-3-oxo-dodecanoyl-L-homoserinelactone

AHL: Acylated homoserine lactone

AMV: Avian Myeloblastosis Virus

ASAH1: Acid ceramidase

ASM: Acid sphingomyelinase/Artificial sputum media

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

BALF: Bronchoalveolar lavage fluid

Bcc: *Burkholderia cepacia* complex

BCCM: Belgian Co-ordinated Collections of Micro-organisms

BHI: Brain heart infusion

BLASTP: Basic Local Alignment Search Tool (Protein)

C_4HSL: N-butyryl-homoserine lactone

cAMP: Cyclic adenosine monophosphate

CCL20: Chemokine (C-C motif) ligand 20

c-di-GMP: Cyclic diguanylic acid

cDNA: Complementary DNA

CF: Cystic fibrosis

CFTR: Cystic fibrosis transmembrane conductance regulator

CO_2: Carbon dioxide

DMAPP: Dimethylallyl pyrophosphate

DMEM: Dubecco's modified Eagle's medium

DMSO: Dimethylsulphoxide

DNA: Deoxyribonucleic acid

eDNA: Extracellular DNA

eNOS: Endothelial nitric oxide synthase

FBS: Foetal bovine serum

FPP: Farnesyl-pyrophosphate

GGPP: Geranylgeranyl-pyrophosphate

GTPase: Guanosine triphosphate hydrolase

H_2O: Water

HHQ: 4-hydroxy-2-heptylquinoline

HMGR: 3-hydroxy-3-methylglutaryl-CoenzymeA reductase

HPRT: Hypoxanthine phosphoribosyltransferase

HSL: Homoserine lactone

IL-8: Interleukin-8

IMG: Integrated Microbial Genomes Database

iNOS: Inducible nitric oxide synthase

IPP: Isopentenyl-pyrophosphate

KEGG: Kyoto Encyclopedia of Genes and Genomics

KLF: Kruppel-like factor

LB: Luria Bertani

LOV: Lovastatin

LPS: Lipopolysaccharide

MEGA: Molecular Evolutionary Genetics Analysis

MEM: Minimal Eagle's medium

MEP/DOXP: 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate

MEV: Mevastatin

MIC: Minimum inhibitory concentration

MOI: Multiplicity of infection

MSM: Mineral salt medium

NFκB: Nuclear factor kappa B

NO: Nitric oxide

O_2: Oxygen

NTA: Nitrilotriacetic acid

OD: Optical density

PAMP: Pathogen-associated molecular pattern

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PI3-K: Phosphatidylinositol 3-kinase

PQS: *Pseudomonas* Quinolone Signal; 2-heptyl-3,4-dihydroxyquinolone

PPARγ: Peroxisome proliferator-activated receptor γ

ProOpDB: Prokaryotic Operon Database

QC: Query coverage

QS: Quorum sensing

qRT-PCR: Quantitative real-time polymerase chain reaction

rDNA: Ribosomal DNA

RMA: Robust Multiarray Average

RNA: Ribonucleic acid

rRNA: Ribosomal RNA

RTI: Respiratory tract infection

SIM: Simvastatin

si-luc: pSUPER-Retro-luc

si-wtKLF6: pSUPER-Retro-wtKLF6

STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

SV: Splice variant

TNFα: Tumour necrosis factor alpha

TLR: Toll-like receptor

TTSS: Type 3 secretion system

WebACT: Web Artemis Comparison Tool

wtKLF6: Wild type KLF6

Units:

aa: Amino acids

bp: Base pairs

CFU: Colony forming units

cm: centimetre

g/L: Grams per litre

hpi: Hours post inoculation/infection

mA: Milli-amps

mg/ml: Milligrams per millilitre

ml: Millilitre

mM: Millimolar

ng: Nanogram

nm: Nanometres

nM: Nanomolar

ºC: Degrees Celsius

rpm: Revolutions per minute

µl: Microlitres

µM: Micromolar

µg/ml: Micrograms per millilitre

V: Volts

v/v: Volume per volume

w/v: Weight per volume

x g: Relative centrifugal force

Abstract

Statins are a class of drug that inhibits cholesterol biosynthesis, and are used to treat patients with high serum cholesterol levels. They exert this function by competitively binding to the enzyme 3-hydroxy-3-methylglutaryl-CoenzymeA reductase (HMGR), which catalyses the formation of mevalonate, a rate-limiting step in cholesterol biosynthesis. In addition, statins have what are called "pleiotropic effects", which include the reduction of inflammation, immunomodulation, and anti-microbial effects. Statins can also improve survival of patients with sepsis and pneumonia. Cystic fibrosis (CF) is the most common recessive inherited disease in the Caucasian population, which is characterised by factors including, but not limited to, excessive lung inflammation and increased susceptibility to infection. Therefore, the overall objective of this study was to examine the effects of statins on CF-associated bacterial pathogens and the host response.

In this work, the prevalence of HMGR was examined in respiratory pathogens, and several CF-associated pathogens were found to possess homologues of this enzyme. HMGR homology was analysed in *Staphylococcus aureus*, *Burkholderia cenocepacia* and *Streptococcus pneumoniae*, and the HMGR of *B. cenocepacia* was found to have significant conservation to that of *Pseudomonas mevalonii*, which is the most widely-characterised bacterial HMGR. However, *in silico* analysis revealed that, unlike *S. aureus* and *S. pneumoniae*, *B. cenocepacia* did not possess homologues of other mevalonate pathway proteins, and that the HMGR of *B. cenocepacia* appeared to be involved in an alternative metabolic pathway. The effect of simvastatin was subsequently tested on the growth and virulence of *S. aureus*, *B. cenocepacia* and *S. pneumoniae.* Simvastatin inhibited the growth of all 3 species in

a dose-dependent manner. In addition, statin treatment also attenuated biofilm formation of all 3 species, and reduced *in vitro* motility of *S. aureus*. Interestingly, simvastatin also increased the potency of the aminoglycoside antibiotic gentamicin against *B. cenocepacia*.

The impact of statins was subsequently tested on the predominant CF-associated pathogen *Pseudomonas aeruginosa*, which does not possess a HMGR homologue. Mevastatin, lovastatin and simvastatin did not influence the growth of this species. However, sub-inhibitory statin concentrations reduced the swarming motility and biofilm formation of *P. aeruginosa*. The influence of statins was also examined on Type 3 toxin secretion, quorum sensing and chemotaxis, and no statin effect was observed on any of these phenotypes. Statins did not appear to have a characteristic effect on the *P. aeruginosa* transcriptome. However, a mutant library screen revealed that the effect of statins on *P. aeruginosa* biofilm was mediated through the PvrR regulator and the Cup fimbrial biosynthesis genes. Furthermore, proteomic analysis demonstrated that 6 proteins were reproducibly induced by simvastatin in the *P. aeruginosa* swarming cells.

The effect of statins on the regulation of the host-*P. aeruginosa* immune response was also investigated. Statin treatment increased expression of the pro-inflammatory cytokines IL-8 and CCL20 in lung epithelial cells, but did not attenuate *P. aeruginosa*-mediated inflammatory gene induction. In fact, simvastatin and *P. aeruginosa* caused a synergistic effect on CCL20 expression. The expression of the transcriptional regulators KLF2 and KLF6 was also increased by statins and *P. aeruginosa*, with the induction of KLF6 by simvastatin proving to be a novel effect. Interestingly, both statins and *P. aeruginosa* were capable of inducing alternative splicing of KLF6. *P. aeruginosa* was found to induce KLF6 alternative splicing by

way of the type 3 secreted toxin ExoS. In addition, a mechanistic role was elucidated for KLF6 in the lung, as it was determined that statin-mediated induction of this protein was responsible for the induction of the host response genes CCL20 and iNOS. Moreover, statin treatment caused a slight increase in infection-related cytotoxicity, and increased bacterial adhesion to cells.

Taken together, these data demonstrate that statins can reduce the virulence of CF-associated bacterial pathogens and alter host response effectors. Furthermore, novel statin effectors were identified in both bacterial and host cells.

Chapter 1

Introduction

1.1. Statins

One of the major undisputed clinical breakthroughs of the 20th century is the discovery of the statin family of drugs. These compounds are renowned for their ability to lower cholesterol levels, and are used to treat approximately 40 million individuals with high cholesterol worldwide. Since the discovery of mevastatin as a metabolic product of *Penicillium citrinum* in 1976 (1, 2) a total of 9 statins have been characterised (Figure 1), 7 of which are approved by the FDA to treat patients with high cholesterol. Most of these are synthetic, although lovastatin occurs naturally as a metabolic product of *Aspergillus terreus* (3). Structurally, statins are characterised by the presence of a conserved lactone ring (4). This structure is present in a hydrolysed (active) form in all statins except for mevastatin, lovastatin and simvastatin, where the lactone ring is hydrolysed in the liver (5).

Statins can be divided into 2 broad classes (Figure 1). Type 1 statins are lipophilic, and possess a butaryl side chain – they are said to structurally resemble mevastatin (4). Lovastatin, pravastatin and simvastatin are type 1 statins. Type 2 statins are classically lipophobic, and are distinguished from type 1 by the replacement of the butaryl side chain with a fluorophenol group and typically larger side chains than type 1 statins (4). Atorvastatin, cerivastatin, fluvastatin, pitavastatin and rosuvastatin are type 2 statins.

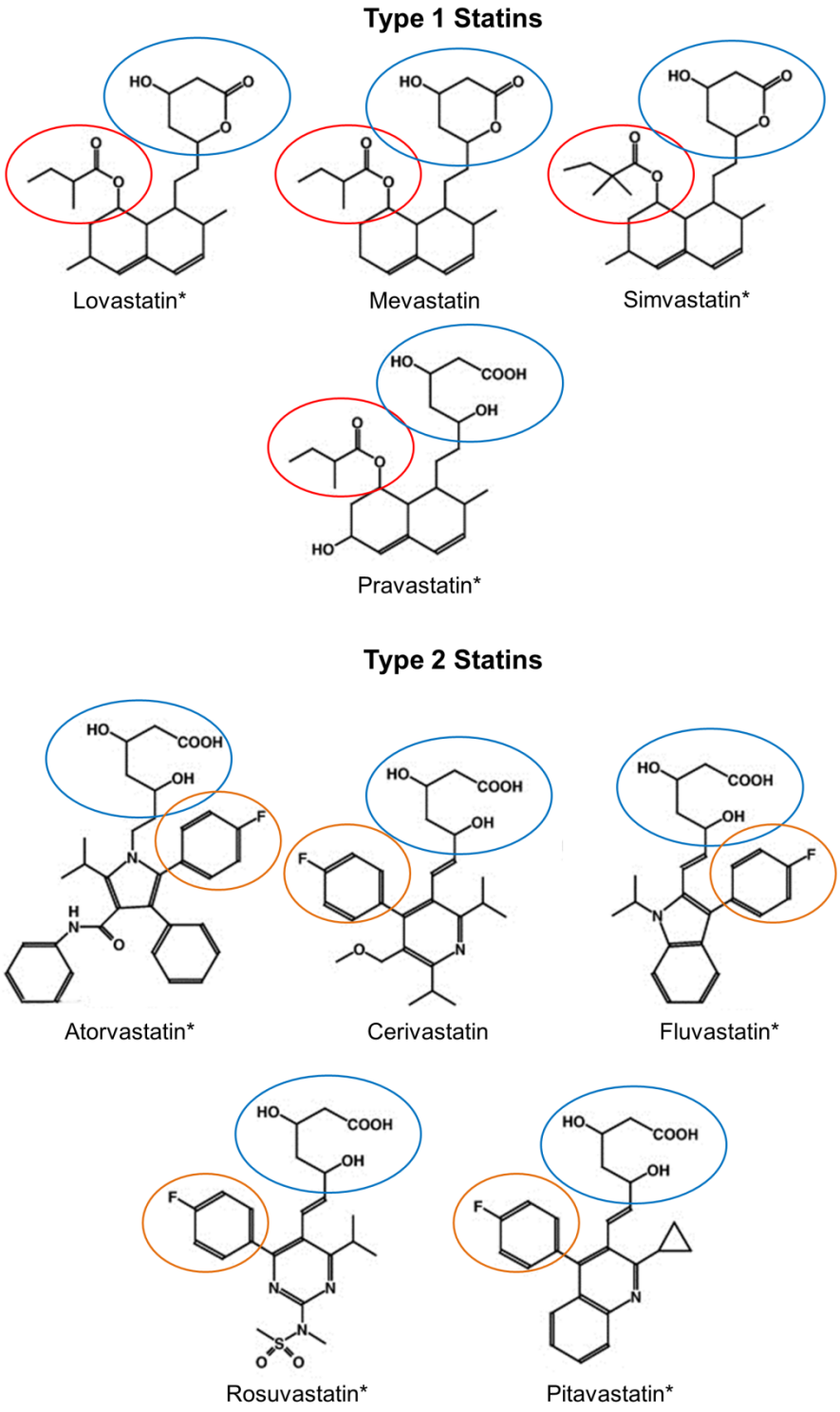


Figure 1: Chemical structures of the statins. Type 1 statins are characterised by a conserved lactone ring (highlighted in blue), a decalin structure (not highlighted) and a butaryl side chain (highlighted in red), which is different in each statin. The type 2 statins differ from type 1 statins due to the replacement of the butaryl side chain with a flurophenyl group (highlighted in orange), and although the lactone ring structure is conserved in all

statins, the decalin group of Type 1 statins is replaced by a longer distinct side chain. Statins marked with an asterisk (*) are licenced to treat high cholesterol. Adapted from Rozman & Monostory, 2010 (6).

1.2. Isoprenoid metabolism

1.2.1. Statins and the mevalonate pathway

Statins exert their cholesterol inhibitory effect by binding to the active site of 3-hydroxy-3-methylglutaryl-CoenzymeA reductase (HMGR), which is a rate-limiting enzyme in cholesterol biosynthesis (4). The reaction that statins inhibit is as follows:

HMG-CoA + 2 NADP(H) + 2 H+ = Mevalonate + CoASH + 2 NAD(P)+

HMGR is an integral part of the mevalonate pathway of isoprenoid biosynthesis. Isoprenoids are lipid compounds, which are essential for cell signalling and structure. Mevalonate dependent-isoprenoid biosynthesis is detailed in Figure 2A. In humans, 3-hydroxy-3-methylglutaryl-CoenzymeA (HMG-CoA) is synthesised from acetoacetate and acetyl-coA by the enzyme HMG-CoA synthase. HMG-CoA is then converted to mevalonate by HMGR, and it is this event that is inhibited by statins (Figure 2B). Mevalonate undergoes 2 phosphorylation events – firstly by mevalonate kinase, producing mevalonate-5-phosphate, and secondly by mevalonate phosphokinase, resulting in the production of mevalonate-pyrophosphate. Mevalonate-pyrophosphate is then converted to the isoprenoid intermediate compound isopentenyl-pyrophosphate (IPP) by mevalonate-pyrophosphate decarboxylase, thus commencing isoprenoid metabolism. IPP is converted to dimethylallyl pyrophosphate (DMAPP) by IPP-isomerase. Farnesyl-pyrophosphate (FPP)-synthase converts DMAPP to FPP, which is subsequently used by geranylgeranyl-pyrophosphate (GGPP)-synthase to produce GGPP.

FPP and GGPP are key intermediate compounds for 2 reasons. Firstly, they are used by the enzyme squalene synthase to produce the sterol squalene, which is one of the

compounds used to synthesize cholesterol. Secondly, FPP and GGPP are also responsible for the prenylation and activation of the Rho GTPase family of proteins (7) and when mevalonate synthesis is inhibited, the corresponding decrease in FPP and GGPP leads to a decrease in Rho GTPase activity. Rho GTPases control a number of cellular functions, including maintenance of the actin cytoskeleton, apoptosis and gene expression. They do this by acting as a "molecular switch", as they cycle between GDP-bound (inactive) and GTP-bound (active) states (8). Furthermore, as well as inhibiting cholesterol biosynthesis by reducing mevalonate production, statin treatment also increases levels of the low density lipoprotein receptor (LDLR), leading to increased cholesterol clearance (7).

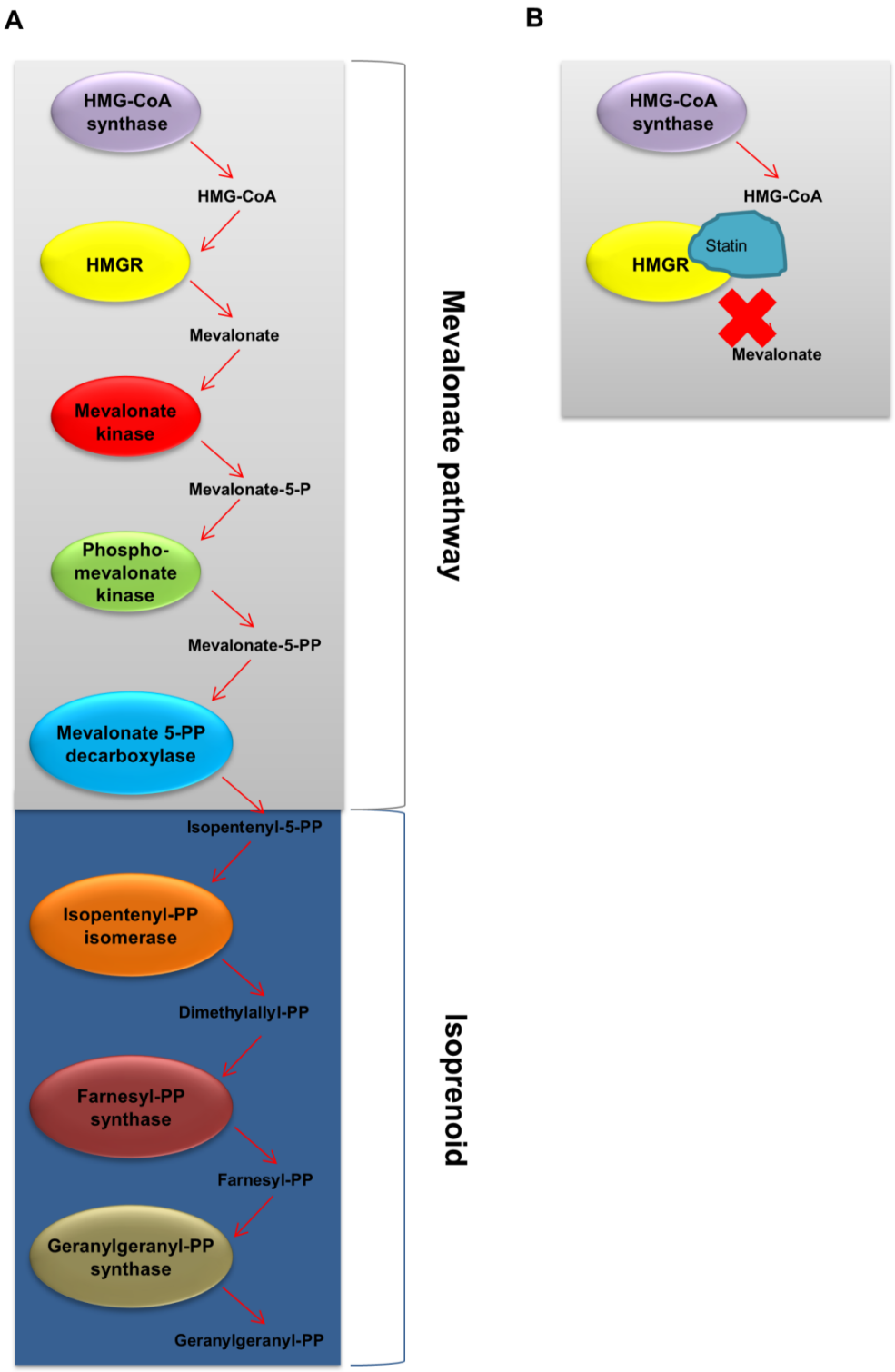


Figure 2: Statins and the mevalonate pathway. (A) In hepatocytes, HMGR binds its substrate HMG-CoA and converts it to mevalonate. This leads to the production of cholesterol via several intermediate compounds, including FPP and GGPP, which are involved in the prenylation and activation of Rho GTPases. **(B)** Where statins are present,

they competitively bind the active site of HMGR, thus inhibiting the production of mevalonate and downstream intermediate compounds.

1.2.2. Isoprenoid production in bacteria – the mevalonate-independent pathway

Several bacterial species undergo HMGR-dependent isoprenoid metabolism. The first bacterial HMGR to be characterised was that of the industrial strain *Pseudomonas mevalonii* in 1989 (9). The HMGR of *P. mevalonii* only possesses 2 regions of significant similarity to mammalian HMGR (9), but it has since been found to have 3 domains that are essential for its catalytic capacity (10). It has since been discovered that statins and statin analogues can bind to and inhibit the function of *P. mevalonii* HMGR (11), thus making it the archetypal form of this enzyme for studies carried out in other bacterial species.

Several other bacterial species are now known to possess functional HMGR analogues, including staphylococci, streptococci, enterococci and *Listeria monocytogenes* (12–15). However, many bacteria do not possess a mevalonate pathway. Instead, these organisms undergo isoprenoid biosynthesis by the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) or mevalonate-independent pathway (16) (outlined in Figure 3). This pathway shares common isoprenoid end products – DMAPP and IPP – with the mevalonate pathway, but different metabolites are used in the course of their synthesis. 1-Deoxy-D-xylulose 5-phosphate synthase (DOXP synthase) produces DOXP from pyruvate and glyceraldehyde-3-phosphate. DOXP reductase then converts DOXP to 2-C-methylerythritol 4-phosphate (MEP), which is then converted to 4-diphosphocytidyl-2-C-methylerythritol (CDP-ME) by CDP-ME synthase. CDP-ME is subsequently phosphorylated by CDP-ME kinase to form methylerythritol cyclodiphosphate (MEcPP), which is subsequently deoxygenated by (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate synthase (HMB-PP synthase). The resultant compound, HMB-

PP, compound is reduced to the isoprenoid intermediate compounds DMAPP and IPP by HMB-PP reductase.

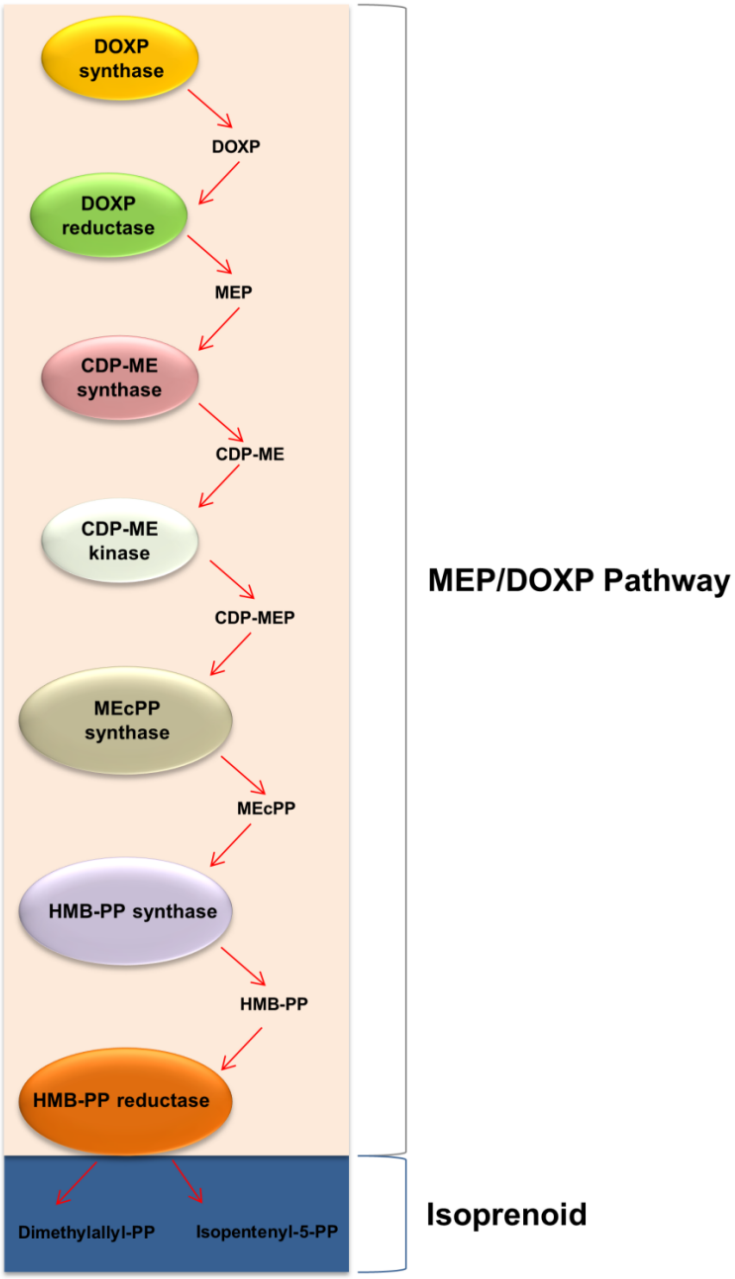


Figure 3: The MEP/DOXP pathway of isoprenoid biosynthesis. DOXP is reduced to MEP. CDP-MEP is synthesised from MEP, and 2 phosphorylation events lead to the formation of MEcPP and HMB-PP. This compound is then reduced to the isoprenoid intermediate compounds DMAPP and IPP.

1.3. Pleiotropic effects of statins

As well as the inhibition of cholesterol, statins have also been found to have a number of cholesterol-independent, so-called "pleiotropic" effects. These include anti-inflammatory effects and immunomodulatory effects on host cell gene expression, but a growing number of studies are demonstrating that statins can also influence growth and behaviour of bacterial pathogens, independent of the host response.

1.3.1. Anti-microbial effects of statins

Statins have been found to directly inhibit the growth of both Gram positive and Gram negative bacterial species which cause a wide range of infections. The growth of the Gram positive nosocomial pathogens *Staphylococcus aureus* and *Streptococcus pneumoniae* has been inhibited by atorvastatin, rosuvastatin and simvastatin (17–20), with 1 study observing a bactericidal effect of simvastatin on *S. pneumoniae* (18). The growth of *S. aureus* is also inhibited by fluvastatin (17). Both type 1 (simvastatin) and type 2 (atorvastatin, fluvastatin and rosuvastatin) statins have also demonstrated a bacteriostatic effect against other Gram positive cocci, notably *Streptococcus pyogenes, Staphylococcus epidermidis* and enterococci (17, 19, 20).

Both type 1 and type 2 statins have also been found to reduce the growth of several clinically important species Gram negative species. The growth of the nosocomial respiratory pathogens *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and *Klebsiella pneumoniae* is reduced by atorvastatin, rosuvastatin and simvastatin (19,

20), while simvastatin is bacteriocidal against *Moraxella catarrhalis,* another causative agent of pulmonary infections (18). It was recently reported that atorvastatin, rosuvastatin and simvastatin have bacteriostatic effects against organisms that cause a range of infections, including *Citrobacter freundii*, *Escherichia coli*, *Enterobacter aerogenes*, *Haemophilus influenzae* and *Proteus mirabilis* (19), although another study found similar simvastatin concentrations to be ineffective against *H. influenzae* (18), suggesting that strain-specificity may be an issue. Furthermore, simvastatin and lovastatin are bactericidal against the spirochete *Borrelia burgdorferi* (the causative agent of Lyme disease) (21).

The minimum inhibitory concentration (MIC) required to inhibit bacterial growth varies based on the type of statin used. Taken together, current data suggests that simvastatin and atorvastatin generally appear to be more effective against *S. aureus, S. pneumoniae* and enterococci than other statins (17–20). The particular bacterial strain employed also seems to be significant. For instance, 3 distinct simvastatin MICs were reported against *S. aureus* clinical isolates from the U. K. and Jordan as well as typed reference strains (17, 19). One common factor is that all efficacious statin concentrations are much higher than the typical levels observed in plasma – for instance, the maximum plasma concentrations for simvastatin (10-20 μg/L) and lovastatin (20-34 μg/L) (22) are much lower than the *in vitro* MIC values observed to date.

Promisingly, statins can inhibit the growth of antibiotic-resistant species such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (17, 19, 20). Given this information, it has been hypothesised that non- or sub-inhibitory doses of statins could be used in combination with antibiotics to increase efficacy of treatment. Current information in this area is conflicting. A synergistic

effect has been reported between penicillin and simvastatin against pneumococcal growth (18) and statins have also been found to increase the potency of anti-malarial agents (23). However, a recent study, which examined the effects of several combination of 6 antibiotics and 5 statins on 4 clinically important Gram negative strains – *P. aeruginosa, A. baumanii*, *E. coli* and *K. pneumoniae* – found that statins neither increase nor decrease the susceptibilty of any of these bacteria to any of the antibiotics tested (24).

1.3.2. Effect of statins on *in vitro* and *in vivo* infection models

Several studies have reported the attenuation of both *in vitro* and *in vivo* bacterial virulence by statin treatment. An in-depth *in vitro* study carried out using *S. pneumoniae* demonstrated that sub-inhibitory concentrations of simvastatin and lovastatin do not influence secretion of the pneumolysin toxin (25). However, the same study analysed the effect of simvastatin on gene expression of *S. pneumoniae*, and found that 24 genes were significantly up-regulated, while 6 genes were significantly decreased. Altered genes mostly fell into the ABC transporter category and did not have clear roles in infection and host interaction.

Statins can reduce the growth and essential internalisation of obligate intercellular bacterial pathogens. Simvastatin and cerivastatin decrease the levels of the intracellular respiratory bacterium *Chlamydiae pneumoniae* in mouse lung cells and macrophages respectively (26, 27). Furthermore, lovastatin reduces both the intracellular growth of the respiratory pathogen *Coxiella burnetti* (which causes Q fever) (28), and plaque formation by the causative agent of Rocky mountain spotted fever, *Rickettsia conorii* (29). The influence of statins on intracellular growth and

internalisation is not limited to obligate intracellular organisms. A recent study involving macrophages demonstrated that simvastatin treatment reduces the ability of the foodborne pathogen *Listeria monocytogenes* to grow inside mouse and primary macrophages, without influencing phagocytosis (30). The intracellular growth of another foodborne bacteria, *Salmonella* Typhimurium, is reduced by lovastatin treatment of murine macrophages, leading to improved infection outcome (31). Furthermore, mevastatin completely inhibits the internalisation of Group B streptococcus into HeLa cells (32), and attenuates the invasion of *E. coli* into bladder epithelial cells (33). Finally, simvastatin can reduce invasion of *S. aureus* into vascular epithelial cells (34) and also reduces the translocation of *P. aeruginosa* across the kidney apical epithelial barrier, although it does not influence its invasive potential (35).

One key mechanism behind the attenuation of bacterial internalisation appears to be the statin-mediated inhibition of lipid raft formation. Lipid rafts are glycoprotein domains present in the cell membrane, which are formed as a result of cholesterol spontaneously interacting with sphingoglycolipids. Bacteria can manipulate lipid rafts in order to invade cells and induce apoptosis (36). However, statins are known to inhibit the formation of lipid rafts due to inhibition of cholesterol biosynthesis (37). It appears that that the effects of statins on *L. monocytogenes* and *R. conorii* may be resultant of the inhibition of lipid raft formation by statins (29, 30).

Statin treatment also appears to increase bacterial killing in host cells. Simvastatin has been found to significantly increase killing of *S. aureus* in a mouse model of pneumonia (38), and reduce *S. aureus* recovery from mouse peritoneal and lung cell (39). Mevastatin significantly reduces the amount of *S. aureus* recovered from intracellular infection of human neutrophils and mouse macrophages (39), and also

reduces the CFU counts of *S. pneumoniae* and *S.* Typhimurium recovered from mouse macrophages (31, 40).

As well as inhibiting bacteria, statins also have protective effects on cells during infection. Firstly, statins can reduce the impact of bacterial toxins on host cells. In a study which utilised *S. aureus* α-toxin, leukocyte recruitment in mice was attenuated by simvastatin treatment (41). Lovastatin can improve the survival of mice which were exposed to another *S. aureus* toxin, enterotoxin B (42). Simvastatin attenuates the cytotoxicity exerted by *S. pneumoniae* pneumolysin in both human endothelial cells and in mouse lungs, leading to improved survival (25). Furthermore, the cytotoxicity of *Bacillus anthracis* lethal toxin against macrophages is reduced by fluvastatin, mevastatin, and simvastatin (43). The regulation of the inflammatory response by statins can also have protective effects. Statin treatment reduces the expression of inflammatory mediators in macrophages infected with *C. pneumoniae*, leading to decreased infective potential (44). The inflammatory response in LPS-treated mice is also reduced by cerivastatin treatment, leading to improved survival (45). Furthermore, simvastatin treatment can reduce both lung injury and the production of pro-inflammatory chemokines in a mouse sepsis model (46).

1.3.3. Effects of statins on the inflammatory and innate immune responses

When a healthy human host encounters infection by a microbial pathogen, the inflammatory and innate immune responses are activated in order to eradicate the threat. Host cells can perceive the presence of bacterial and viral pathogens by the Toll-like receptor (TLR) family of sensor proteins. Each member of this family recognises a specific ligand termed a "pathogen-associated molecular pattern"

(PAMP) such as lipopolysaccharide (LPS) (TLR4) (47) and flagellin (TLR5) (48). The binding of a particular PAMP to its respective TLR causes the activation of the MyD88 signalling cascade, resulting in the activation of the master regulator of inflammation (and indeed numerous other processes), NFκB (49). Activated NFκB is composed of 5 protein subunits – p50, p52, p65 (relA), relB, and relC – and this complex translocates to the nucleus, where it activates the transcription of pro-inflammatory cytokines and chemokines (49, 50). This mechanism is described in detail in Figure 4.

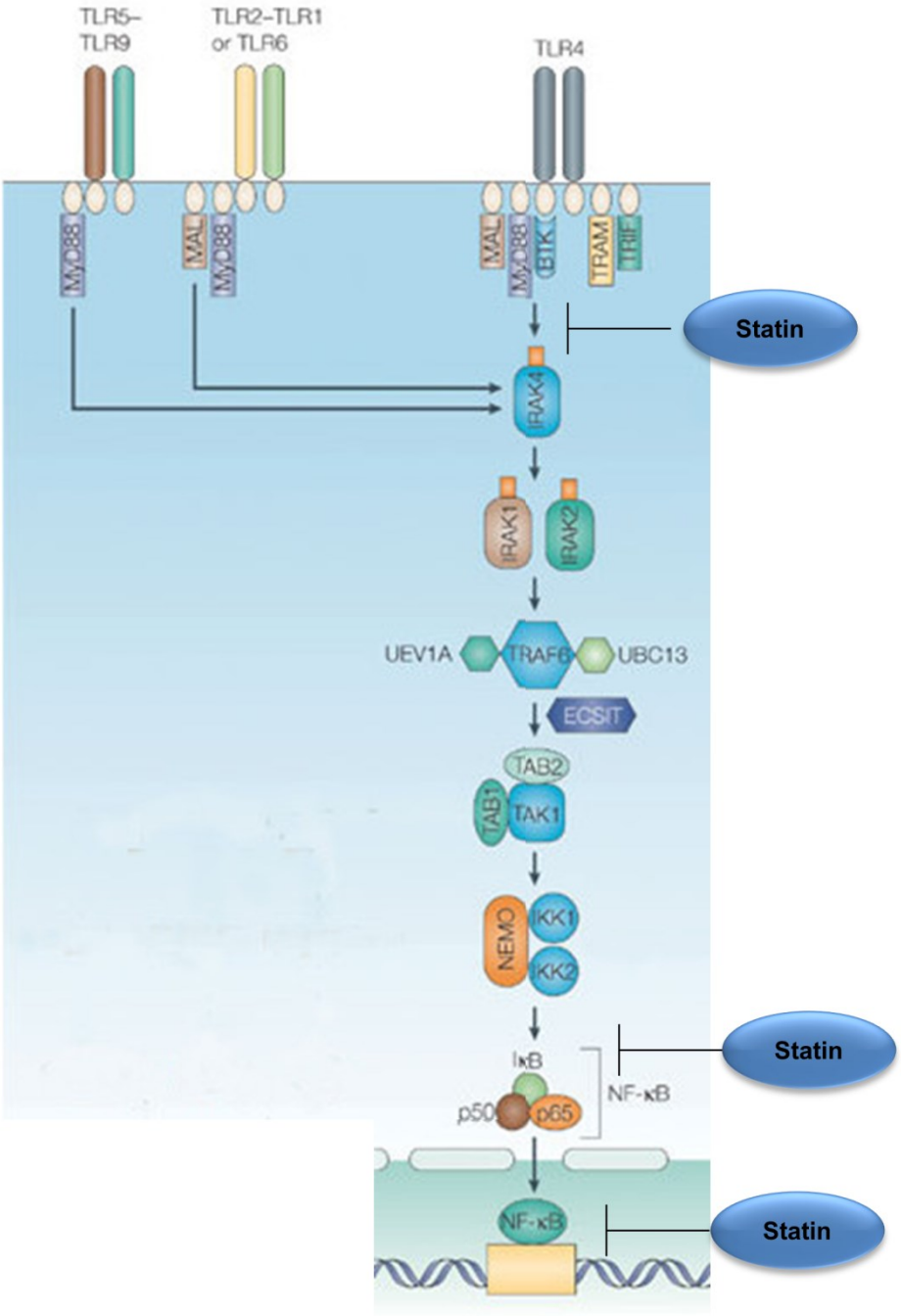


Figure 4: TLR-mediated pro-inflammatory signalling in response to bacterial infections. TLR4 and TLR5 recognise and bind to LPS and flagellin, respectively. MyD88 interacts with the intercellular component of TLRs and recruits the IL-1 receptor (IL-1R)-associated kinase family members IRAK1/2 and IRAK4. IRAK1/2 phosphorylates TNF receptor associated factor 6 (TRAF6), which ubiquinates TAB2/3, allowing transforming growth factor-activated protein kinase 1 (TAK1) to ubiquinate NFκB essential modifier

(NEMO). This activates IKKα and IKKβ, which phosphorylate IκK, causing it to be ubiquinated and degraded. This results in the formation of NFκB from its 5 subunits. NFκB translocates to the nucleus where it activates the expression of pro-inflammatory modulators. Statins have been found to inhibit MyD88-dependent signalling, NFκB complex assembly and NFκB-mediated expression of pro-inflammatory genes. Adapted from Liew *et al*., 2005 (50).

Statins can attenuate the activation of the innate immune and inflammatory responses in a number of tissue types. They exert these properties by inducing anti-inflammatory regulators and effectors, and by repressing pro-inflammatory signalling and the production of cytokines. Statins can induce expression of the anti-inflammatory enzymes endothelial nitric oxide synthase (eNOS) (51) and thrombomodulin (52), leading to a reduction in vascular inflammation and coagulation-mediated inflammation. Additionally, statins can activate expression of anti-inflammatory transcriptional regulators such as Kruppel-like Factor 2 (KLF2) (53–55), conferring a general anti-inflammatory state on the cell. The effects of KLF2 are discussed in more detail in 4.2.1. Another anti-inflammatory transcription factor, peroxisome proliferator-activated receptor γ (PPARγ) was also found to be activated by statin treatment of monocytes (56).

Statins may also act as inhibitors of pro-inflammatory mediators, and have several mechanisms by which they reduce these targets. Firstly, statins act by inhibiting the expression of the pro-inflammatory transcriptional regulator NFκB (57–59). They do this by inhibiting mevalonate-dependent protein prenylation, which in turn reduces activation of NFκB by the pro-inflammatory cytokine tumour necrosis factor-α (TNFα) and the hormone angiotensin II in lymphocytes and vascular and mesangial cells (58–60). Statins also impact NFκB by inhibiting the translocation of its p65 subunit from the cytoplasm to the nucleus (61). In addition, the induction of the anti-inflammatory regulator PPARγ by statins reduces TNFα production, which in turn attenuates NFκB activity (56).

Statins inhibit the production of the pro-inflammatory cytokine Interleukin-8 (IL-8) by stimulated *ex vivo* blood cells (62), endothelial cells (63), granulocytes (64) and rat and rabbit airway cells (65, 66). The reduction of IL-8 by statins attenuates

neutrophil infiltration into stimulated lung cells (66) and chemotactic migration by granulocytes (64). The statin-mediated reduction of IL-8 production by primary bronchial epithelial cells is channelled through inhibition of IL-17, another pro-inflammatory cytokine (67). Statins can also regulate the activation of macrophages. The differentiation of LPS-stimulated monocytes into macrophages is inhibited by mevastatin (68), whereas lovastatin reduces migration and phagocytic activity of differentiated rabbit alveolar macrophages (65). These effects can be associated with the impact of statins on pro-inflammatory cytokines. For instance, statins inhibit the production of Chemokine (C-C motif) ligand 20 (CCL20) by keratinocytes (69) and endothelial cells (70), and also reduce the migration of T cells towards CCL20 (69). Like IL-8, the effect of statins on CCL20 is mediated through the inhibition of IL-17 (70), and inhibition of TNFα by statins also causes a reduction in CCL20 production by fibroblasts (71). As well as inhibiting pro-inflammatory cytokine production, statins can also inhibit both the chemotactic migration of leukocytes towards IL-8 (72) and the release of free radicals (the so-called "respiratory burst") from leukocytes (64, 73). Furthermore, statins also inhibit neutrophil recruitment and migration in the lung (46).

1.3.4. Improvement of patient morbidity and mortality by statins

Increasing interest has been shown in using the pleiotropic effects of statins in order to combat multi-system microbial infections, such as sepsis and pneumonia. Sepsis is an infection-induced whole body inflammatory state, and as statins act in an anti-inflammatory manner, it has been proposed that statins could be used to both prevent and treat sepsis (74). An early study in this field noted that individuals who had

undergone statin therapy prior to the onset of bacterial infection have a reduced risk of developing severe sepsis (75). Furthermore, a clinical review found evidence to suggest that not only do statins reduce morbidity and mortality associated with sepsis, but they also have the potential to be used as therapeutic agents for the management of sepsis (76). In accordance with the effects seen on sepsis, patients with bacteriaemia who have undergone prior statin treatment have a decreased risk of mortality (77). Another study of bacteriaemic patients also observed a decreased mortality rate in patients who had prior statin exposure, and also observed an inverse correlation between the length of statin treatment and risk of mortality (78).

The use of statins has also been associated with a reduced risk of morbidity and mortality resulting from pneumonia. A retrospective study of patients in the U. K. found that prior statin treatment reduces pneumonia-associated mortality, and the risk of developing pneumonia (79). Prior statin treatment also reduced mortality rates in patients in the U. S. A. with community-acquired pneumonia (80). Furthermore, data from the Justification for the Use of Statin in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) trial, which was initially undertaken to determine whether rosuvastatin could reduce the risk of cardiac disease in people without hyperlidemia (81) were retrospectively analysed in 2012. This analysis suggested that rosuvastatin treatment may decrease the occurrence of pneumonia (82).

Some conflicting evidence has also been reported regarding statins and infections. A prospective cohort study which examined adults in 6 Canadian hospitals concluded that prior statin treatment does not yield reduced mortality from pneumonia, or a reduced risk of developing the condition (83). Additionally, retrospective analysis of the JUPITER trial data demonstrated that statin treatment has no impact on the risk

of developing sepsis (82). Therefore, while several studies have shown extremely promising potential for the use of statins to treat sepsis and pneumonia, further research is needed to evaluate the efficacy and safety of the use of statins as antimicrobial agents.

2.1. Respiratory Tract Infections

Despite the discovery of anti-microbial compounds throughout much of the last century, respiratory tract infections (RTIs) caused by bacteria, fungi and viruses are still a significant cause of morbidity and mortality in the modern world. According to the most recent World Health Organisation (WHO) estimates, over 3.5 million people died as a result of RTIs in 2008 alone, meaning that RTIs accounted for 6.2 % of all deaths worldwide (84). Bacterial RTIs can be caused by many different microorganisms. In the developing world, the predominant causes of bacterial RTIs are organisms like *Mycobacterium tuberculosis*, *Bordetella pertussis*, and pneumonia caused by *Haemophilus influenzae* and *Streptococcus pneumoniae*. However, in regions where vaccinations are routinely carried out against such organisms – such as Europe and North America – the most common causes of bacterial RTIs are opportunistic and nosocomial species. This correlates with the increased prevalence of RTIs in young, elderly and immunocompromised individuals compared to other members of the general population in these regions. Bacterial pneumonia and chronic obstructive pulmonary disease (COPD) and their associated complications are a major cause of death in the developed world. However, a number of genetic conditions with dysfunctional respiratory tract components are exacerbated by bacterial RTIs. One example of this is the genetic condition cystic fibrosis.

2.2. RTIs and Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disorder, which occurs due to a mutation in the gene that encodes the cystic fibrosis transmembrane conductance

regulator (CFTR). The term "cystic fibrosis" was devised by Dorothy Hansine Anderson in 1938, who described the symptoms of CF disease in the lungs, pancreas and gastrointestinal tract (85). The European incidence of CF is 1:2000-3000 and it is more common amongst Caucasians than any other ethnic group (86). Ireland has the highest rate of CF in the world – over 1200 individuals in Ireland have CF, giving it an incidence of 1:1353 (87). Furthermore, 1/19 individuals in Ireland are heterozygous carriers of the defective *cftr* gene, and this again is higher than the EU average of 1/25 (88). The risk of CF in Ireland is so severe in comparison to other regions that pre-natal screening was introduced in 2009.

Five different classes of *cftr* mutations have been documented (reviewed in (89)), with a frameshift mutation known as Δ508 being the most commonly-documented *cftr* mutation. This 3 base pair (bp) deletion is found in approximately 70 % of CF patients and is characterised by a missing phenylalanine residue at position 508 in the CFTR protein (90), causing it to be degraded by ubiquination (91). Another *cftr* mutation which is of particular interest in Ireland is the G551D mutation, in which the glycine residue at position 551 in CFTR is replaced by aspartic acid (92). G551D has become known as the Celtic mutation, due to Irish CF patients reportedly having the highest incidence of this mutation in the world (93).

In individuals without CF, CFTR functions as a chloride ion channel which secretes Cl^- out of cells (Figure 5). This inhibits the neighbouring epithelial Na^+ channel (ENaC), of which the function is the absorption of Na^+ into cells. The inhibition of ENaC results in the presence of Na^+ in the airways, which causes water to leave epithelial cells by osmosis. This water plays a vital role as it keeps the respiratory mucus layer and epithelia apart, ensuring that beating of cilia occurs in order to effectively trap inhaled pathogens. In CF patients, the lack of functional CFTR leads

to deregulation of ENaC, causing excess Na^+ to be absorbed by epithelial cells. This leads to increased amounts of water being drawn into the cells. In terms of CF lung disease, the lack of water in the airway results in adhesion of mucus to epithelial cells, and a higher concentration of mucus which prevents the beating of cilia, thus leading to increased risk of RTIs. However, CF patients also suffer from pancreatic and gastro-intestinal disease as a result of defective CFTR, leading to the development of conditions including gastro-oesophageal reflux, diabetes, and pancreatitis and cysts.

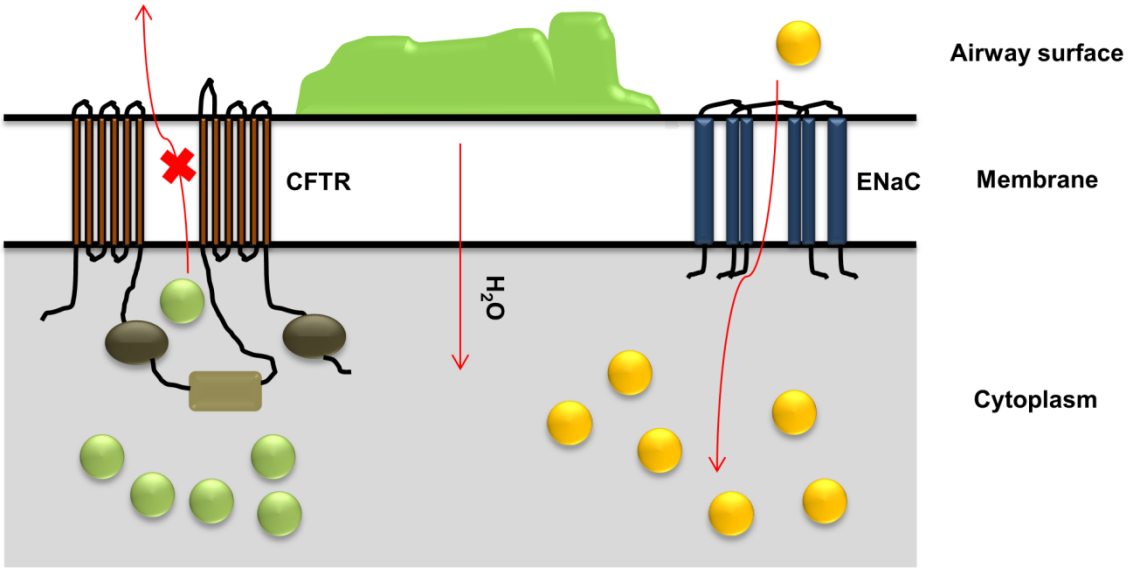


Figure 5: Mechanism of CFTR function in CF cells. The CFTR and ENaC channels are located adjacent to each other in the cell membrane. In CF, mutated CFTR does not export Cl- ions (green circles) out of the cell. The accumulation of Cl- in the cell causes the import of Na+ (yellow circles) via the ENaC channel. This leads to water entering the cell via osmosis, resulting in increased viscosity of pulmonary mucus, which is then colonised by bacterial and fungal species.

2.3. Epidemiology of CF associated pathogens – the CF microbiome

Bacteria and fungi are able to effectively colonise the CF lung due to the concentrated mucus of CF patients, which provides a nutrient-rich environment where the bacteria can grow and evade the host immune response. People with CF are now known to possess a distinct microbial community composed of opportunistic and nosocomial pathogens – termed the "CF microbiome" – within their lungs. Traditionally, the members of this community were identified from bronchoalveolar lavage fluid (BALF) using culture based methods (Figure 6A). However, more recently, 16S ribosomal DNA (rDNA) or rRNA sequencing has allowed for the identification of unculturable organisms within the CF lung (Figure 6B) (94).

Children with CF are commonly colonised with *Staphylococcus aureus, Pseudomonas aeruginosa* and *Haemophilus influenzae* (95). However, *S. aureus* is the dominant species in the lungs of paediatric CF patients (96). Neonatal screening has detected *S. aureus* in up to 76 % of patients (97) and this species is found in over 60 % of CF patients aged between 6 and 17 years old (96). Even though *S. aureus* is no longer dominant in adult CF patients, it remains a major component of the CF microbiome, infecting approximately 50 % of adults with CF (98). The maximum prevalence of *H. influenzae* is 32 % in CF patients aged between 2 and 5 years, after which its prevalence decreases to below 10 % of adult CF patients (96). The Gram positive species *Streptococcus pneumoniae* is also found in paediatric CF patients and is reportedly dominant in the lungs of infant CF patients (99). The official incidence of *S. pneumoniae* in CF patients is unknown, although it has been reported that approximately 5.5-6.9 % of people with CF are colonised with this organism

(100). In addition, over 61 % of CF patients were found to possess antibodies to *S. pneumoniae* (101).

The Gram negative species *Pseudomonas aeruginosa* remains the predominant microorganism in the lungs of adult CF patients, chronically infecting approximately 70-80 % of people with this disease (98). In addition, *P. aeruginosa* infection is correlated with the decline of lung function in CF patients (102). Colonisation with *P. aeruginosa* begins as a series of intermittent acute infections which eventually becomes a chronic infection (96). *P. aeruginosa* is also present in paediatric CF patients, but at a lower abundance than *S. aureus* (96). The mechanism by which *P. aeruginosa* out-competes *S. aureus* in adult CF patients remains unclear. *Burkholderia cepacia* complex (Bcc) is another significant Gram negative CF-associated pathogen which causes chronic infections. Bcc is known to be composed of at least 17 *Burkholderia* species (103, 104). Bcc strains have been recovered from approximately 10 % of adult CF patients (105). *B. multivorans* and *B. cenocepacia* are the most common Bcc species found in CF patients, causing approximately 85-97 % of all CF-related infections (106). However, the most prevalent Bcc species varies between CF patients in different regions – for instance, *B. multivorans* is now the most common Bcc species found in the UK, but *B. cenocepacia* is the most frequently-occurring species in the USA and Canada (105). CF patients are also at risk of chronic infection with non-tuberculous *Mycobacterium* species, which infect between 2 and 28 % of CF patients (96). As well as chronic infections, CF patients also experience transient infections from bacteria such as *Stenotrophomonas maltophilia,* which infects between 4 % and 12.4 % of CF patients (96), and *Acinetobacter* species (96).

The advent of sequence-based methods has allowed for the identification of unculturable and novel strains in the CF microbiome (Figure 6B). Analysis of ribosomal RNA (rRNA) in BALF from children with CF allowed for the identification of non-typical and non-culturable CF-associated bacteria, including novel α-Proteobacteria and *Lysobacter* species (107). rRNA sequencing has also uncovered *Neisseria* and *Leptotrichia* species, as well as Fusobacteria sequences, which were undetectable using culture-based methods (108). Furthermore, pyrosequencing of 16S ribosomal DNA (rDNA) from sputum samples has demonstrated the presence of non-canonical CF-associated bacteria, including *Gemella, Prevotella* and *Veillonella* (96). Sequencing developments have resulted in improved taxonomical classification of CF isolates. Sequencing and PCR-based analysis of 16S rDNA from clinical isolates which were originally classified as *Burkholderia* or *Ralstonia* led to the discovery and classification of the genus *Pandoraea* (96, 109). The methodology used in this area is consistently evolving and becoming more specific. For instance, a recently-described novel deep sequencing method revealed greater species specificity of unculturable samples and reduced error (110).

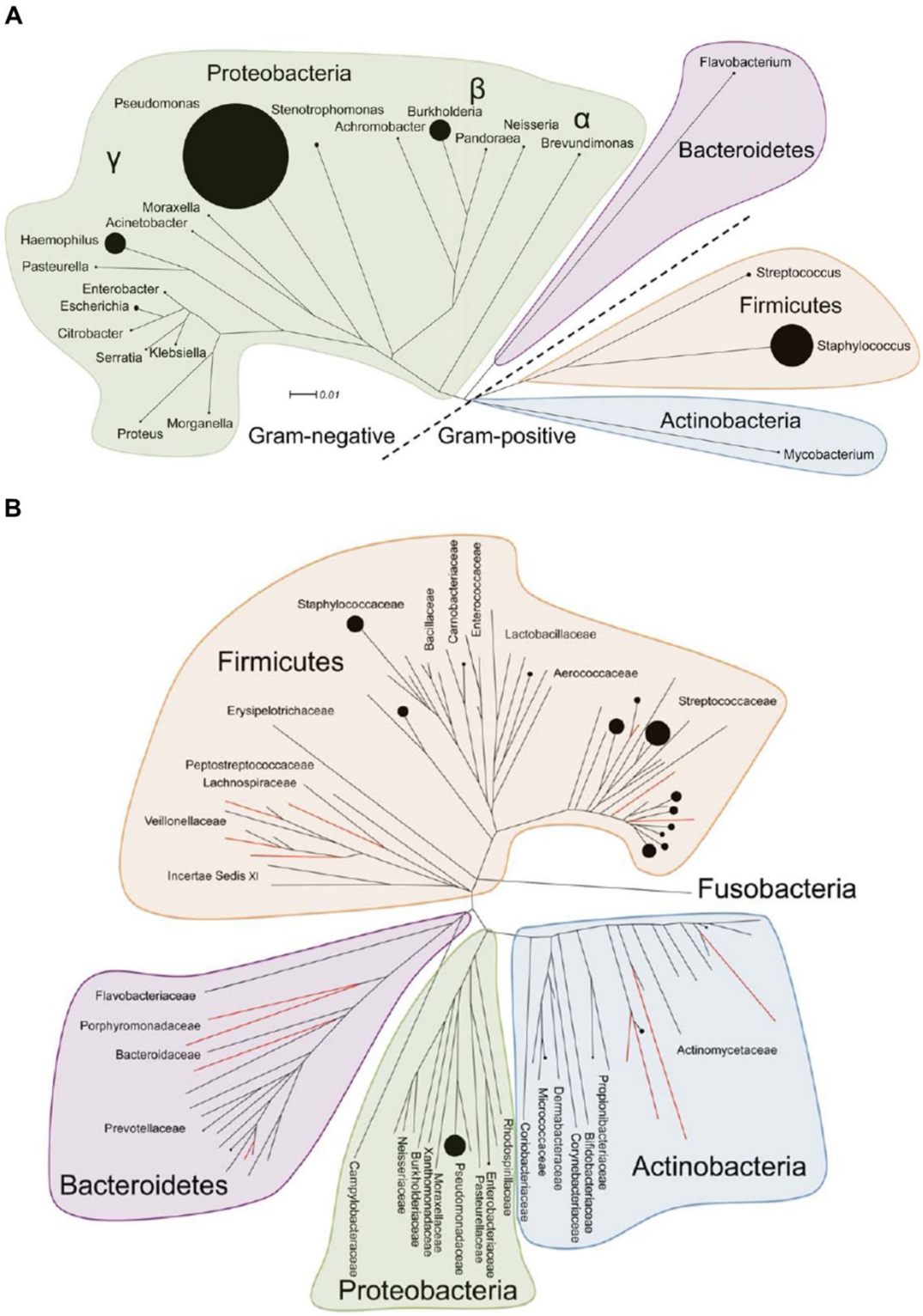


Figure 6: Bacterial ecology of the CF lung. (A) Bacteria which have been isolated from CF patients using culture-based methods. **(B)** Species which have been identified using sequencing technology. Adapted from Sibley *et al*., 2011 (108).

3.1. Virulence of *Pseudomonas aeruginosa* – the predominant CF-associated pathogen

P. aeruginosa is a Gram negative, rod-shaped bacterial species, which is free-living and naturally occurs ubiquitously in the environment. This bacterium is classed as an opportunistic and nosocomial pathogen, and in addition to RTIs can also cause corneal and wound infections. *P. aeruginosa* is not only the most prevalent CF-associated organism, but is arguably the most widely-characterised too, as the genomes of 13 *P. aeruginosa* strains have been sequenced to date (111). This makes *P. aeruginosa* an ideal model organism to study the mechanisms by which infections occur. *P. aeruginosa* has a number of behavioural traits, termed "virulence factors", which facilitate its colonisation and long term survival in several different and often hostile host environments, including the lungs of people with CF. These factors and their regulation are detailed in Figure 7. Several of these factors are of interest in this study, and these are discussed below.

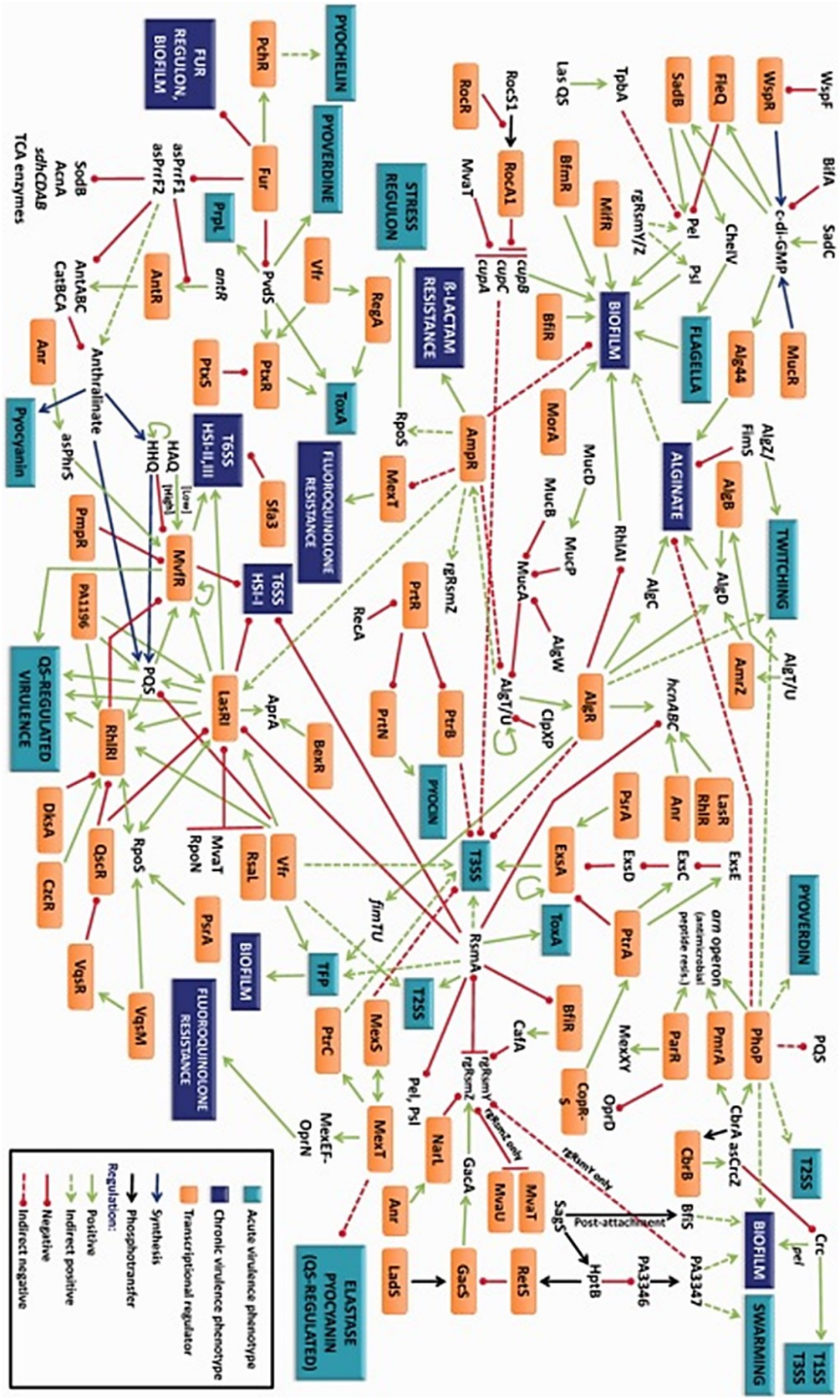


Figure 7: Regulation of *P. aeruginosa* virulence. *P. aeruginosa* possesses numerous virulence factors which are required for both acute and chronic infections. These factors are

regulated by a plethora of transcriptional and post-translational pathways. Many virulence factors share a regulatory pathway, or are inversely regulated by the same pathway. From Balasubramanian *et al.*, 2013 (112).

3.2. Type III Toxin Secretion

A key virulence trait of *P. aeruginosa* is toxin secretion by way of a Type III secretion system (TTSS), and this virulence mechanism is crucial during the early acute phase of *P. aeruginosa* infection (113, 114). *P. aeruginosa* produces 4 type III exotoxins – ExoS, T, U and Y. ExoS and ExoT are bi-functional proteins, which contain an N-terminal GTPase-activation (GAP) domain, and C-terminal ADP-ribosyltransferase activity (115–118). ExoU is a phospholipase (119), while ExoY possesses adenylate cyclase activity (120). Both ExoS and ExoU are primarily responsible for actin depolymerisation and cytotoxicity (121, 122). These proteins are mutually exclusive amongst *P. aeruginosa* strains, and strains which possess ExoU are generally more virulent and cytotoxic than strains which possess ExoS (121). Although ExoT also possesses GAP activity, it has been found to induce actin depolymerisation without causing cell death (123). ExoY causes cyclic-AMP-mediated actin depolymerisation, but does not appear to influence cell death (120, 124).

The TTSS is activated when *P. aeruginosa* binds to host cell receptors. This induces the formation of a needle complex, which penetrates the host cell membrane and injects toxins into the cytoplasm (113). Host cell contact activates the ExsCDE regulatory cascade, which induces the transcription factor ExsA (113, 125, 126). ExsCDE is also activated under conditions of low calcium (Ca^{2+}) (125, 126). Low Ca^{2+} also induces synthesis of cyclic AMP (cAMP), which complexes with the Vfr transcription factor, leading to Vfr-mediated TTSS activation (126). The direct expression of all TTSS toxins and secretion apparatus is regulated by ExsA (125, 126). However, the transcriptional regulation of ExsA-mediated TTSS induction is extremely multifaceted (Figure 8).

The production of TTSS effectors by *P. aeruginosa* is positively regulated by the small RNA-binding protein RsmA (127). RsmA is in turn regulated by the small RNAs RsmY and RsmZ, which bind to RsmA, thus inhibiting its activity (128–132). The transcription of RsmY and RsmZ is directly regulated by the GacA regulatory protein (133), which is activated by its cognate sensor kinase GacS (134). GacA also interacts with the LadS and RetS sensors (135). RetS positively regulates the expression of the TTSS, while LadS has the opposite effect (125). However, RetS also indirectly induces TTSS by reducing intracellular levels of the signalling compound cyclic-di-GMP (c-di-GMP) in a RsmY/Z-dependent manner (136). The AlgR regulator also manipulates RsmY/Z expression in order to inhibit TTSS expression (137). Furthermore, the long chain fatty acid sensor PsrA can also regulate TTSS, as ligand binding to PsrA represses TTSS by inhibiting expression of *exsC* (138). TTSS expression can also be regulated by several environmental stress factors. For instance, we determined that limiting O_2 induces the GltS/R sensor-regulator system, which activates the Anr regulator, leading to induction of another regulator, NarL, which induces TTSS via modulation of RsmAYZ (139, 140). TTSS is also inhibited by metabolic cues such as long chain fatty acids and tryptophan, and by stress factors including DNA damage, copper and osmotic stress (125, 126).

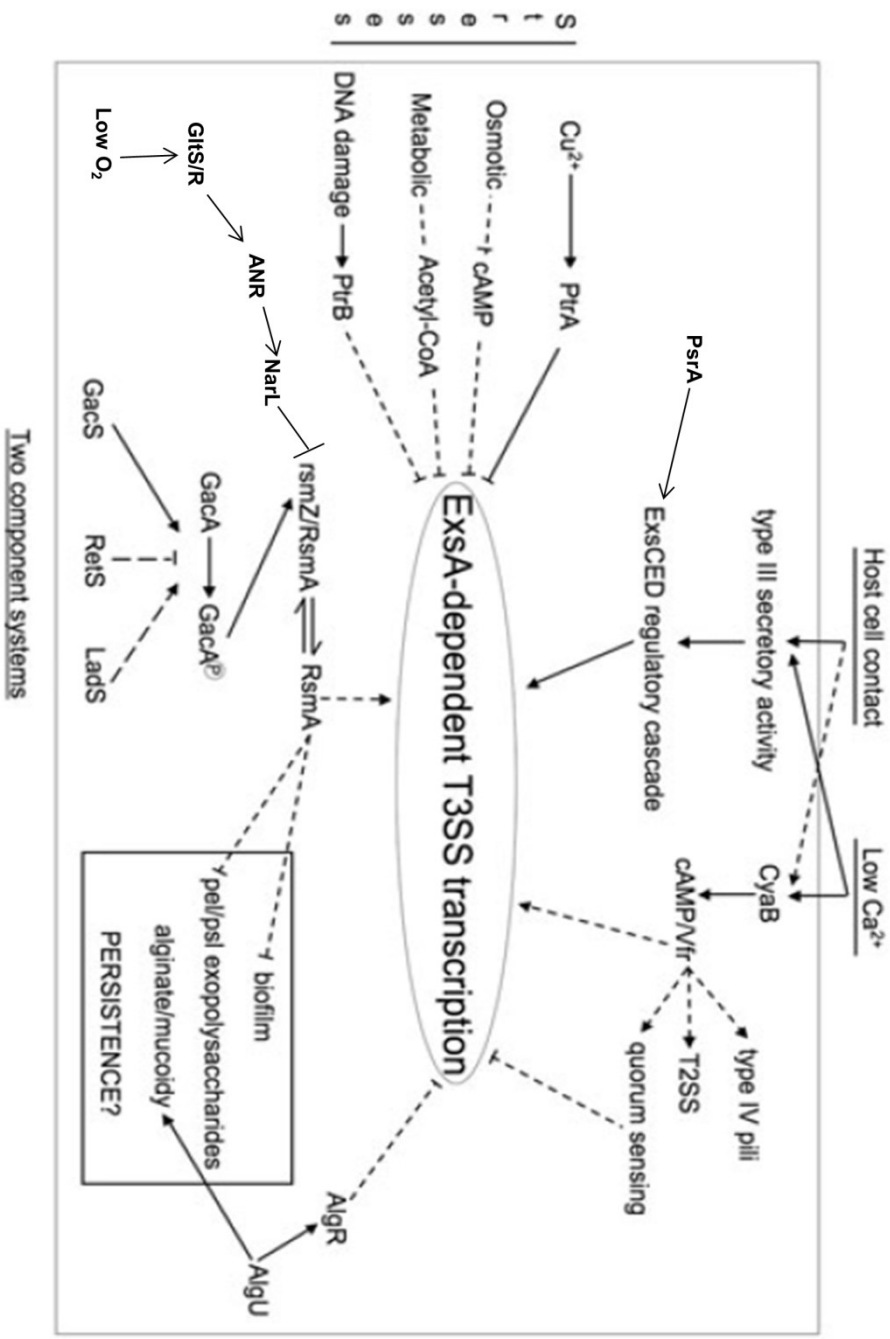


Figure 8: Regulation of the *P. aeruginosa* TTSS. Expression of TTSS effectors is transcriptionally regulated by a number of conditions and proteins. However, ExsA is indisputably required for the expression of TTSS-related genes. Adapted from Yahr & Wolfgang, 2006 (126).

3.3. Quorum sensing

Quorum sensing (QS) is a mechanism of intercellular bacterial communication, which is mediated through distinct molecular signals. *P. aeruginosa* QS is modulated via 2 key groups of chemical compounds, homoserine lactones (HSLs) and quinolones, and 3 QS systems. The regulation of these systems is detailed in Figure 9. One key system of HSL-mediated QS regulation in *P. aeruginosa* is the LasI/R 2 component sensor/regulator system. LasI is responsible for the synthesis of the HSL N-3-oxo-dodecanoyl-L-homoserine lactone ($3OC_{12}$-HSL), which is recognised by the receptor LasR (141–143). Once the number of bacteria in culture passes a certain threshold, $3OC_{12}$-HSL binds to LasR, leading to the abundant activation of genes involved in a number of processes, including the activation of 2 other *P. aeruginosa* QS systems – the 2 component RhlI/R system, and 2-heptyl-3,4-dihydroxyquinolone (*Pseudomonas* quinolone signal; PQS). RhlI synthesises another HSL, N-butaryl-homoserine lactone (C_4-HSL), which binds to RhlR, the regulator of the RhlI/R 2 component system (144–146). The production of C_4-HSL is also positively regulated by GacA and RsmY/Z (131).

PQS is one of approximately 50 quinolone-based QS molecules that *P. aeruginosa* possesses, and it and its precursor 4-hydroxy-2-heptylquinoline (HHQ) regulate the expression of virulence factors including motility, biofilm formation and antibiotic resistance. PQS and HHQ are produced by the *pqsABCDE, phnAB* and *pqsH* genes (147, 148) during the late stationary phase of infection (149). *pqsABCDE* and *phnAB* are regulated by the PqsR transcription factor (147, 150). Interestingly, some cross-regulation exists between the *P. aeruginosa* QS systems. PQS can induce RhlI to the same extent as C_4-HSL (149). Furthermore, *pqsR* transcription is positively regulated by LasR and inhibited by RhlR (150). In addition to the LasI/R, RhlI/R and PQS

systems, a putative third QS regulator – QscR – has been identified in *P. aeruginosa* (151). This regulator can bind $3OC_{12}$-HSL (152) and can consequently inhibit LasI and RhlR (153).

The QS systems of *P. aeruginosa* collectively regulate virulence, causing a coordinated shift from acute to chronic infection phenotypes. HSL signalling is integral to *P. aeruginosa* virulence as it regulates myriad virulence factors, including production of Exotoxin A, elastase and lipase, and motility (154). Most importantly, HSL-mediated QS induces biofilm formation, arguably the most significant virulence factor of chronic *P. aeruginosa* infections (155). Las-mediated QS is required for initial attachment, while RhlI/R are implicated in maturation of biofilms (156). LasI is also essential for the formation of structurally-correct biofilms (157), and LasR is required for the production of microcolonies (158). Furthermore, LasI and RhlI are required for release of LPS and extracellular DNA (eDNA) during stationary phase, which are crucial for biofilm formation (159). Other virulence factors are also influenced by QS. The TTSS is inhibited by RhlI/R-mediated QS (160), and PQS inhibits the secretion of TTSS effectors in a concentration-dependent manner (161). The *P. aeruginosa* Type VI secretion system (Figure 7) is also modulated by LasI/R and RhlI/R-mediated QS – 2 Type VI-associated gene clusters (HSI-II and HSI-III) are increased by LasI/R and RhlI/R, while the remaining cluster (HSI-I) is decreased by Las QS (162). Furthermore, all 3 QS systems inhibit flagella-associated motility (163, 164). In addition, RhlR directly regulates the production of *P. aeruginosa* rhamnolipid surfactant compounds, and PQS signalling is also essential for production of these compounds (165).

As QS regulates such a wide array of virulence factors, it has become a target in the development of novel antimicrobials. Several inhibitory mechanisms of QS – termed

"Quorum Quenching" (QQ) – have been identified. Firstly, QQ can be accomplished using compounds that mimic QS molecules. Algae, plants and fungi produce compounds which resemble AHLs (166). Secondly, many bacteria possess enzymes which degrade or modify QS molecules, such as the PvdQ acylase of *P. aeruginosa*, which catalyses the degradation of AHLs by hydrolysis of the peptide bond between the acyl chain and the homoserine lactone nucleus (167, 168). Degradation and modification of QS compounds renders them unrecognisable, leading to reduced virulence and infectivity (169).

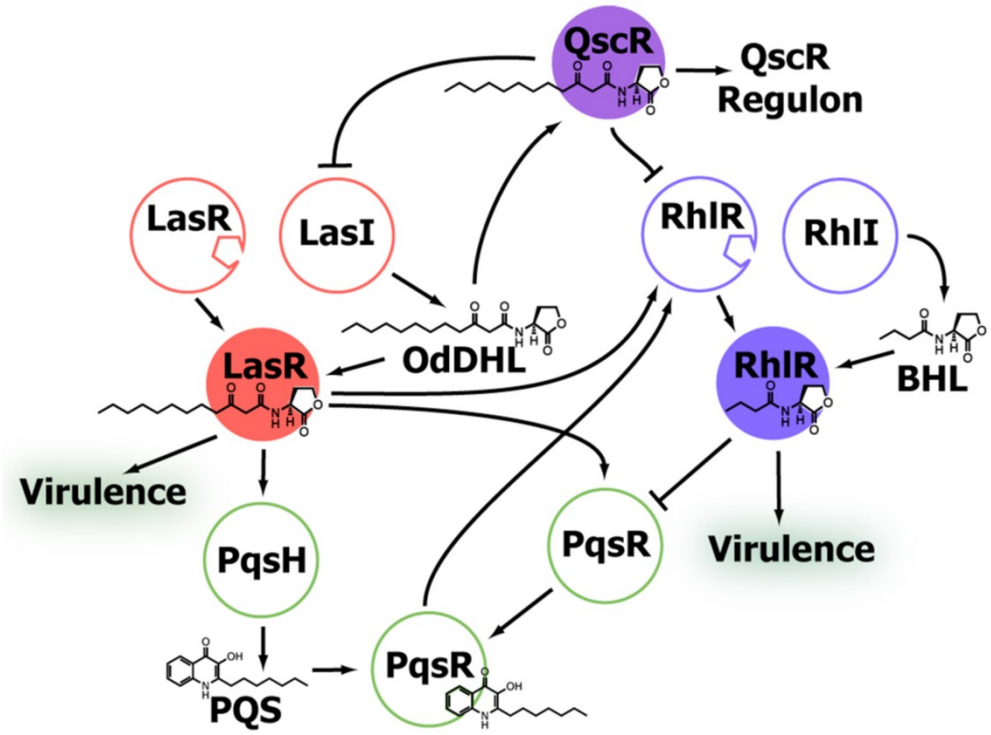


Figure 9: Regulation of quorum sensing in *P. aeruginosa*. OdDHL ($3OC_{12}$-HSL) is produced by LasI and binds to LasR, inducing virulence factors. Activation of LasI/R signalling induces expression of the RhlI/R system and its signalling molecule BHL (C_4-HSL). LasI/R signalling also stimulates the production of PQS, which positively regulates RhlR via interacting with the PqsR regulator. The orphan regulator QscR can inhibit both LasI and RhlR. From Mattmann & Blackwell, 2010 (153).

3.4. Motility

Bacterial motility is a key factor in both initiation of infection and the switch from the acute to chronic phase of infection. Three types of *in vitro* motility – swimming, swarming and twitching – have been observed in *P. aeruginosa.* Each of these phenotypes is controlled by an extracellular appendage of the bacterial cell wall. Swimming motility is controlled by the flagellum, and bacteria form a circular colony on plates (170). Twitching motility is characterised as bacterial adherence to and movement across a surface, and is mediated through Type IV pili (171). Swarming motility requires the co-expression of pili and flagella (172) and is characterised by the formation of snowflake-like pattern of tendrils on semisolid agar. However, each type of *P. aeruginosa* motility is also dependent upon several other factors, including chemotaxis, nutrient availability and QS.

Swarming is an extremely multi-faceted phenotype. Over 200 *P. aeruginosa* genes with roles in a wide array of functions, including metabolism, transport and secretion, have been found to play a role in swarming (173). The regulation of this behaviour is similarly disparate, and is mediated through several regulatory and QS-associated pathways. Both LasI/R and RhlI/R are required for full swarming motility (172). RsmA and HHQ promote swarming motility (130, 174), while it is inhibited by PQS (163) and the signalling molecule c-di-GMP (175). The latter effect is regulated by amino acid-mediated induction of c-di-GMP, which promotes biofilm formation (176). Because swarming is regulated by both LasI/R and RhlI/R, which are involved in attachment and biofilm maturation (156), it is thought that swarming motility is associated with the switch from acute to chronic infection. This hypothesis is supported by the observation that the gene expression and morphology of cells in the swarm centre are biofilm-like, while cells at the swarm tip are

planktonic and primarily express genes involved in nutrient acquisition and metabolism (172, 177). In addition, rhamnolipids are not only required for swarming motility, but also regulate the manner in which the bacteria move (178), highlighting the importance of RhlI/R in swarming motility. Inorganic phosphate deficiency has been correlated with inhibition of swarming (as well as swimming and twitching) (179) while swarming motility is inhibited by ammonium and excess iron (180).

Swimming motility is regulated in a less complex manner than swarming, and is dependent upon the expression of the flagellum (172). This phenotype is associated with chemotaxis, and is mediated through the *che* genes, which regulate the direction of the flagellum (181, 182), and the Mot proteins, which form the flagellar motor (183). In addition, it also appears to be inhibited by LasR- and RhlR-mediated QS, as swimming is enhanced in the absence of LasR and RhlR (164). In contrast to swarming, swimming does not require rhamnolipids (178), but does require inorganic phosphate (179), and is also increased by tryptophan (184).

Twitching motility is linked with biofilm formation (discussed in 3.5.)(185). It is a significant virulence factor, as it is required for the adhesion of *P. aeruginosa* to host surfaces (186). Twitching is regulated by the FimS/AlgR sensor-regulator system, and also requires the transcription factor AlgU, demonstrating that production of the exopolysaccharide alginate is associated with twitching motility (187). In addition, twitching, like TTSS, is positively regulated by the RetS sensor (188) and, in contrast to swarming, is increased by c-di-GMP (189). Under optimal nutrient conditions, twitching occurs independently of LasI/R- and RhlI/R-mediated QS (190), but interestingly is PqsR-dependent (163). However, under limiting iron, *P. aeruginosa* requires RhlI expression to carry out twitching motility (191). Other

nutritional requirements of twitching include inorganic phosphate (179) and carbon catabolite repression (192).

3.5. Biofilm production

The key trait which allows bacteria to chronically colonise the mucus of CF patients is biofilm formation. This phenomenon generally occurs in phases (described in (193) and in Figure 10). Firstly, bacteria reversibly attach to biotic or abiotic surfaces (Figure 10A). Initial attachment is linked to the motility of *P. aeruginosa*, which can utilise flagella and type IV pili to attach to surfaces (185). Attachment is also reciprocally associated with swarming motility – an attachment-deficient mutant has been found to display increased swarming motility (194). The switch from motile to sessile bacteria is mediated through c-di-GMP, which promotes aggregation and attachment of *P. aeruginosa* (195). Attachment becomes irreversible when Las-mediated QS is activated (156). Interestingly, under anaerobic conditions, such as those encountered in the thick mucus of the CF lung, biofilms can develop as a structure called a microcolony, where bacteria do not attach directly to a surface, but instead adhere to themselves and material within sputum (196, 197). LasR is also required for microcolony formation (158).

Once irreversibly attached to a surface, the bacteria enter a sessile state, termed maturation (193). Two phases of maturation – maturation-1 and -2 – have been proposed based on differences in gene expression at different intervals in *in vitro* mature biofilms (156). Maturation-1 is regulated by Rhl QS (156). In this phase, the density of the biofilm begins to thicken, as bacteria become surrounded by a tough extracellular matrix composed of extracellular DNA (eDNA), proteins and

exopolysaccharide. Inside this structure, bacteria are essentially protected from external stresses and become extremely resistant to most antibiotics. eDNA is known to be essential for the establishment of biofilms in *P. aeruginosa* (198). The main exopolysaccharide component produced by *P. aeruginosa* is alginate, which is associated with bacteria in a mucoid state (199) and is produced as a response to low oxygen/anaerobic environments (197, 200). Two other exopolysaccharides – the products of the *pel* and *psl* genes – are also essential for *P. aeruginosa* biofilm formation (201). The regulatory protein AlgR inhibits the transcription of RhlI, meaning that it may promote development from maturation-1 into maturation-2 (202). This phase is characterised by maximum biofilm density, meaning that most of the cells within the biofilm are in a low oxygen or anaerobic environment (156).

The final stage of biofilm formation is dispersal (Figure 10B), whereby the structure ruptures and the bacteria become disseminated to distal infection sites. This occurs as a result of changes in the external environment – *P. aeruginosa* chemotaxis transducer proteins have been found to regulate nutrient-induced dispersal (203). c-di-GMP is also associated with biofilm dispersal. Phosphodiesterases which degrade c-di-GMP, such as DipA and PvrR, have been found to induce biofilm dispersal (204–206). In addition, the production of *cis-2*-decenoic acid by the DspI protein has recently been found to play a role in biofilm dispersal (207).

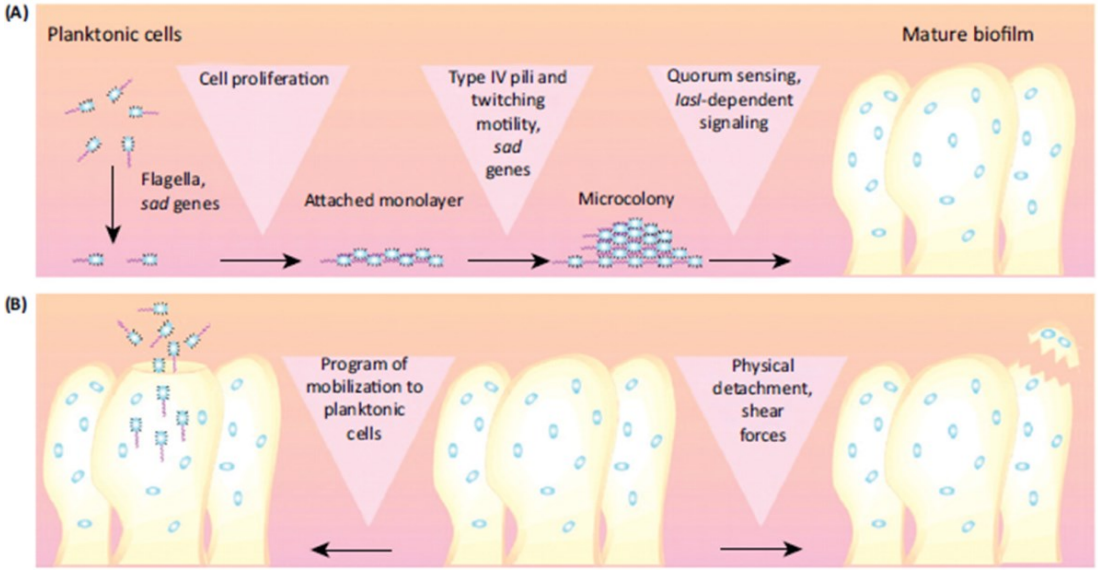


Figure 10: Biofilm formation by *P. aeruginosa*. (A) Planktonic cells attach to a surface and form a monolayer. The production of Type IV pili leads to microcolony formation. $3OC_{12}$-HSL mediates the development into a mature biofilm (maturation-1 and 2). **(B)** Mature biofilms can disperse by either internal stimulation from bacterial signalling or by physical stress from the external environment. From Bjarnsholt *et al.,* 2013 (193).

4. Regulation of host cell gene expression by *P. aeruginosa*

4.1. Induction of inflammation by *P. aeruginosa*

Infection of host cells by *P. aeruginosa* triggers the activation of genes involved in inflammation, immunity and cell death. Increased inflammation is a major cause for concern in CF patients, as the chronic inflammation caused by *P. aeruginosa* and other colonising organisms leads to impaired pulmonary function and permanent damage (89). *P. aeruginosa*-mediated inflammation is activated via a number of pathways, notably by the Toll-Like Receptors TLR4 and TLR5 (described in 1.4.3).

The pro-inflammatory chemokine IL-8 (also known as CXCL8) is a key and widely-characterised target protein of *P. aeruginosa* infection (208). IL-8 is a 99 amino acid (aa) protein whose genetic locus is located on Chromosome 4 in humans. It is produced by several tissue types including airway epithelial cells (209) and alveolar macrophages (210) with the purpose of attracting neutrophils to the site of infection (209). IL-8 expression is induced by TLR4 and TLR5 signalling (47, 211) in response to the LPS and flagellin of numerous Gram negative species, including *Salmonella* and *E. coli* (47). However, IL-8 is aberrantly produced in the CF lung by an altered neutrophil response, and is a contributory factor to the chronic inflammation and lung damage experienced by CF patients (212–215). CF-specific *P. aeruginosa*-mediated IL-8 activation was first reported by Schuster and colleagues (216). In addition, TLR/MyD88 signalling is known to induce NFκB-mediated IL-8 expression in CF cells (217). IL-8 is chronically up-regulated in people with CF (218), and this is known to be a consequence of increased NFκB expression. The induction of inflammation by *P. aeruginosa* also causes deregulation of the macrophage response, as the pro-inflammatory cytokine

Chemokine (C-C motif) ligand 20 (CCL20) is upregulated by *P. aeruginosa* via TLR5 signalling (219, 220). This 95 aa protein (also known as Macrophage Inflammatory Protein-3; MIP3α) is also induced by LPS-stimulated TLR4 signalling (221) and is produced by several tissues, including but not limited to, airway epithelial cells (222), mast cells (219) neutrophils (223) and macrophages (224). CCL20 is chemoattractant to dendritic cells (225), T cells (226) and peripheral blood mononuclear cells (227), and stimulates monocyte differentiation into macrophages (228).

P. aeruginosa colonisation also interferes with eukaryotic signalling systems in CF. Two signalling molecules of interest in this study are nitric oxide (NO) and ceramide. NO is a free radical compound which is produced in the lung by inducible nitric oxide synthase (iNOS) (229). NO contributes to the immune and inflammatory responses, as it is produced by phagocytes for respiratory burst-mediated bacterial killing (230). NO production is decreased in the CF lung, increasing the susceptibility to *P. aeruginosa* infection (231). Ceramide is a sphingolipid which is synthesised by the acid sphingomyelinase protein (ASM) (232). *P. aeruginosa* induces ASM activity in the lung, leading to increased ceramide production (233) and this contributes to the chronic inflammation experienced by CF patients (234). The mechanism by which ceramide increases inflammation is unclear; however, ceramide accumulation is correlated with an increased presence of macrophages and neutrophils in the lungs of *cftr*-deficient mice (234)

The CF inflammasome is under study with regard to developing novel treatment strategies for people with CF. Recently, decoy molecules which mimic NFκB were found to bind to the promoters and reduce the expression of several pro-inflammatory genes, including IL-8 (235). Screening is also underway to identify

potential novel anti-inflammatory therapies such as the plant compound corilagin, which has been found to reduce expression and secretion of IL-8 (236).

4.2. Kruppel-like factors – a detailed example of a *P. aeruginosa* host cell target

Kruppel-like factors (KLFs) are a family of DNA-binding transcription factors which are characterised by the presence of 3 highly conserved Cys_2His_2 zinc fingers of size 82 aa (237). To date, 17 members of the KLF family have been characterised. KLFs bind to similar GT-box (5'-GGGGCGGGG-3') or CACCC motifs in DNA in order to exert their function (238). KLFs regulate many diverse and important functions, including apoptosis, development, inflammation and proliferation (239). Therefore, it is reasonable to expect that they are prime targets in bacterial-host cell interactions. The family members KLF2 and KLF6 are of particular interest in this study, as they have been found to be induced in CF-affected tracheal epithelia by *P. aeruginosa* (240). The structures of these proteins are detailed in Figure 11.

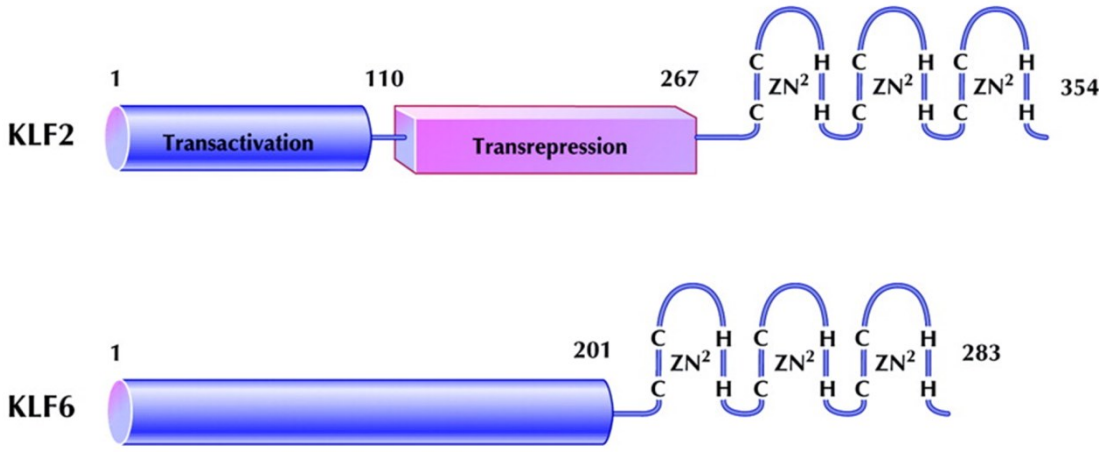


Figure 11: Protein structure of KLF2 and KLF6. Both proteins possess an N-terminal activation domain (blue). In addition, KLF2 possesses a transrepression domain (pink) between aa 110 and 267. The 3 characteristic C_2H_2 zinc fingers are conserved in both KLF2 and KLF6. Adapted from Atkins & Jain, 2007 (241).

4.2.1. KLF2

KLF2 is a 354 aa protein and is expressed in the lungs, the endothelial lining of blood vessels and lymphocytes (242), and is composed of an N-terminal activation domain between amino acids 1 and 110 and an inhibitory domain between amino acids 110 and 267 (243). KLF2 has mainly been characterised as an anti-inflammatory transcription factor (241). As previously described, KLF2 is induced by statins in endothelial cells and macrophages (53–55), and interestingly, KLF2 can act as a repressor of inflammation by regulating similar processes to statins. It binds to and inhibits the promoter of p65, which is a subunit of NFκB (244). The primary mechanism of KLF2 induction is the binding of the MEF2 transcription factor to the KLF2 promoter (245). Interestingly, p65 is known to inhibit KLF2 promoter activity by forming a trimeric complex with MEF2 and HDAC4 (245). KLF2 also inhibits the promoter activity of AP-1, another pro-inflammatory regulator (244), and has been found to reduce the p65 and TNFα-mediated induction of pro-inflammatory adhesion molecules (246). Furthermore, it has been proposed that KLF2 plays a role in the CF lung. KLF2 can inhibit IL-8 release in an *in vitro* model of neutrophil-mediated inflammation, and has also been found to be absent in tissue from patients with severe respiratory inflammation, thus suggesting that IL-8 in chronic inflammation is modulated by KLF2 (247).

As well as inhibiting promoters of inflammation, like statins KLF2 promotes the expression of anti-inflammatory proteins. For example, eNOS expression is strongly induced by KLF2, and this confers an anti-inflammatory phenotype on endothelial cells (246). Additionally, KLF2 can induce thrombomodulin in endothelium, leading to a decrease in blood clotting (248). In fact, the induction of both eNOS and thrombomodulin by statins are mediated through KLF2 (53).

Given KLF2's regulatory role in inflammation and immunity, it is not surprising that it is influenced by bacterial infection. We identified that the *P. aeruginosa* ExoS toxin is responsible for transcriptional induction of KLF2 in *cftr*$^{-}$ tracheal epithelia (240), but other bacterial species have also been found to induce KLF2 (249). The Gram negative gastro-intestinal pathogen *Yersinia enterocolitica* induces KLF2 expression in HeLa cells (250) and mouse macrophages (251, 252) and this expression is dependent on presence of the cysteine protease YopT (251). KLF2 induction is not limited to Gram negative toxins – the *Clostridium botulinum* C3 toxin can induce KLF2 expression in vascular endothelial cells (54). Furthermore, Moreilhon *et al.* demonstrated that *S. aureus* induces KLF2 in tracheal gland cells, although this effect is not linked to the production of a particular virulence factor, as both live bacteria and supernatant could increase KLF2 to a similar extent (253). KLF2 expression subsequently appears to influence the impact of bacterial infections. KLF2 was found to reduce *P. aeruginosa*-mediated NFκB activation in primary airway cells (247). It has been hypothesised that bacterial toxins induce KLF2 by inhibiting the downstream effects of RhoA, and this was suggested to be an immunosuppressive mechanism to prevent the damaging over-activation of the inflammatory response (254). Another report appears to confirm this hypothesis, as it showed that KLF2 deficiency causes unregulated activation of inflammatory components such as IL-17 and TNFα, resulting in increased bactericidal activity in polymicrobial infection of myeloid cells (255).

4.2.2. KLF6

KLF6 is a ubiquitously expressed 283 aa protein, which contains a serine- and proline–rich N-terminal activation domain (256). It is also known as core promoter element-binding protein (COPEB), GC-rich site binding factor (GBF) and zinc finger 9 (ZF9) (257–259). The main known function of KLF6 is regulation of cell proliferation and apoptosis. KLF6 halts cell growth by positively inducing the cyclin-dependent kinase p21, which has a strong inhibitory effect on cell proliferation (260). KLF6 induces apoptosis via a TGF-β-dependent mechanism (261). Due to the regulatory effects of KLF6 on apoptosis and proliferation, it has been described as a tumour suppressor gene. Loss of heterozygosity (LOH), promoter hypermethylation and alternative splicing of KLF6 have all been implicated in several types of cancers. KLF6 inactivation by LOH has been identified in tumours from patients with prostate (262), colorectal (263), lung (264) and ovarian (265) cancers. Moreover, silencing of KLF6 expression due to promoter hypermethylation has been implicated in oesphageal squamous cell (266) and hepatocellular (267) carcinomas.

Despite its role as a tumour suppressor, it appears that KLF6 may have a role in inflammation and immunity. KLF6 indirectly induces CCL20 by inhibiting PPARγ in kidney cells (268), and may also induce TNFα (269). Another target of KLF6 is acid ceramidase (270), a protein which regulates the degradation of ceramide. Taken together, these reports suggest that KLF6 may increase inflammation. Furthermore, KLF6 has been induced by oxidative stress in a hepatocellular carcinoma cell line (269), and contributes to the production of NO by directly inducing expression of iNOS in kidney cells (271).

Intriguingly, given its potential role as an inflammatory regulator, KLF6 is induced during bacterial and viral infections. KLF6 expression is induced in CF tracheal epithelia by the *P. aeruginosa* toxins ExoS and ExoY (240). Furthermore, KLF6 was also found to be induced in mouse macrophages by *S. aureus* toxins (253) and in HeLa cells by *Y. enterocolitica* (250). The induction of iNOS by KLF6 also promotes apoptosis of cells infected with influenza A (272) and respiratory syncytial virus (273). Therefore, although KLF6 is not yet fully characterised as a regulator of infection, it is clear that it has a function in the immune response.

4.2.3. Alternative splicing and KLF6

As previously stated, KLF6 is 283 aa in size, and is coded by an mRNA of 4679 base pairs (bp). However, KLF6 is also regulated by alternative splicing. Alternative splicing is a process whereby an mRNA transcript of a gene can be spliced across a number of sites, generating 2 or more alternative RNA and protein products from the same transcript. Approximately 70 % of human genes are routinely regulated by alternative splicing, but this process can also occur abberantly, and as such is implicated in inflammatory disorders and cancer. KLF6 alternative splicing falls into the abberant category.

KLF6 is spliced into 3 splice variants, and these are termed KLF6-SV1, KLF6-SV2 and KLF6-SV3, with respective mRNA transcript lengths of 4525, 4553 and 4555 bp) (274). These transcripts share the proline- and serine-rich N-terminal domain with full-length KLF6 (also known as wild type KLF6 [wtKLF6]), but, due to the deletion of nucleotides, the C-terminal zinc finger is either altered or not present in the splice variants (274). The structures of the KLF6 splice variants are detailed in

Figure 12. SV1, the shortest transcript, does not possess any zinc fingers, but instead contains a unique C-terminal sequence due to out of frame splicing. The first of the 3 zinc fingers is absent from SV2, and SV3 contains the first zinc finger, but due to out-of-frame splicing the second and third zinc fingers are replaced by a novel C-terminus sequence (274).

With regard to mechanism, KLF6 alternative splicing is mediated through the splicing enhancer proteins ASF/SF2, which promotes dominant splicing of wtKLF6 (275) and SRp40, which causes increased expression of the variants relative to the wild type (274). Increased expression of Ras, and activation of downstream proteins also increases production of splice variants (275). Furthermore, inheritance of *IVSΔA*, a particular KLF6 allele corresponding to a single nucleotide polymorphism upstream of exon 2, is linked to increased production of splice variants, as well as a reduction in p21 induction (274).

As the splice variants lack the 3 conserved zinc fingers of the KLF family, they are unable to exert the same anti-proliferative effects as wtKLF6, and are consequently implicated in several types of cancers, with SV1 being most commonly observed. SV1 has been seen to be increased in prostate (274), liver (275), lung (276) and ovarian cancers (265), and overexpression of SV1 has been linked to decreased p21, and increased expression of the anti-apoptotic protein Bcl-2 (274, 277). At first, SV1 was thought to be primarily localised in the cytoplasm (274) but it was demonstrated that SV1 can move between the nucleus and cytoplasm (278), and can bind the pro-apoptotic protein NOXA, targeting it for degradation (279). Interestingly, SV1 may ablate KLF6 activity, as it has been found to bind KLF6, consequently inhibiting its ability to activate p21 and accelerating its cytoplasmic degradation (280). Furthermore, SV1 may also have a role in inflammation, as it is induced by oxidative

stress (269). However, unlike wtKLF6, SV1 may have an anti-inflammatory role, as it was found to reduce TNFα promoter activation (269).

SV2 has been shown to be localised in the cytoplasm (274), and has been implicated in liver (281), ovarian (265) and prostate (274) cancers. Like SV1, overexpression of SV2 is known to decrease wtKLF6-mediated induction of p21 (274). To date, no additional information is known regarding SV3, and there is no current evidence to suggest that this transcript is translated to a protein.

With regard to infection, a clear context has not yet been established for KLF6 splice variants. One report demonstrated that both wtKLF6 and SV2 are increased to a similar extent in human lung cells by $3OC_{12}$-HSL from *P. aeruginosa* (282). However, the transcriptional induction of wtKLF6 by bacterial toxins suggests that KLF6 splice variants may be induced during infections. Bacteria have been found to alter alternative splicing of human genes, either by causing simultaneous expression of more than 1 variant, or by promoting dominant transcription of one transcript variant over any others. Invasion of both *Salmonella* Typhimurium and *Y. enterocolitica* increases alternative splicing of the HLA-B27 gene – which is implicated in immunity and reactive arthritis – into a pro-arthritic soluble form (283). Furthermore, *Mycobacterium tuberculosis* can induce alternative splicing of the β1 subunit of IL-12 (*il12rb1*), producing a novel isoform which is linked to increased immune activation (284).

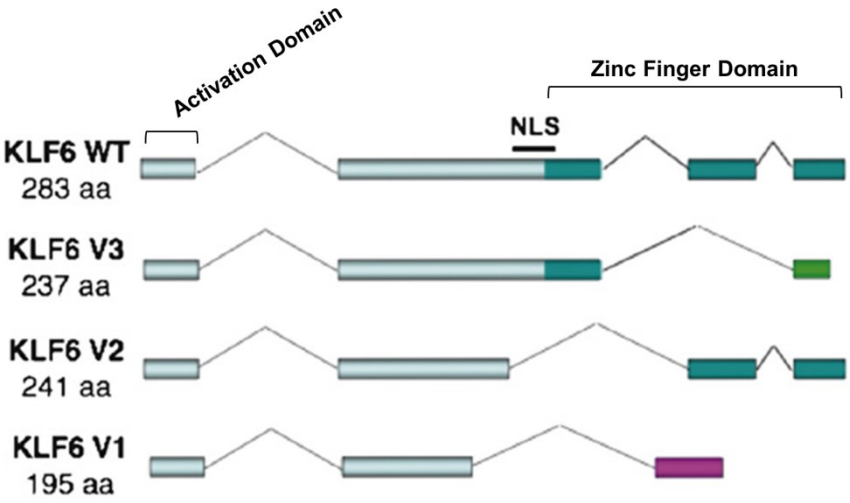


Figure 12: Structure of the KLF6 splice variants. wtKLF6 possess an N-terminal activation domain, a nuclear localisation signal (NLS) and 3 zinc fingers (blue). The N-terminal domain is conserved in all splice variants, but they differ from the wt and each other at the C-terminus. SV3 possesses the NLS, 1 zinc finger, and a novel 12 aa domain which arises from out-of-frame splicing (green). The first zinc finger and NLS are absent from SV2, while the SV1 C-terminus is composed of a 25 aa domain resulting from out-of-frame splicing (purple). Adapted from DiFeo *et al*., 2009 (285).

5.1. Statins, RTIs and cystic fibrosis: Objectives of this study

Given the anti-inflammatory and anti-microbial effects of statins, it is possible that these compounds may play a role in attenuating bacterial virulence and morbidity associated with CF. Therefore, the aim of this research was to examine the effects of statins on the causative agents of CF-associated respiratory tract infections. In Chapter 2, this first part of this analysis entailed examining the prevalence of the mevalonate pathway in RTI-associated bacteria, and examining the effects of statins on these bacteria. Chapter 3 involved studying the effects of statins on *in vitro* virulence behaviour and transcriptomic and translational expression of *Pseudomonas aeruginosa*. Chapter 4 was undertaken to examine the mechanisms by which statins influenced *P. aeruginosa* infection, by examining specific infection-related target genes, including elucidating the role of KLF6 splice variants in *P. aeruginosa* infection. In summary, the effects of statins on both the host and the pathogen were examined, to determine whether these compounds could be used as therapeutic agents against CF-associated bacterial pathogens.

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Chapter 2

Identifying the potential for the use of statins against respiratory bacterial pathogens

Abstract

The mevalonate pathway is a metabolic pathway which feeds into cholesterol biosynthesis in mammals. This pathway is inhibited by a class of drug called statins, which bind to a rate-limiting enzyme within the pathway, 3-hydroxy-3-methylglutaryl-CoenzymeA reductase (HMGR). Statins have also been found to have pleiotropic effects, including the inhibition of bacterial growth and improved survival of patients with pneumonia and sepsis. Based on this information, the prevalence of HMGR in respiratory bacterial pathogens was examined. Three cystic fibrosis-associated pathogens – *Staphylococcus aureus, Streptococcus pneumoniae* and *Burkholderia cenocepacia* – were found to possess HMGR homologues, but interestingly *B. cenocepacia* did not possess a mevalonate pathway. Bioinformatic analysis suggested that the HMGR of *B. cenocepacia* may have an alternative metabolic role. The impact of simvastatin on the growth and virulence of the selected species was subsequently examined. Simvastatin reduced the growth of all 3 species in a dose dependent manner. In addition, statin treatment inhibited the spreading motility of *S. aureus*, and the biofilm formation of all 3 species. Furthermore, simvastatin also potentiated the effect of the aminoglycoside antibiotic gentamicin against *B. cenocepacia*.

Introduction

Statins are a class of pharmaceuticals that reduces cholesterol biosynthesis, by inhibiting the rate-limiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) enzyme in the human mevalonate pathway, of which cholesterol is one of the synthetic end products (1). This inhibitory effect occurs via the competitive binding of statins to the active site of HMGR, blocking the binding of its substrate 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (1). In addition to this inhibitory effect, statins have also been found to have a number of cholesterol-independent, pleiotropic effects. Two key pleiotropic effects are reduction of inflammation (reviewed in (2)), and inhibition of microbial growth. With regard to the latter phenomenon, over the course of the past decade statins have increasingly been found to reduce the *in vitro* growth of bacterial pathogens. The affected species include both Gram positive organisms such as *Staphylococcus aureus* (3), and *Streptococcus pneumoniae* (4), and the Gram negative bacteria *Escherichia coli* (5)*, Pseudomonas aeruginosa* (5) and *Moraxella catarrhalis* (4). Statins may also influence bacterial virulence in infections. Shibata and colleagues demonstrated that simvastatin treatment can reduce the translocation of *Pseudomonas aeruginosa* across epithelial cell monolayers (6) and statins have also been found to attenuate the adhesion of *Streptococcus pneumoniae* to lung cells in an *in vivo* infection model (7). In addition, without influencing toxin production, statins can reduce the cytotoxicity of *Bacillus anthracis* lethal toxin and the inflammatory effects of *S. aureus* toxins against host cells (8, 9). Furthermore, epidemiological data suggest that patients with prior statin treatment are less likely to develop conditions such as sepsis (10) and pneumonia (11, 12) compared to un-treated patients, and are also more likely to survive these conditions (11, 13).

Due to their anti-inflammatory and anti-microbial effects, it has been proposed that statins may have beneficial effects for patients with the recessive genetic disorder cystic fibrosis (CF). This disease is caused by a mutation that renders the CFTR Cl^- transporter protein non-functional (14) and is characterised by impaired lung function as a result of viscous mucous production, leading to chronic inflammation and infection with opportunistic pathogens. Statins have previously been found to reduce both basal and LPS-induced production of the pro-inflammatory cytokine Interleukin 8 (IL-8) in blood cells from CF patients (15). Furthermore, Chen *et al*. demonstrated that statins can reduce mucin production and pro-inflammatory cytokine production in an *in vivo* airway model (16). The potential advantages of statin treatment for CF patients are so promising that in 2010, researchers at the University of British Columbia proposed plans for a randomised clinical trial to test the efficacy of statin treatment of CF patients (17), although this trial was later withdrawn due to lack of funding.

To date, the mechanisms by which statins inhibit bacterial growth are not yet clear, although it was proposed by Wilding and colleagues that the HMGR of *S. aureus* (MvaA) is essential for the growth of this organism, thus implying that statins may inhibit bacterial growth due to their interaction with this enzyme (18). HMGR enzymes are common in mammalian species, and are present in mice, dogs and primates amongst other species; these are termed Class I HMGR. However, there is also a second category of HMGR enzymes which is present only in prokaryotic species, termed Class II HMGR. The first Class II HMGR to be identified was that of the environmental bacterium *Pseudomonas mevalonii* (19), and homologues of this enzyme were subsequently identified in *Staphylococcus aureus* (18), *Streptococcus pneumoniae* (20) and *Enterococcus faecalis* (21). The distinction of

Class I and Class II HMGR was proposed by Bochar *et al.*, based on protein sequence dissimilarities between eukaryotic HMGR and prokaryotic HMGR (22), and this distinction is also based upon the coenzyme specificity (either NADH, NAD(P)H or NADPH) required for the reaction to occur (reviewed in (23)). Although statins are classically seen as inhibitors of Class I HMGR, they have also been found to inhibit the activity of Class II HMGR of *Pseudomonas mevalonii* (24), *Enterococcus faecalis* (21) and *Streptococcus pneumoniae* (25), and have a weak effect on the HMGR of *Listeria monocytogenes* (26). Interestingly, statins also appear to have an inhibitory effect on bacteria which do not possess HMGR, such as *E. coli* and *P. aeruginosa* (5).

In humans and other mammals, the mevalonate pathway is characterised by the presence of 5 key enzymes, including HMGR (described in Figure 1), and is dependent upon the synthesis of mevalonate, which is then phosphorylated, and converted to the isoprenoid intermediate isopentenyl-5-pyrophosphate (IPP). This compound is subsequently converted to the important intermediate compounds farnesyl-pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP); these compounds are implicated in diverse physiological processes such as proliferation and inflammation due to their interaction with Rho GTPase family proteins (27, 28). Interestingly, an analogous mevalonate pathway to humans has also been identified in *S. aureus*, including the upstream HMG-CoA synthase, and the downstream mevalonate kinase, phosphomevalonate kinase and mevalonate diphosphate decarboxylase (29) (Figure 1). The mevalonate pathway contributes to the synthesis of a family of lipid compounds termed isoprenoids (or terpenoids), which, in bacteria are involved in the synthesis of peptidoglycan and teichoic acid (30, 31) and electron transport (32). In humans, the intermediate compounds isopentenyl-pyrophosphate

(IPP), dimethylallyl-pyrophosphate (DMAPP) and the aforementioned FPP and GPP are produced as a result of mevalonate-dependent isoprenoid biosynthesis and analogues of the enzymes responsible for their production have been described in *S. aureus* isoprenoid biosynthesis (33). However, not all bacteria possess a mevalonate pathway, and in these species (and in plants) isoprenoid metabolism is mediated through the 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP-DOXP) pathway, which is mevalonate-independent (34, 35) (Figure 2). In this pathway, DOXP is synthesised and reduced to MEP, which is eventually converted to IPP and utilised in subsequent isoprenoid biosynthesis.

The objectives of this study were as follows. Firstly, bioinformatic methodology was employed to investigate the prevalence of HMGR in respiratory bacteria, and examine the conservation and organisation of isoprenoid biosynthesis genes in 3 CF-associated species – *Staphylococcus aureus, Streptococcus pneumoniae* and *Burkholderia cenocepacia*. The effect of statins on growth and virulence phenotypes of these species was then characterised using *in vitro* assays. Finally, the potential for statin use in combination therapy with antibiotics was investigated.

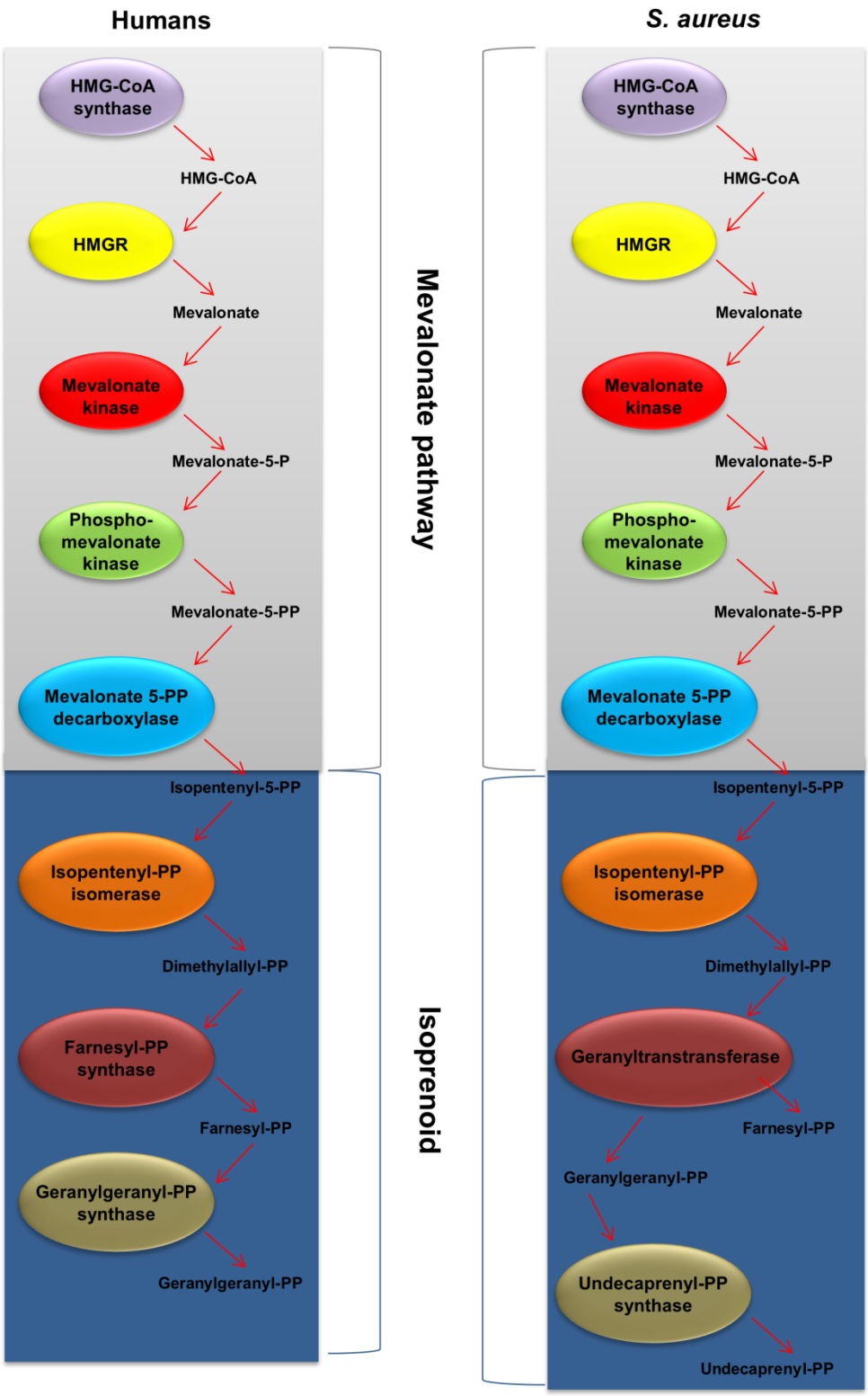


Figure 1: Comparison of the human and bacterial mevalonate pathways. The pathways are presented according to reference genomes for *Homo sapiens* and *S. aureus*. The core mevalonate pathway enzymes are functionally conserved between humans and bacteria. However, differences arise at the level of isoprenoid biosynthesis. In humans,

geranylgeranyl-pyrophosphate (GGPP) is synthesised as a product of farnesyl-pyrophosphate (FPP) catabolism, but in bacteria, these intermediate isoprenoid compounds can be synthesised by the same protein (geranyltranstransferase). Furthermore, in bacteria, GGPP is converted to another isoprenoid compound, undecaprenyl-pyrophosphate.

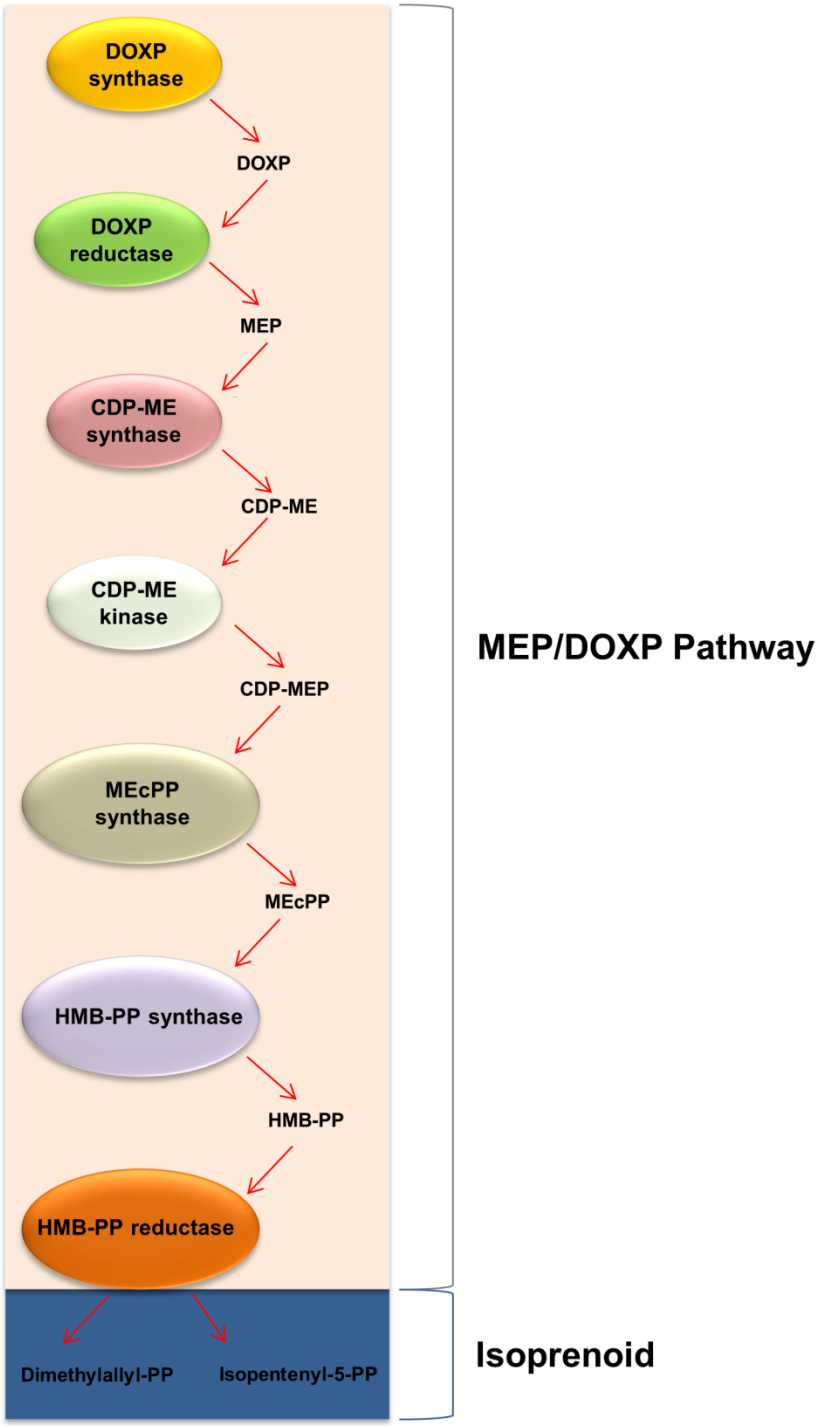


Figure 2: The MEP/DOXP (mevalonate-independent) pathway. This pathway is present in species which do not possess a mevalonate pathway, including plants and many bacterial species. 1-Deoxy-D-xylulose 5-phosphate (DOXP) is synthesised from pyruvate and glyceraldehyde-3-phosphate, following which it is reduced to 2-C-methylerythritol 4-phosphate (MEP). 4-diphosphocytidyl-2-C-methylerythritol (CDP-ME) is synthesised from MEP, and is subsequently phosphorylated (MEcPP) and converted to (E)-4-Hydroxy-3-

methyl-but-2-enyl pyrophosphate (HMB-PP). This compound is then reduced to the isoprenoid intermediate compounds DMAPP and IPP.

Materials and methods

Bacterial strains and growth conditions

Staphylococcus aureus NCDO949 (Shinfield, U. K.) and *Burkholderia cenocepacia* J2315 (BCCM) were cultured in LB broth (tryptone: 10 g/L, yeast extract: 5 g/L, sodium chloride: 5 g/L) at 37 ºC, and *Streptococcus pneumoniae* TIGR4 (ATCC) was cultured in Brain-Heart Infusion (BHI; Sigma-Aldrich, U.K.) at 37 ºC and 5 % CO_2, unless otherwise stated. Simvastatin (Sigma-Aldrich) was resuspended in DMSO at a concentration of 20 mg/ml and stored at 4 ºC. Gentamicin sulphate (Sigma-Aldrich) was resuspended in water at 50 mg/ml. Stock solutions of gentamicin were stored at – 20 ºC and working solutions were stored at 4 ºC.

Bioinformatic analysis

Protein sequences of bacterial HMGR were obtained from NCBI Protein Database (http://www.ncbi.nlm.nih.gov/protein), and were imported into MEGA 5.2.1 (36). Sequences were aligned using ClustalW alignment (37), and the tree-building function of MEGA 5.2.1. was utilised to construct a maximum likelihood tree, using 1000 Bootstrap replications. Mevalonate pathway proteins were defined according to those present in the Kyoto Encyclopedia of Genes and Genomics (KEGG) (38) pathway entry M00095. Homologues of these proteins were identified in *S. pneumoniae* and *B. cenocepacia* using the NCBI BLASTP algorithm, with an E-value cut-off of 1. Genomic organisation of the mevalonate pathway was compared using WebACT (39). The Integrated Microbial Genomes (IMG) Database (40), Prokaryotic Operon Database (ProOpDB) (41) and Search Tool for the Retrieval of

Interacting Genes/Proteins (STRING) Database (42) were all employed in the examination of *B. cenocepacia* HMGR.

Analysis of bacterial growth

S. aureus and *B. cenocepacia* were cultured shaking overnight at 37 ºC, following which overnight cultures were centrifuged at 3220 x g, and washed twice in phosphate-buffered saline (PBS). Pelleted bacteria were then resuspended in Mueller-Hinton broth (Oxoid) and the absorbance at 600 nm (OD_{600nm}) was measured using a Beckman-Coulter UD530 spectrophotometer. Bacteria were then inoculated into a 100 well honeycomb plate (Oy Growth Curves Ab Ltd) at an OD_{600nm} of 0.01, containing 200 µL of Mueller-Hinton with either simvastatin (1 mM, 500 µM, 100 µM, 50 µM, 25 µM, 10 µM and 1 µM) or DMSO (2.09 % (v/v)), or medium only. Plates were then incubated for 24 hours at 37 ºC on a Bioscreen-C automated growth curve system, and the OD_{600nm} of each well was measured at 15 minute intervals for 24 hours. Five technical replicates of each condition were utilised.

For *S. pneumoniae*, bacteria were incubated static in BHI broth at 37 ºC and 5 % CO_2 overnight. The OD_{600nm} of the overnight culture was measured and bacteria were inoculated into a 100 ml conical flask containing 20 ml of BHI supplemented with simvastatin (1 mM, 500 µM, 100 µM, 50 µM, 25 µM, 10 µM and 1 µM) or an equivalent volume of DMSO (2.09 % (v/v)) at an OD_{600nm} of 0.01. A BHI only flask was also prepared in this manner. Bacteria were incubated static at 37 ºC and 5 % CO_2 for 24 hours, after which bacterial growth was examined by measuring the OD_{600nm} of each flask on a Beckman-Coulter UD530 spectrophotometer.

Gentamicin potentiation analysis of *B. cenocepacia* was carried out using 50 μg/ml gentamicin and 50 μM simvastatin. This assay was carried out using the Bioscreen-C system and medium and growth conditions as described above.

Motility assays

Swarming motility of *B. cenocepacia* was analysed using 0.6 % (w/v) Eiken agar (6 g/L Eiken agar, 8 g/L Eiken broth (both from Eiken Chemical, Japan)), supplemented with 0.5 % (w/v) glucose (Sigma-Aldrich). Swimming motility was analysed using 0.4 % (w/v) agar, containing 5 g/L peptone, 5 g/L yeast extract and 2 % (v/v) glycerol. Colony spreading motility of *S. aureus* was examined using 0.24 % (w/v) tryptic soy broth in accordance with the protocol described in (43). All plates were incubated at 37 ºC in an upright position for 24 hours, following which they were photographed with an Epson v750 scanner.

Analysis of bacterial attachment

Bacteria were cultured overnight at 37 ºC. The OD_{600nm} of overnight cultures was measured, and bacteria were inoculated into a 24 well plate containing 1 ml LB broth (*S. aureus* and *B. cenocepacia*) or BHI broth (*S. pneumoniae*) supplemented with simvastatin or DMSO where appropriate, at an OD_{600nm} of 0.1. Plates were incubated static at 37 ºC for 8 hours (*S. aureus*) or 24 hours (*B. cenocepacia* and *S. pneumoniae*), after which medium was removed. Wells were washed 3 times using LB broth or BHI broth as appropriate, and attached bacteria were stained using crystal violet (0.1 % w/v) for 15 minutes. Excess dye was removed by washing wells

5 times with water, and remaining dye was solubilised using 96 % ethanol for 15 minutes. 100 µL of solubilised dye was transferred to a 96 well plate, and attachment was measured by quantifying the OD_{570nm} using a SpectraMax Plus 384 96 well plate reader (Molecular Devices).

Statistical analysis

Statistical significance was calculated using a paired T-test where appropriate.

Results

Distribution of HMGR in respiratory bacterial and fungal pathogens

The objective in this study was to test the effects of statins on a selection of CF-associated bacterial species. In order to identify target organisms for this investigation, the prevalence of the rate-limiting mevalonate pathway enzyme HMGR in respiratory bacterial and fungal pathogens was analysed. Statins inhibit HMGR function, and it was hypothesised that bacteria possessing an analogue of this enzyme would be susceptible to statin treatment. The NCBI Protein Database (http://www.ncbi.nlm.nih.gov/protein) was mined to determine which respiratory bacteria possessed HMGR. Protein sequences were aligned using ClustalW and aligned data was used to construct a Neighbour-joining phylogenetic tree (Figure 3). The sequence of HMGR from *Pseudomonas mevalonii* was also inputted into this analysis, because the HMGR of this species has been found to be inhibited by statins binding to its active site in a manner similar to human HMGR (24), and it was therefore hypothesised that bacteria possessing HMGR with closely conserved sequences to that of *P. mevalonii* may be inhibited by statins.

According to this analysis, the respiratory pathogen with the closest conserved HMGR to that of *P. mevalonii* was that of *Burkholderia cenocepacia*. Interestingly, 4 other species of the genus *Burkholderia* were tested, and these were grouped together, but this cluster did not include *B. cenocepacia*. HMGR was highly conserved within this cluster of *Burkholderia* strains, suggesting that *B. cenocepacia* has a distinctive HMGR sequence and structure compared to other members of its genus, and thus it may be more susceptible to statin treatment. The HMGR enzymes of staphylococci and streptococci were found to form 2 clusters; however,

examination of adjacent nodes suggested that the HMGR of *Enterococcus faecalis* was related to staphylococcal HMGR, while the HMGR of the Gram negative rod *Leptotrichia buccalis* was grouped with streptococcal HMGR. The HMGR of *Nocardiopsis alba* (Gram positive) and *Bordetella petrii* (Gram negative) were also present on another node, thus suggesting that there is no significant sequential difference between the HMGR of Gram positive and Gram negative bacteria. Three other nodes were noted – fungal species, *Legionella* species, and *Coxiella burnetti* and *Nocardia brasiliensis*. The significance of this analysis lies in examining the proximity of putative and uncharacterised HMGR to those that are known to be functional. For instance, the adjacent locations of the *N. alba*/*B. petrii* node and the *P. mevalonii*/*B. cenocepacia* node suggests that statins may be able to efficiently inhibit function of the uncharacterised HMGR from these former species.

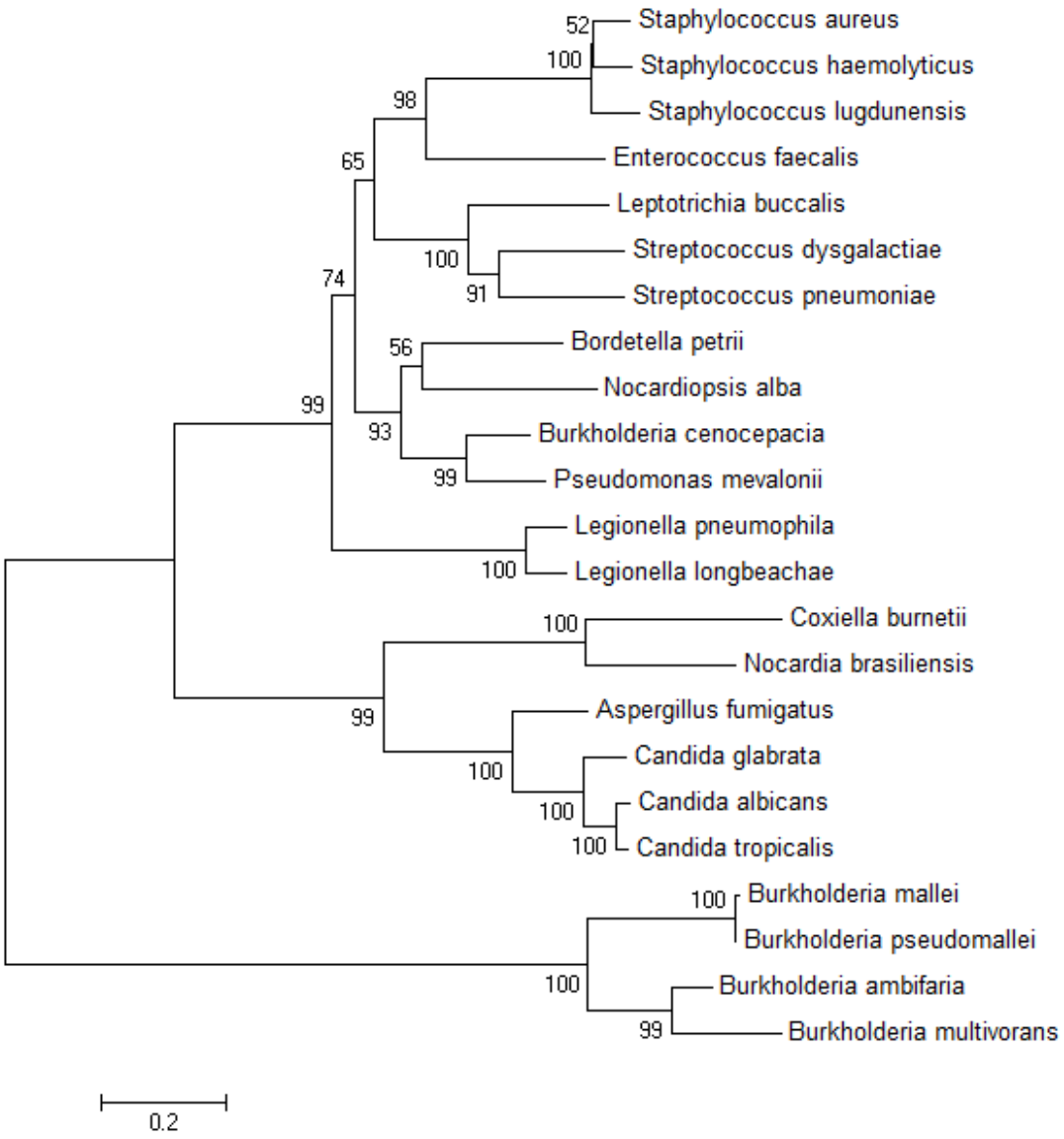


Figure 3*:* Neighbour-joining phylogenetic tree of HMGR protein sequences from bacterial and fungal respiratory pathogens. The HMGR sequences of respiratory pathogens were acquired using the NCBI protein database and aligned using a neighbour-joining algorithm and 1000 Bootstrap replications. The evolutionary distances observed in this tree demonstrate that a high level of conservation is generally exhibited amongst bacterial and fungal HMGR proteins.

Phylogenetic analysis also demonstrated the notable absence of HMGR in several significant respiratory bacteria, amongst them *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Bordetella pertussis*. The KEGG Pathway Database (http://www.kegg.jp/kegg/pathway.html) was employed to examine whether respiratory bacteria favoured mevalonate-dependent or mevalonate-independent metabolism, yielding some interesting information (Table 1). Firstly, it appeared that Gram positive respiratory pathogens mostly possess a mevalonate pathway, but Gram negative bacteria tend to favour mevalonate-independent isoprenoid metabolism. However, some species which do not possess a mevalonate pathway but utilise the MEP/DOXP pathway – *B. petrii, B. cenocepacia,* and *N. alba* – were also found to possess HMGR. The role of mevalonate in these organisms is currently unclear; it has been suggested that the HMGR of *Aspergillus fumigatus* (which possesses a complete mevalonate pathway) is linked to siderophore production (44), and it is possible that the "orphan" HMGR of these species could play a similar role. Finally, despite the fact that the mevalonate and MEP/DOXP pathways generally appeared to be mutually exclusive, *Nocardia brasiliensis* was found to possess homologues of both pathway genes, suggesting that statins may not be particularly effective against this species.

Table 1: Isoprenoid metabolism of respiratory bacterial pathogens. The KEGG database was mined to examine the mechanisms of isoprenoid biosynthesis present in respiratory bacteria. Some species were found to possess HMGR as well as non-mevalonate pathway genes.

Species	Mevalonate	Non-mevalonate	HMGR
Gram positive			
Coxiella burnetti	+	-	+
Enterococcus faecalis	+	-	+
Legionella pneumophila	+	-	+
Mycobacterium tuberculosis	-	+	-
Nocardia brasiliensis	+	+	+
Nocardiopsis alba	-	+	+
Staphylococcus aureus	+	-	+
Streptococcus pneumoniae	+	-	+
Gram negative			
Acinetobacter baumannii	-	+	-
Bordetella pertussis	-	+	-
Bordetella petrii	-	+	+
Burkholderia cenocepacia	-	+	+
Haemophilus influenzae	-	+	-
Leptotrichia buccalis	+	-	+
Pseudomonas aeruginosa	-	+	-
Stenotrophomonas maltophilia	-	+	-

Conservation and genomic organisation of the mevalonate pathway in CF-associated pathogens

Based on phylogenetic analysis, it appeared that HMGR was present in a number of pathogens which cause chronic respiratory infection in individuals with the recessive genetic disorder cystic fibrosis (CF). From this subset of CF-associated pathogens, 3 respiratory bacterial pathogens were chosen for further analysis – *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Burkholderia cenocepacia.* All 3 of these species are prominent causes of opportunistic and nosocomial infections in cystic fibrosis (CF) patients (45–47). It was hypothesised that statins could potentially be used as a novel treatment against these bacteria, and the potential impact of statins on these micro-organisms was initially examined by exploiting bioinformatic methodologies.

Initially, the sequence conservation of HMGR in the test strains was analysed and compared to the sequence of the 428 amino acid (aa) *P. mevalonii* HMGR. Protein sequences of HMGR were obtained from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein), and were aligned using the ClustalW algorithm. This analysis demonstrated that there was a high degree of homology between the HMGR protein sequences, with 103 aa being present in the same predicted locations in all 4 sequences. Class II HMGR are known to have at least 3 regions which are essential for their catalytic function (48, 49). In *P. mevalonii*, these catalytic domains are present as follows: ENVIG (starting at aa position 52), DAMGAN (starting at aa 183) and GLVGG (starting at aa 326). *S. aureus* HMGR is also known to possess these catalytic domains (18), and the sequence conservation of HMGR in *S. pneumoniae* and *B. cenocepacia* was examined to determine whether these essential domains were conserved within these sequences. This analysis

revealed that regions with high homology to the 3 conserved catalytic domains of *S. aureus* and *P. mevalonii* were also present in the HMGRs of *S. pneumoniae* and *B. cenocepacia* (Figure 4). The sequences of the putative catalytic domains of *B. cenocepacia* HMGR were identical to those of *P. mevalonii*, and were located in the same aa position within the sequences. The 3 putative catalytic domains of *S. pneumoniae* HMGR were altered by at least 1 aa change compared to both *S. aureus* and *P. mevalonii*. The first domain observed was ENVVG, compared to ENVIA of *S. aureus* and ENVIG of *P. mevalonii*. The DAMGAN domain present in the other test strains was altered to EAMGAN in *S. pneumoniae* and the third *S. pneumoniae* domain (ATKGG) differed by 2 aa from those identified in *S. aureus* (AIVGG) and *P. mevalonii*/*B. cenocepacia* (GLVGG), but was present at the same aa position (324) as the third *S. aureus* domain. As well as the catalytic domains, a high degree of homology was observed between the HMGR protein sequences, with 24 % of aa (103 aa) being present in the same predicted locations in all 4 sequences. In addition, conserved long motifs at positions 81-92 and 255-275, and shorter conserved regions were also present towards the C-terminus region of the sequences were also identified. Significantly, it was noted that the HMGR of *B. cenocepacia* had a high degree of homology to that of *P. mevalonii* (72 %), while the HMGR of *S. pneumoniae* had much lower, but approximately equal levels of identity to those of *S. aureus* (43 %) and *P. mevalonii* (42 %). Given that 42 % sequence identity exists between *S. aureus* and *P. mevalonii* HMGR, and that statins can inhibit the HMGR of both *P. mevalonii* and *S. pneumoniae* (24, 25), it was thus proposed that statins may potentially have an equal binding affinity for *B. cenocepacia*.

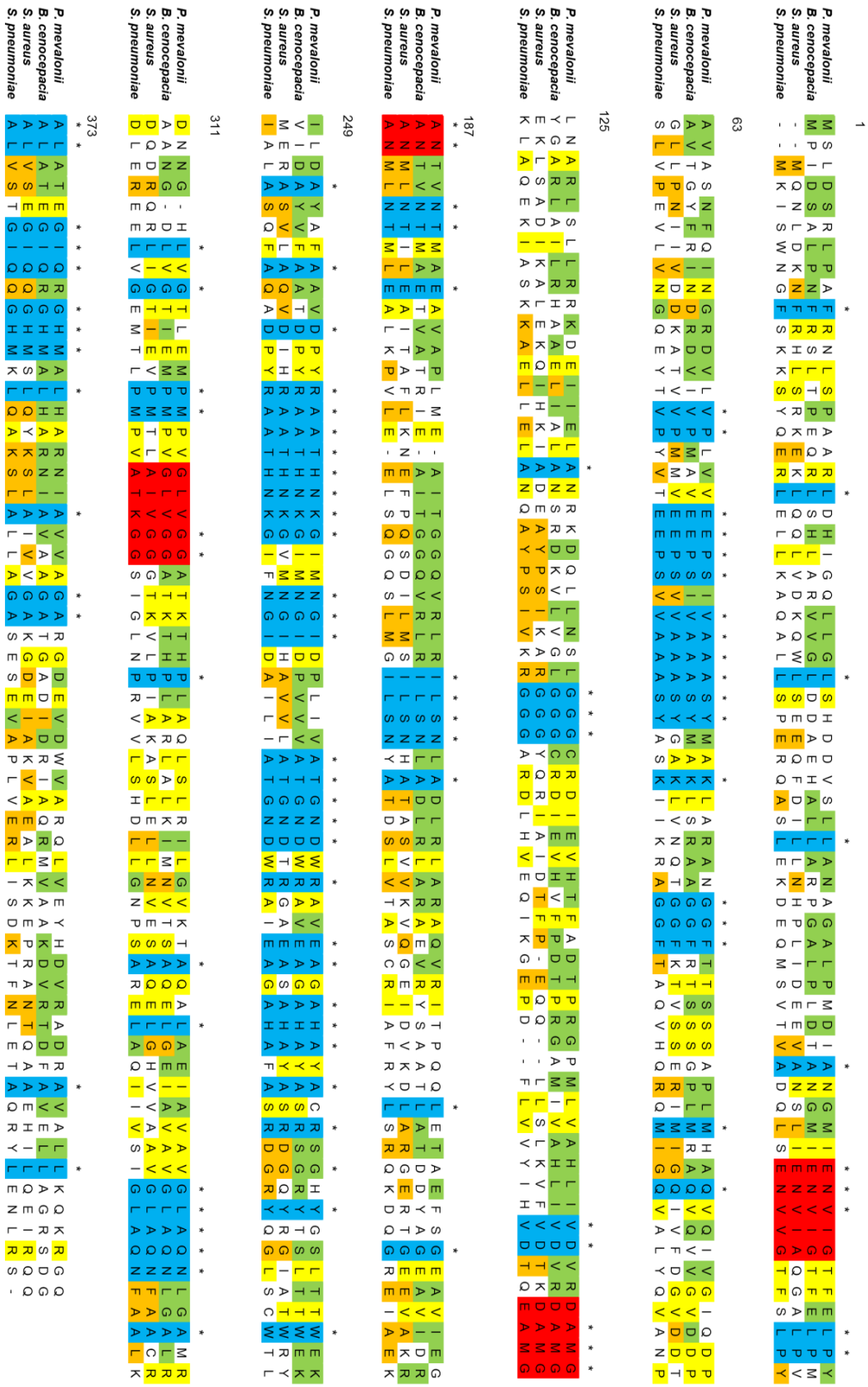


Figure 4: Conservation of catalytic domains and amino acid residues of bacterial HMGR. The HMGR enzymes of *P. mevalonii* and *S. aureus* contain 3 conserved domains

which are essential for their catalytic activity. These residues are also conserved in the HMGR of *S. pneumoniae* and *B. cenocepacia*. Key to colours: red = catalytic domains, blue = conserved residue in all 4 sequences, yellow = conserved residue in 3 sequences, orange and green = conserved residue in 2 sequences. * indicates conserved aa in all 4 sequences.

The conservation of mevalonate pathway proteins in the selected species was subsequently examined. Several mevalonate pathway and isoprenoid biosynthesis genes have previously been characterised in *S. aureus* and *S. pneumoniae* (29, 33). However, the pathway of *B. cenocepacia* has not yet been studied, and sequence information is not available on the *P. mevalonii* mevalonate pathway. The NCBI BLASTP algorithm was used to identify homologues of *S. aureus* mevalonate pathway proteins from the reference genome NCTC 8325 in *S. pneumoniae* TIGR4 and *B. cenocepacia* J2315. The proteins involved in the mevalonate pathway were defined according to those listed in the KEGG Reference pathway M00095 and by Balibar *et al.,* (33); in total 8 proteins were analysed including HMGR. The results of this analysis are detailed in Table 2. *S. pneumoniae* was found to possess homologues of all 8 *S. aureus* mevalonate pathway and isoprenoid biosynthesis proteins. Examination of *B. cenocepacia* did not yield any obvious mevalonate pathway homologues; however, interestingly, this species was found to possess homologues of the isoprenoid biosynthesis proteins geranyltranstransferase and undecaprenyl-pyrophosphate synthase. The mevalonate pathway was previously found to be absent in *B. cenocepacia* (Table 1), and it is likely that the *B. cenocepacia* isoprenoid biosynthesis homologues metabolise the products of the MEP/DOXP pathway in this species.

Table 2: Homology of mevalonate pathway genes in *S. pneumoniae* and *B. cenocepacia*.

The 8 mevalonate pathway genes of *S. aureus* were tested against the genomes of *S. pneumoniae* and *B. cenocepacia* using BLASTP, and the top hit for each gene is detailed here along with its query coverage (QC), E-value and identity.

	S. pneumoniae				***B. cenocepacia***			
	Name	**QC**	**E-value**	**Identity**	**Name**	**QC**	**E-value**	**Identity**
HMG-CoA synthase	HMG-CoA synthase (NP_346164.1)	100 %	2e-116	48 %	3-oxoacyl-(acyl carrier protein) synthase III (YP_002230146.1)	28%	0.005	26%
HMG-CoA reductase	HMG-CoA reductase (NP_346163.1)	97 %	5e-110	43%	HMG-CoA reductase (YP_002233154.1)	93%	7e-93	41%
Mevalonate kinase	Mevalonate kinase (NP_344908.1)	99 %	5e-45	35%	Putative patatin-like phospholipase (YP_002231254.1)	16 %	1.0	36%
Phosphomevalonate kinase	Phosphomevalonate kinase (NP_344910.1)	97 %	2e-27	28%	Putative transport membrane protein (YP_002235068.1)	18 %	0.27	26 %
Diphosphomevalonate decarboxylase	Diphosphomevalonate decarboxylase (NP_344909.1)	89 %	2e-63	40%	CMK (B4EAR4.1)	25 %	0.27	25%
Isopentenyl PP isomerase	Isopentenyl PP isomerase (NP_344911.1)	91 %	3e-84	40%	Putative FMN-dependent dehydrogenase (YP_002234876.1)	23 %	0.019	26%
Geranyltranstransferase	Geranyltranstransferase (NP_345672.1)	88 %	4e-61	46 %	Putative geranyltranstransferase (YP_002233528.1)	97 %	2e-57	39 %
Undecaprenyl-PP synthase	Undecaprenyl pyrophosphate synthase (NP_344799.1)	90 %	4e-90	53%	Undecaprenyl pyrophosphate synthetase (YP_002231213.1)	92 %	2e-70	41%

Genomic localisation and organisation of the genes involved in the mevalonate pathway in *S. aureus*, *S. pneumoniae* and *B. cenocepacia* were compared using the genomic comparison tool WebACT. This analysis (detailed in Figure 5) yielded some surprising information, in that the relative genomic organisation of mevalonate pathway and isoprenoid biosynthesis genes differed between *S. pneumoniae* and *S. aureus*, despite their sequential similarities. Mevalonate synthase (*mvaS*) and HMGR (*mvaA)* were located furthest downstream in both species, but while these genes appeared to be present in an operon on the complement strand of the *S. pneumoniae* genome, in *S. aureus* they did not appear to be co-regulated, and were divergently expressed. A striking similarity between both species was that mevalonate kinase (*mvaK1*), phosphomevalonate kinase (*mvaK2*) and mevalonate diphosphate decarboxylase (*mvaD*) were present in predicted operons on the positive strand in both species. However, a key difference was that the *S. pneumoniae* operon contained a fourth gene, isopentenyl-diphosphate isomerase (*fni*), whereas this gene was located upstream of *mvaA* (by 217288 base pairs (bp)), and was present in the opposite orientation to the predicted *mvaK* operon in *S. aureus*. A difference was also observed in the relative locations of *fni* and farnesyl-pyrophosphate synthase (*ipsA*) – in *S. aureus*, *fni* was present in the same orientation as *ipsA* with a large distance (868753 bp) separating them; however in *S. pneumoniae* these genes were present in opposite orientations (*ipsA* on the complement strand) 773003 bp apart. The final isoprenoid gene studied, undecaprenyl pyrophosphate synthase (*uppS*) was was located 127178 bp upstream of the *mvaK1* operon of *S. pneumoniae*, but was located 601974 bp downstream of the *S. aureus mvaK1* operon. For *B. cenocepacia,* the 3 homologous proteins identified in Table 2 were present on 2 different chromosomes. *uppS* was located on Chromosome 1, whereas *mvaA* and *ispA* were

located 410011 bp apart on the positive strand on Chromosome 2. This correlated with the *S. pneumoniae mvaA* and *ipsA* homologues, which were separated by a similar distance (490800 bp). The differences in relative genomic locations of mevalonate pathway and isoprenoid biosynthesis genes suggested that the expression of these genes was differentially regulated between each species, which in turn suggested that each species might have a different susceptibility to any effects exerted by statins.

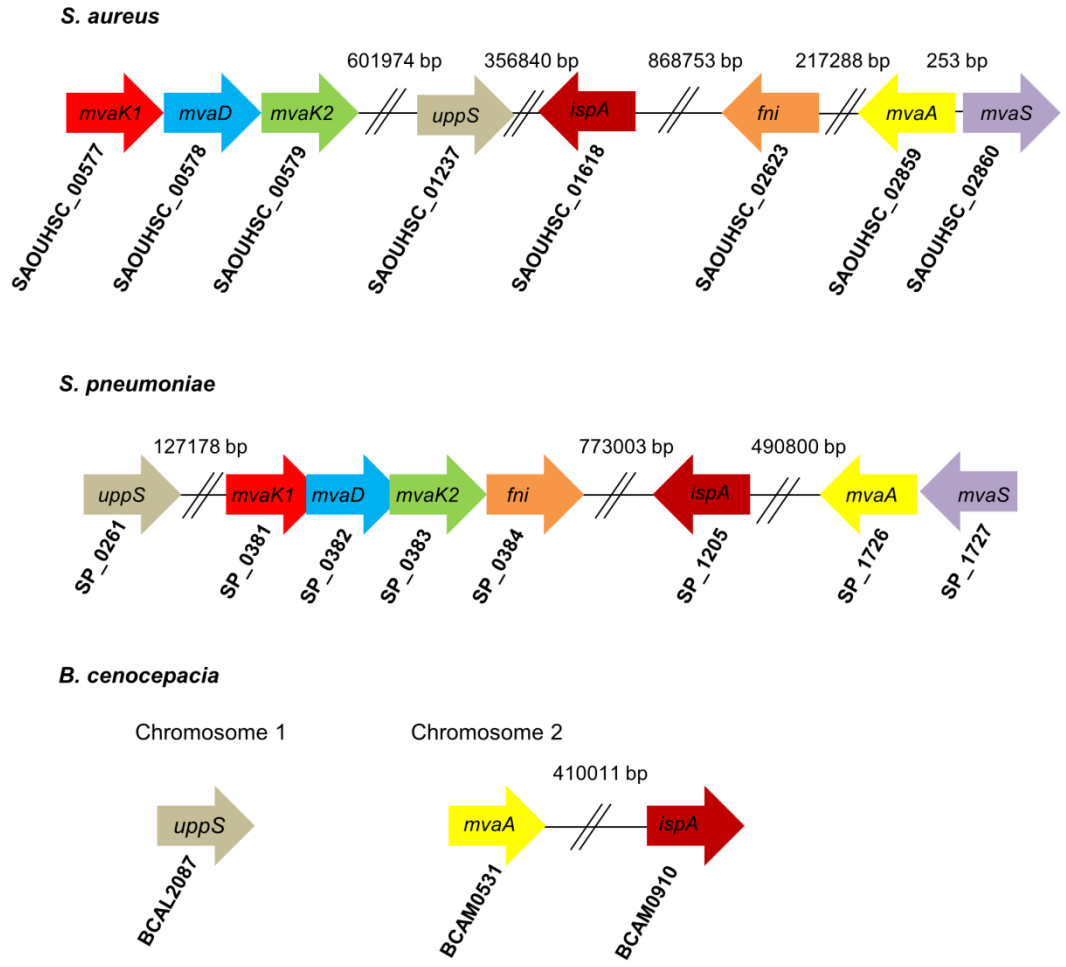


Figure 5: Genomic organisation of mevalonate pathway genes in *S. aureus*, *S. pneumoniae* and *B. cenocepacia*. The mevalonate pathway in *S. aureus* is composed of 1 operon and 4 downstream genes which appear to be under their own regulatory systems. *S. pneumoniae* possesses 2 mevalonate pathway operons, and 1 distally-located upstream gene. The 3 mevalonate pathway homologues of *B. cenocepacia* are located in different orientations on different chromosome. Gaps are not presented to scale.

As *B. cenocepacia* possesses an "orphan" HMGR (without other mevalonate pathway genes), further tools were utilised in an attempt to elucidate the function of HMGR and mevalonate in this organism. Firstly, the Gene Neighbourhood function of the IMG database was used to examine the chromosomal area surrounding the *B. cenocepacia mvaA* gene (BCAM0531) (Figure 6A). BCAM0531 lies in close proximity to 2 downstream hypothetical proteins (BCAM0532 and BCAM0533), while the upstream adjacent protein (BCAM0530) is a GntR-type regulator. BCAM0532 has no predicted products, whereas BCAM0533 has homology to a tricarboxylate transporter protein. The ProOpDB tool has predicted that BCAM0531 is not part of an operon with any of its neighbouring genes.

The STRING database was utilised to examine putative interaction networks of *B. cenocepacia mvaA*. This analysis yielded a number of potential functional partners of BCAM0531, the results of which are detailed in Figure 6B. The top 10 predicted partners were identified based on their proximity to BCAM0531 (neighbourhood) and textmining (the co-occurrence of protein names in literature). This algorithm predicted that BCAM0531 was part of a circuit with its neighbouring genes BCAM0530, BCAM0532 and BCAM0533. BCAM0531 was also predicted to be part of 2 tripartite regulatory loops. The first of these was with HisB and HisD, 2 proteins which are involved in the biosynthesis of histidine, while the second encompassed PheA (responsible for phenylalanine biosynthesis) and the ribosomal protein RplT. Other predicted partners of BCAM0531 were the gluconate reductase DkgB, the tRNA synthase BCAL2169, and BCAM1883, of which the function is unknown. These putative interactions seem to illustrate a metabolic role for BCAM0531, particularly in the context of amino acid biosynthesis.

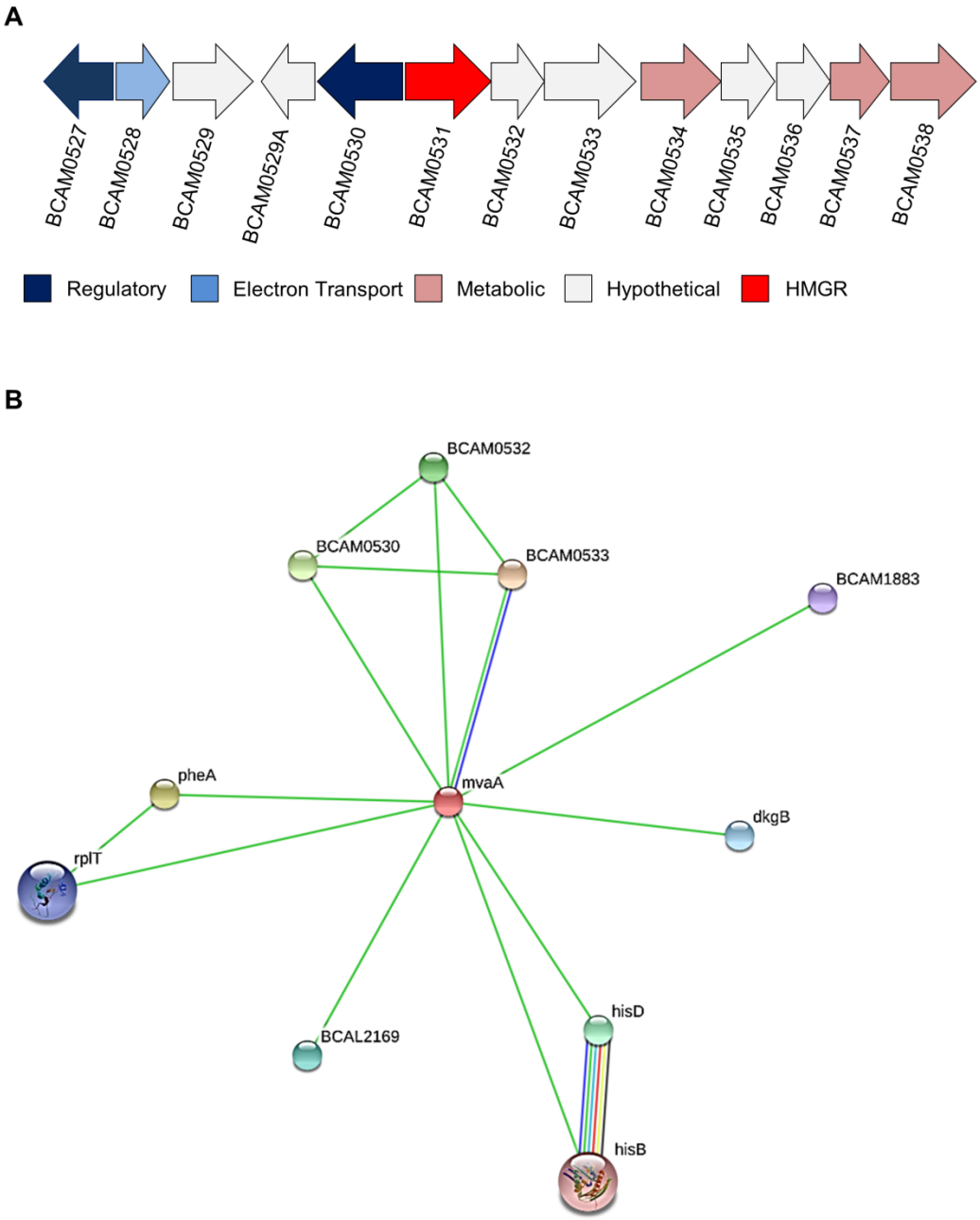


Figure 6: Analysis of the *B. cenocepacia* HMGR BCAM0531. (A) BCAM0531 is present on Chromosome 2 of J2315, where it is surrounded by mostly hypothetical or metabolic genes. **(B)** STRING database demonstrated putative interactions of *mvaA* (BCAM0531) with its neighbouring genes BCAM0530, BCAM0532 and BCAM0533, as well as several metabolic proteins (notably PheA, HisB, HisD and DkgB).

Simvastatin has distinct effects on bacterial growth levels and kinetics

Based on bioinformatic analysis of the mevalonate pathway in *B. cenocepacia*, *S. aureus* and *S. pneumoniae,* it was hypothesised that statins may have differing effects on the growth of these species. Therefore, the effect of simvastatin on bacterial growth was analysed by culturing bacteria in the presence of varying simvastatin concentrations (1 mM, 500 μM, 100 μM, 50 μM, 25 μM, 10 μM and 1 μM) for 24 hours, and comparing their growth to that of bacteria cultured in the presence of an equivalent DMSO vehicle control, and in medium alone. The first observation made in this study was that DMSO (2.09 % (v/v)) reduced the growth of *B. cenocepacia* (Figure 7A), *S. aureus* (Figure 8A) and *S. pneumoniae* (Figure 9A) compared to levels observed in medium alone. After 24 hours of growth, this difference was statistically significant for *B. cenocepacia* (P = 0.015), but a significant difference was not detected for *S. aureus* and *S. pneumoniae*.

The growth of *B. cenocepacia* was inhibited by 1 mM, 500 μM and 100 μM simvastatin, but this effect was not bactericidal. 1 mM simvastatin inhibited *B. cenocepacia* growth at 8 hours post inoculation (hpi) and all subsequent timepoints, with statistically significant effects exerted at 16 hpi (P = 0.004) and 24 hpi (P =0.043) (Figure 7B). Simvastatin was still effective against *B. cenocepacia* at half of this concentration (500 μM), which reduced bacterial growth at 12 hpi and subsequent timepoints, and similarly to 1 mM simvastatin, a significant effect was exhibited by 500 μM at 16 hpi (P = 0.01) (Figure 7C). 100 μM simvastatin was also capable of significantly decreasing *B. cenocepacia* at 16 hpi (P = 0.03), and also caused moderate but not significant decreases in growth at 12, 20 and 24 hpi (Figure 7D). 50 μM and lower simvastatin concentrations had no statistically significant effect on the kinetics of *B. cenocepacia* growth (Figure 7E-H). 25 μM simvastatin

had a slight bacteriostatic effect at 12, 16, 20 and 24 hpi, but this effect was not statistically significant and was less reproducible than the effects achieved at higher concentrations (Figure 7F). These data demonstrated that high statin concentrations were required to achieve significant inhibition of *B. cenocepacia* growth, and that inhibitory concentrations of simvastatin appeared to be most effective against *B. cenocepacia* at 16 hpi, which equated to mid to late log phase in the context of this study.

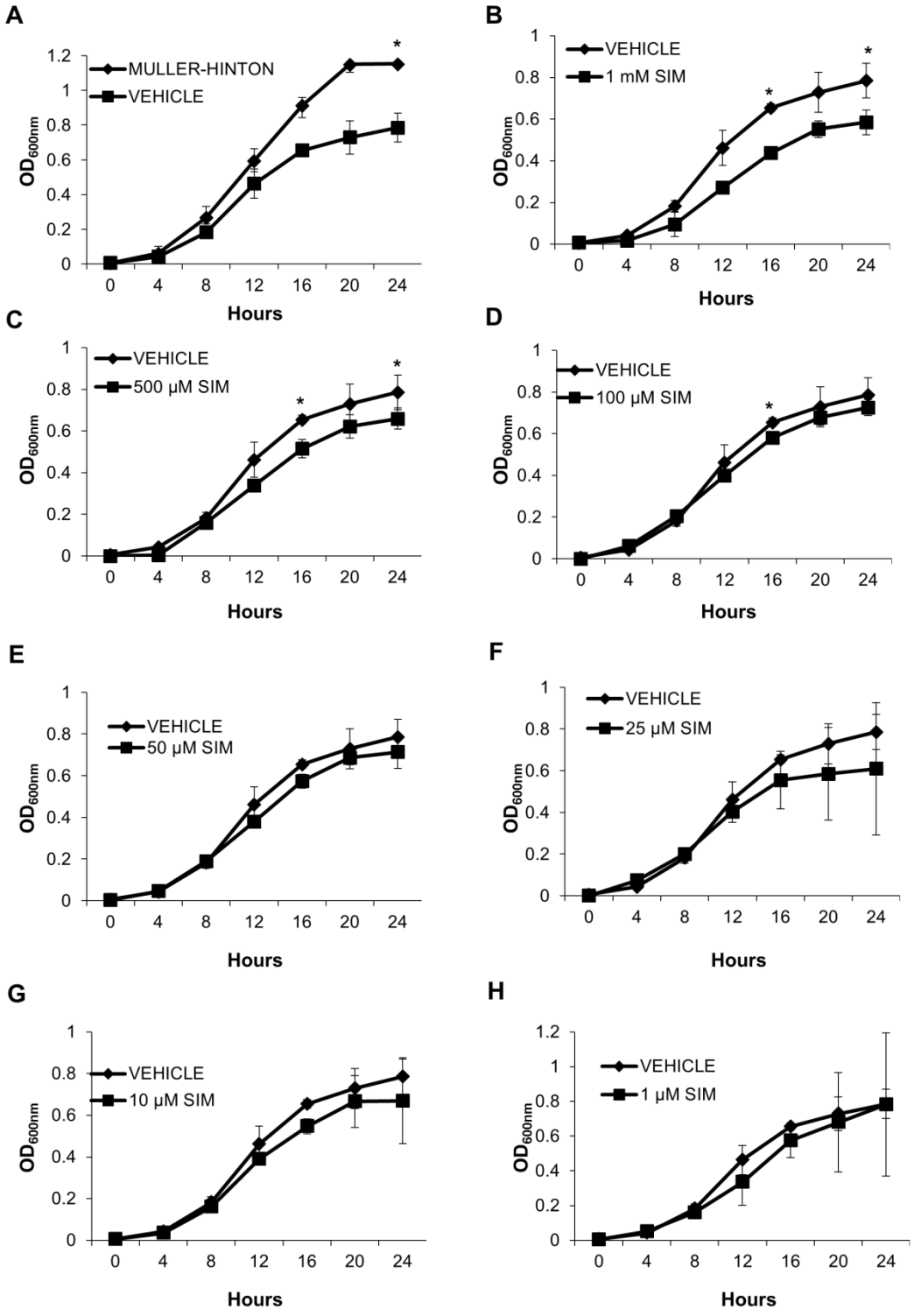


Figure 7: Effect of simvastatin on growth of *B. cenocepacia*. (A) Effect of vehicle on bacterial growth. Bacteria were cultured in Muller-Hinton only, or were treated with a DMSO volume (2.09 % (v/v)) which was equivalent to that of 1 mM simvastatin. **(B) to (H)** Bacteria were cultured in the presence of 1 mM, 500 µM, 100 µM, 50 µM, 25 µM, 10 µM

and 1 μM simvastatin and the level of growth achieved at 4 hour intervals was compared to that of bacteria treated with the vehicle. Data presented are the average and standard deviation of 3 biological replicates. * indicates a statistically significant difference between conditions.

Simvastatin also reduced the growth of *S. aureus*, although the kinetic pattern seen here was different to that of *B. cenocepacia*. 1 mM simvastatin significantly inhibited the growth of *S. aureus* compared to the DMSO control at 4 hpi, (P = 0.047) and 8 hpi (P = 0.04), and non-significantly reduced growth at 12 hpi, 16 hpi and 20 hpi; however, no change was observed at 24 hpi (Figure 8B). An interesting trend was observed in the presence of 500 μM simvastatin – at this concentration, significant inhibitory effects were observed at 4 hpi (P = 0.016) and 8 hpi (P = 0.013) and a non-significant reduction was observed at 12 hpi, but no change was observed at 16 hpi, and at subsequent intervals statin-treated bacteria actually grew to marginally greater – albeit non-significant – levels than vehicle-treated bacteria (Figure 8C). The same trend was noted in bacteria cultured with 100 μM simvastatin, but in this instance a significant inhibitory effect was observed at 4 hpi only (P = 0.018) (Figure 8D). These data suggested that simvastatin could inhibit the progression of the exponential phase of *S. aureus* growth in a dose-dependent manner.

Another striking alteration in *S. aureus* growth kinetics was observed in the presence of 50 μM and 25 μM simvastatin. At these concentrations, the growth of *S. aureus* appeared to be *enhanced* by simvastatin compared to the vehicle (Figure 8E and 8F). From 8 hpi onwards, a noticeable and significant increase in growth was observed in bacteria treated with 50 μM simvastatin. An increase was also observed with 25 μM simvastatin at 12 hpi and subsequent timepoints, but a significant increase was present at 12 hpi (P = 0.036) and 16 hpi (P = 0.024) only. Finally, 10 μM and 1 μM simvastatin had no significant effect on the kinetics of *S. aureus* growth (Figure 8G and 8H). Taken together, these data demonstrated that, as opposed to having a bacteriocidal effect on *S. aureus*, simvastatin instead appeared to delay the

progression of bacterial growth into exponential phase at high concentrations (1 mM, 500 μM and 100 μM), but certain sub-inhibitory simvastatin concentrations (50 μM and 25 μM) actually appeared to enhance *S. aureus* growth.

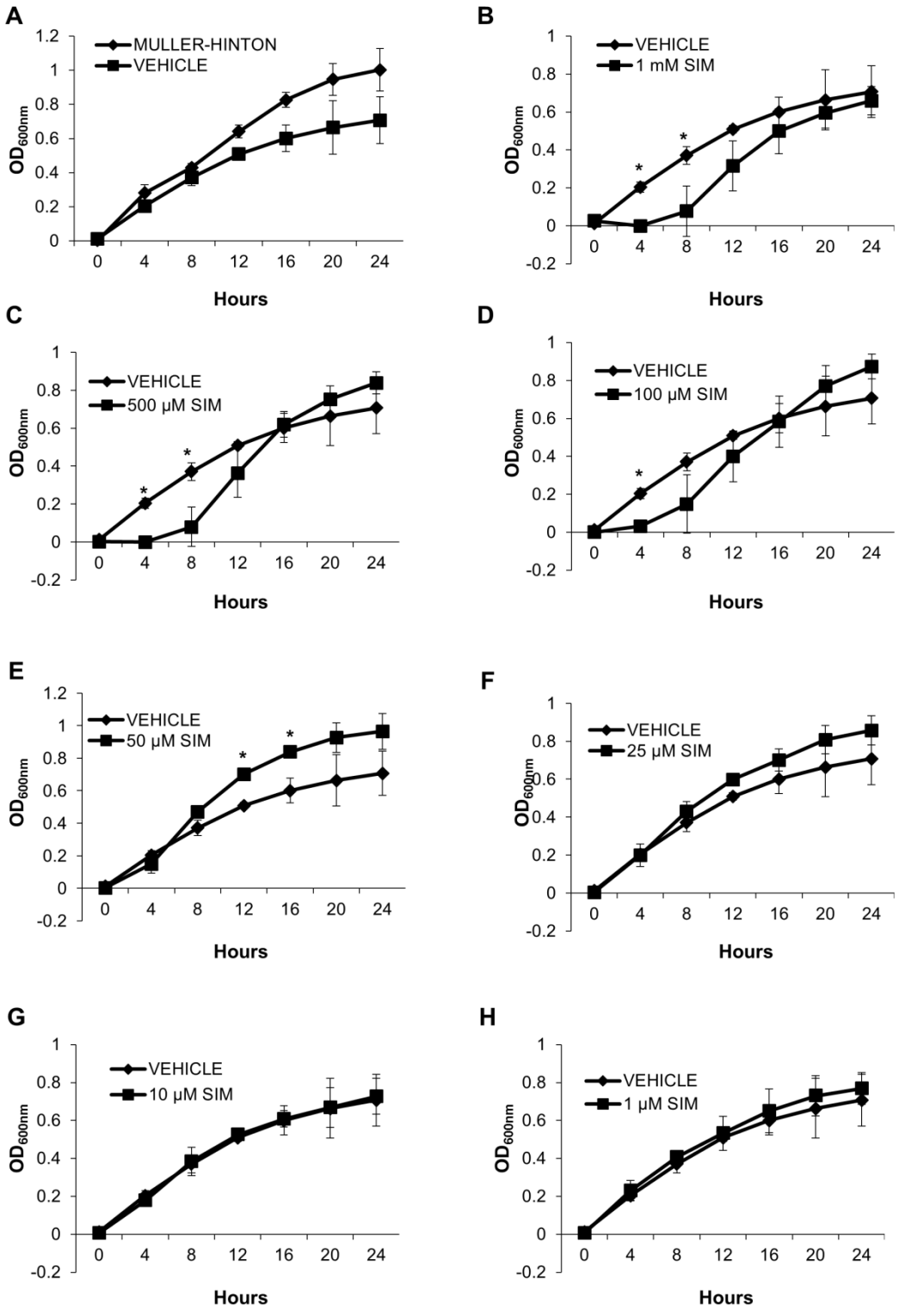


Figure 8: Effect of simvastatin on growth of *S. aureus*. (A) Effect of vehicle on bacterial growth. Bacteria were cultured in Muller-Hinton only, or were treated with a DMSO volume (2.09 % (v/v)) which was equivalent to that of 1 mM simvastatin. **(B) to (H)** Bacteria were cultured in the presence of 1 mM, 500 µM, 100 µM, 50 µM, 25 µM, 10 µM and 1 µM

simvastatin and the level of growth achieved at 4 hour intervals was compared to that of bacteria treated with the vehicle. Data presented are the average and standard deviation of 3 biological replicates. * denotes a statistically significant difference between conditions.

The third organism tested in this study was *S. pneumoniae.* Interestingly, simvastatin was found to have a much greater antibacterial effect on this pathogen than on *B. cenocepacia* and *S. aureus*. In fact, after 24 hours of incubation, simvastatin concentrations of 1 mM, 500 μM, 100 μM and 50 μM had a significant bacteriocidal effect on the growth of *S. pneumoniae* (Figure 9B), with p-values of 0.005 (1 mM), 0.008 (500 μM), 0.004 (100 μM) and 0.0045 (50 μM). 25 μM and 10 μM doses of simvastatin also reduced the growth of *S. pneumoniae* compared to the DMSO control, although these effects were not statistically significant. 1 μM simvastatin did not affect bacterial growth compared to the vehicle control. The inhibition of pneumococcal growth observed in this study was at lower concentrations than have been previously reported.

These data indicated that statins could reduce the growth of all 3 test strains, but demonstrated a markedly increased effect on *S. pneumoniae* compared to *B. cenocepacia* and *S. aureus*. Furthermore, the statin effects appeared to be concentration dependent and to target different phases of bacterial growth.

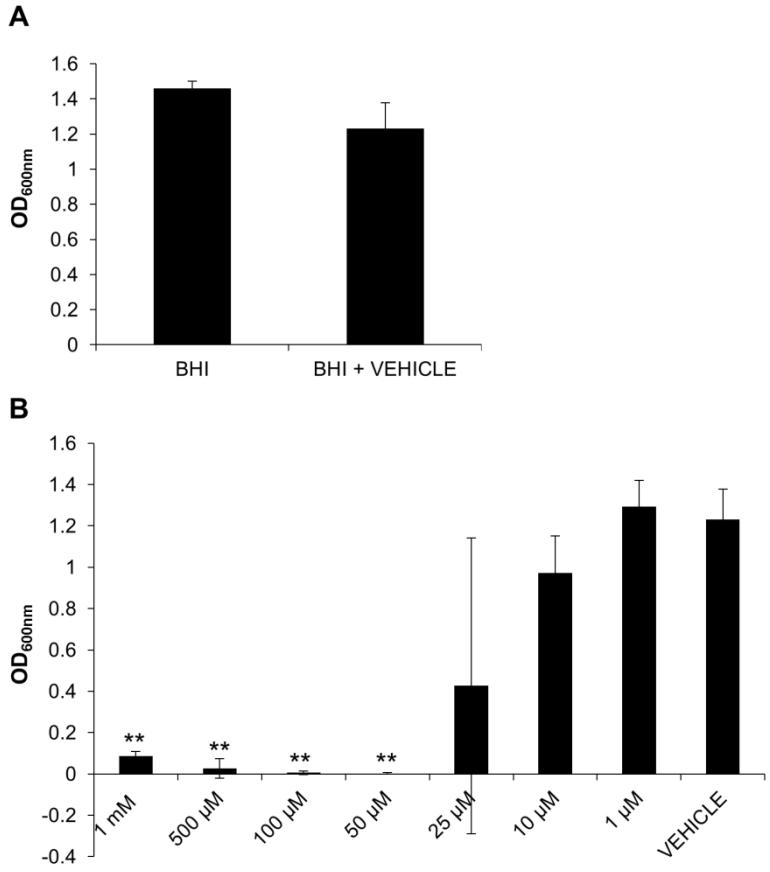


Figure 9: Effect of simvastatin on growth of *S. pneumoniae*. Bacteria were cultured for 24 hours in media alone, in the presence of 1 mM, 500 µM, 100 µM, 50 µM, 25 µM, 10 µM and 1 µM simvastatin or an equivalent vehicle control (2.09 % (v/v) DMSO). **(A)** DMSO did not influence the growth of *S. pneumoniae.* **(B)** 1 mM, 500 µM, 100 µM, and 50 µM simvastatin had a significant bacteriocidal effect on *S. pneumoniae* compared to the vehicle. 25 µM and 10 µM were capable of reducing bacterial growth, while 1 µM had no effect. Data presented are the average and standard deviation of 3 biological replicates. $^{**}P \leq 0.01$.

Effect of simvastatin on bacterial motility

It is well-known that certain compounds can influence the behaviour of bacteria at sub-inhibitory concentrations. For instance, the induction of quorum sensing by sub-inhibitory concentrations of the peptide colistin has previously been demonstrated (50). Therefore, the effect of simvastatin on the virulence phenotypes of the chosen species was investigated, beginning with the examination of motility of *B. cenocepacia* and *S. aureus*. *S. pneumoniae* motility was not examined as the strain utilised in this study was non-motile. *B. cenocepacia* displays 2 types of *in vitro* motility; swimming, which is mediated through the polar flagellum of the bacteria (51), and swarming, which is linked to several factors including quorum sensing and biofilm formation as well as flagellar activity (52). Both of these motility phenotypes were tested at the sub-inhibitory simvastatin concentrations of 10 μM and 25 μM. Neither type of motility was inhibited by simvastatin compared to the vehicle control (Figure 10A). To further elucidate whether statins could affect *B. cenocepacia* motility, assays were also carried out in the presence of 100 μM and 50 μM simvastatin. None of these statin concentrations impacted on the motility of *B. cenocepacia*, indisputably demonstrating that simvastatin does not influence this phenotype.

S. aureus, while strictly non-motile *in situ*, displays an *in vitro* colony spreading motility phenotype which is associated with techoic acid production (43). Colony spreading was tested in the presence of 50 μM, 25 μM and 10 μM simvastatin, which were all found to be sub-inhibitory during growth analysis. 50 μM simvastatin caused a reduction in spreading motility (Figure 10B). However, 25 μM and 10 μM simvastatin did not affect motility, demonstrating that its statin-mediated attenuation was dose-dependent.

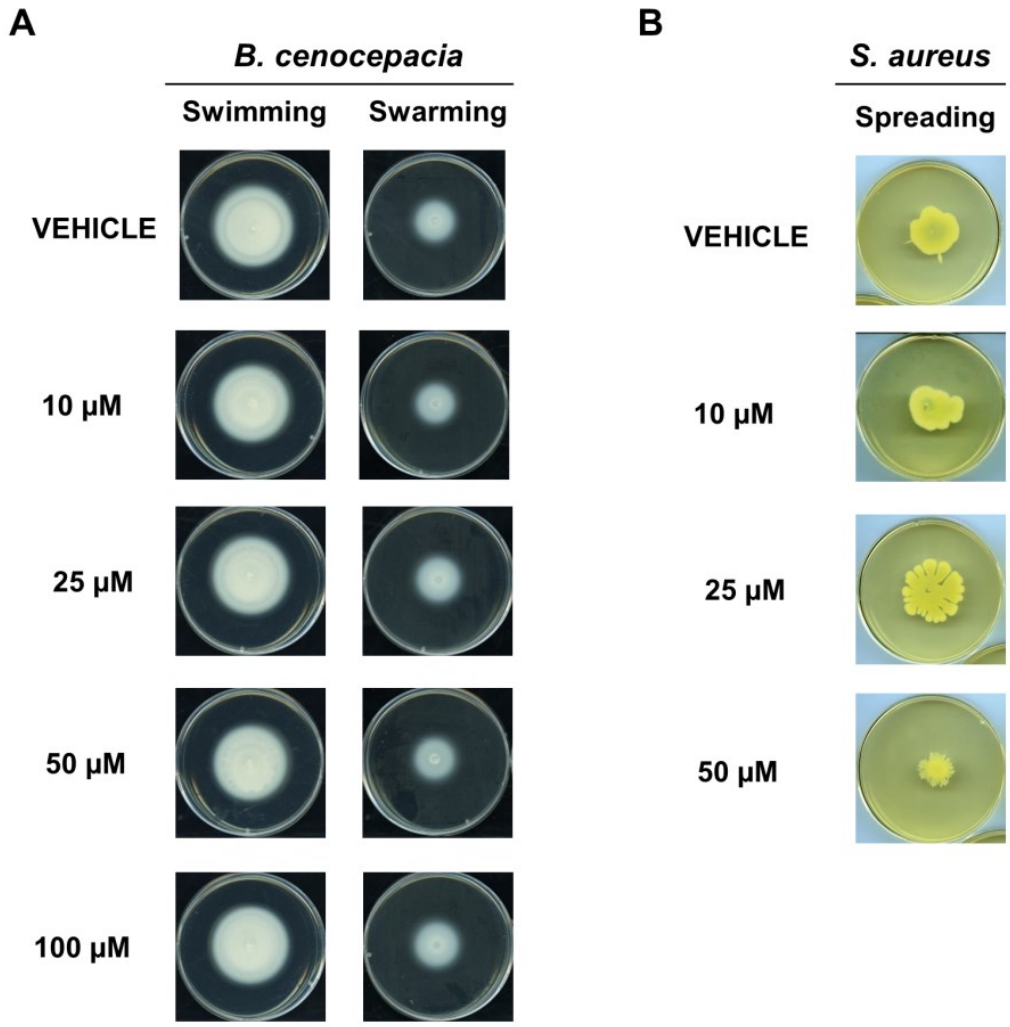


Figure 10: Effect of simvastatin on bacteria motility. *B. cenocepacia* and *S. aureus* were cultured on soft agar in the presence of inhibitory and sub-inhibitory concentrations of simvastatin. **(A)** Swimming and swarming motility of *B. cenocepacia* were not affected by any concentration of simvastatin. **(B)** 50 µM simvastatin reduced spreading motility of *S. aureus*; however lower concentrations were not effective. Data are representative of 3 biological replicates.

Simvastatin influences biofilm production of CF-associated bacteria

Biofilm is another key virulence factor of *B. cenocepacia*, *S. aureus* and *S. pneumoniae,* which contributes to bacterial persistence in CF (recently reviewed in (53)). The effect of simvastatin on biofilm formation was investigated by examining attachment of the 3 test strains in the presence of sub-inhibitory simvastatin concentration compared to an appropriate DMSO vehicle control. In order to fully realise the anti-bacterial potential of simvastatin, its effect on biofilms was tested in accordance with the optimal incubation lengths observed in growth kinetic analysis; these data showed that simvastatin exerted its greatest anti-*Staphylococcus* effect at the beginning of log phase, but had a greater impact on *B. cenocepacia* at late log phase, and impacted *S. pneumoniae* at 24 hours. Therefore, *S. aureus* biofilm formation was tested after 8 hours, while *B. cenocepacia* and *S. pneumoniae* biofilms were cultured for 24 hours. After these periods of incubation attached bacteria were stained using crystal violet, which was resuspended in ethanol in order to quantify the magnitude of attachment. 100 μM simvastatin reduced the attachment of *B. cenocepacia* relative to the vehicle, although this effect was not statistically significant ($P = 0.065$) (Figure 11A). 50 μM did not decrease biofilm formation, and actually appeared to slightly increase attachment compared to the vehicle, but again this effect was not significant. Given that 100 μM simvastatin could also non-significantly reduce the growth of *B. cenocepacia*, the reduction in biofilm at this concentration may have been a result of the growth inhibitory effect of simvastatin. However, the effects observed on *S. aureus* attachment in the presence of simvastatin appeared to occur independently of growth. 100 μM simvastatin (a growth-inhibitory concentration) caused an approximate 5-fold decrease in attachment compared to the vehicle ($P = 0.0077$) (Figure 11B). However, 50 μM simvastatin (sub-inhibitory)

also had an inhibitory, albeit non-statistically significant, effect on *S. aureus* biofilm. 25 μM and 10 μM simvastatin were used in the analysis of *S. pneumoniae* attachment, given that these were the highest statin concentrations which did not obliterate bacterial growth. 25 μM simvastatin significantly attenuated the attachment of *S. pneumoniae* ($P = 0.001$), while 10 μM had a slight inhibitory effect (Figure 11C). However, as these concentrations had a bacteriostatic effect on *S. pneumoniae* growth, the anti-biofilm effect may be attributed to attenuation of growth. Taken together, these data demonstrated that statins had a dose-dependent effect on biofilm formation of CF-associated pathogens, but that sub-inhibitory concentrations were not significantly efficacious.

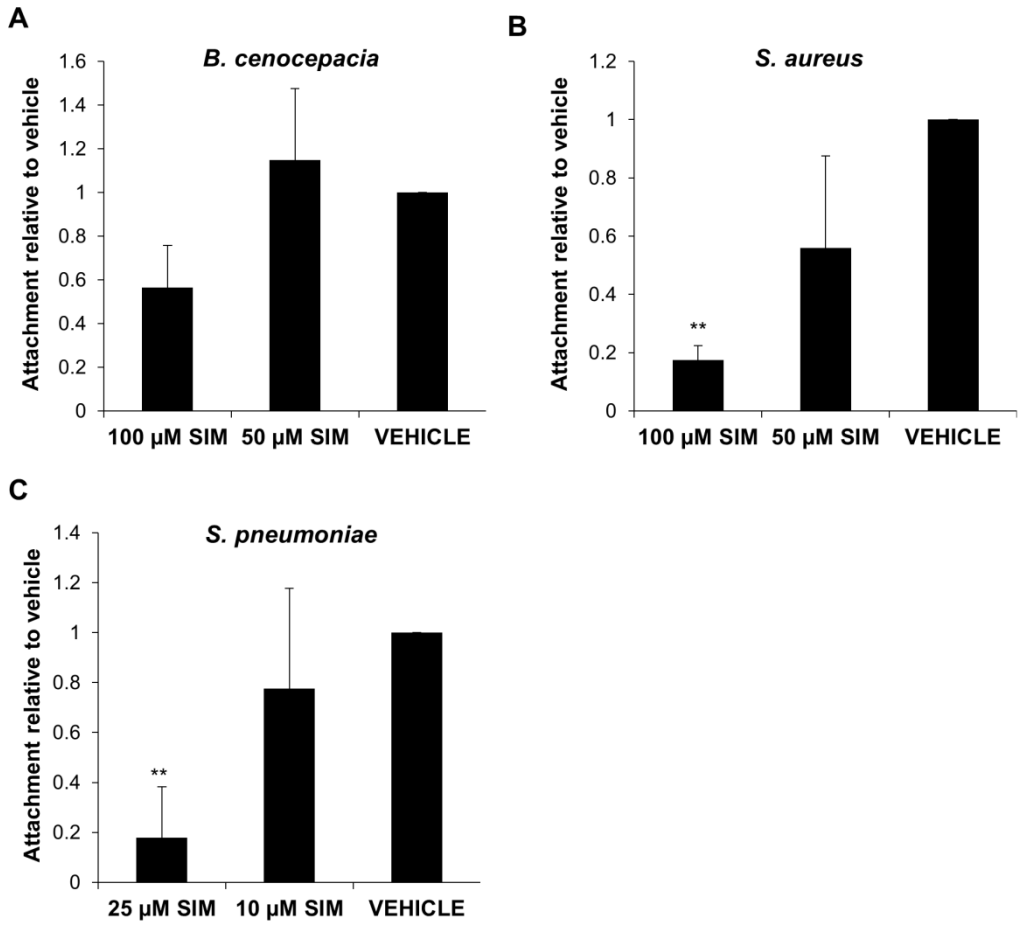


Figure 11: Impact of simvastatin on attachment of CF-associated bacteria. Attachment of A) *S. aureus*, B) *B. cenocepacia* and C) *S. pneumoniae* was measured by crystal violet staining after 8 hours (*S. aureus*) or 24 hours (*B. cenocepacia*, *S. pneumoniae*) of incubation with simvastatin or vehicle control (0.209 % (v/v) DMSO for *B. cenocepacia* and *S. aureus*, 0.052 % (v/v) for *S. pneumoniae*). **(A)** 100 µM reduced *B. cenocepacia* attachment, while 50 µM had no inhibitory effect. **(B)** *S. aureus* attachment was reduced by both 100 µM and 50 µM simvastatin. 100 µM had a significant effect ($P = 0.0077$). **(C)** 25 µM simvastatin significantly reduced *S. pneumoniae* attachment ($P = 0.001$), and 10 µM caused a slight, non-significant reduction. All data are representative of 3 biological replicates. $^{**}P \leq 0.01$.

Simvastatin potentiates the effect of gentamicin against *B. cenocepacia*

Given that statins caused the reduction of bacterial growth and virulence, it was hypothesised that statins could potentially increase the inhibitory effect of antibiotic treatment. The aminoglycoside antibiotic gentamicin was selected for this analysis, as this and other aminoglycosides are commonly used to treat infections and exacerbations in CF patients (54). *B. cenocepacia* was selected for this experiment, as clinical isolates of *Burkholderia* have been found to have a particularly high level of resistance against aminoglycosides (55). Furthermore, not only was this organism affected the least by simvastatin in this study (Figure 7), but preliminary analysis demonstrated that gentamicin had a markedly reduced effect against *B. cenocepacia* J2315 compared to *S. aureus* NCDO949, on which a largely bacteriocidal effect was exerted (Appendix Figure A1). Therefore, *B. cenocepacia* J2315 was cultured in the presence of sub-inhibitory concentrations of gentamicin (50 μg/ml) and simvastatin (50 μM) individually, and a combination of gentamicin + simvastatin. Bacteria were also cultured under control conditions of the vehicle (DMSO), and a combination of DMSO and gentamicin was also utilised to ensure that any effect observed was not due to cross-reaction between DMSO and gentamicin. Throughout early exponential phase (between 4 hpi and 8 hpi), no difference was observed in the growth of *B. cenocepacia* under different treatment conditions (Figure 12). However, by 12 hpi, the growth of bacteria treated with both simvastatin and gentamicin was modestly decreased compared to other conditions. When growth progressed from late log to early stationary phase (16, 20 and 24 hpi), a significant difference was observed in the growth of bacteria cultured with simvastatin and gentamicin combined compared to those cultured with the vehicle (16 hpi: $P = 0.01$, 20 hpi: $P = 0.015$, 24 hpi: $P = 0.008$). Furthermore, at 20 and 24 hpi, the growth of bacteria treated with simvastatin

+ gentamicin was markedly decreased compared to all other conditions, and was significantly less than the growth of bacteria + simvastatin (20 hpi: $P = 0.008$, 24 hpi: $P = 0.004$). These novel and exciting data demonstrated that simvastatin could significantly increase the inhibitory effect exerted by gentamicin on *B. cenocepacia* during log phase growth, suggesting that there could be potential for the use of these compounds as part of a combination therapy approach.

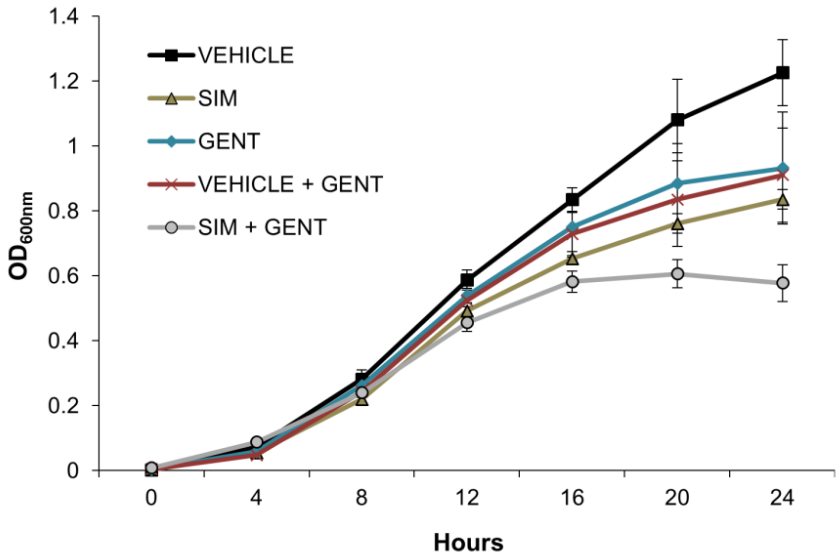


Figure 12: Effect of statins on the inhibitory potential of gentamicin. *B. cenocepacia* was treated with vehicle (black square markers), 50 µM simvastatin (green triangles), 50 µg/ml gentamicin (blue diamonds), vehicle + 50 µg/ml gentamicin (red crosses) and 50 µM simvastatin + 50 µg/ml gentamicin (grey circles). Simvastatin and gentamicin combined reduced the bacterial growth compared to all other conditions. Data are representative of the average and standard deviation of 3 biological replicates.

Discussion

Inhibition of bacterial growth and reduced inflammation, morbidity and mortality of patients with bacterial infections are key pleiotropic effects of statins. Therefore, it was hypothesised that statins could have an impact on bacterial pathogens associated with RTIs of people with CF, a condition characterised by chronic inflammation and infection. The first step in the identification of target organisms with possible susceptibility to statins was to search for the presence of HMGR, the enzyme which statins inhibit, in RTI-associated bacterial species. Here, it was observed that 10 out of 16 bacterial species examined possess a homologue of the enzyme that statins inhibit, HMGR, but only 7 of these organisms possess other mevalonate pathway genes. From the organisms surveyed, it generally appeared that Gram positive organisms tend to possess a mevalonate pathway, whereas Gram negative bacteria appear to predominantly utilise mevalonate-independent isoprenoid biosynthesis. It has been proposed that the mevalonate pathway was present in a common ancestor of prokaryotes, eukaryotes and archaea, but was subsequently replaced in most bacterial phyla by the MEP/DOXP pathway (56), and this may explain the higher occurrence of mevalonate pathway genes in Gram negative bacteria observed in this study.

In *B. cenocepacia*, a HMGR homologue with no corresponding mevalonate pathway genes was identified. It is currently unknown whether this protein is functional in this species but given its close conservation to the functional HMGR of *P. mevalonii* (Figure 4) it was proposed that not only may *B. cenocepacia* HMGR be functional, but it may also be inhibited by statins. However, this still begs the question of the role of mevalonate in an organism where MEP/DOXP genes are present. Bioinformatic prediction of interactions suggested that the HMGR of *B. cenocepacia*

(BCAM0531) may have a metabolic role. Two proteins in the predicted BCAM0531 network – PheA and HisB/HisD – may be of particular importance. PheA is responsible for biosynthesis of phenylalanine, which is known to inhibit Class I HMGR function (57), and therefore PheA may be part of a negative regulatory loop of BCAM0531 activity. Conversely, histidine has been found to increase mevalonate production (58), and as HisB and HisD are involved in biosynthesis of histidine, there may be a positive interaction between these and BCAM0531. Further work is needed to elucidate the role of HMGR in *B. cenocepacia*, and in other species lacking a mevalonate pathway.

Although the effects of statins on bacterial growth and virulence were tested in a comparable manner between *B. cenocepacia*, *S. aureus* and *S. pneumoniae*, the magnitude of the effects observed was variable between the 3 species. Simvastatin reduced the growth of all 3 species, but much higher concentrations were required for the reduction of growth of *B. cenocepacia* and *S. aureus* than *S. pneumoniae*. It is possible that the differences observed in inhibitory statin concentrations may be linked to the differential organisation and regulation of the mevalonate pathway in these species. As the *S. pneumoniae fni* gene, which produces the Fni protein responsible for synthesis of the intermediate DMAPP, is located within the predicted *mvaK1* operon, this strain may be more susceptible to statins than *S. aureus*, where *fni* appears to be independently regulated. An intriguing prospect uncovered in this study was that the growth of *S. aureus* was enhanced compared to the vehicle at 50 μM and 25 μM simvastatin (Figure 8E and 8F). Biofilm formation and motility of *S. aureus* were decreased at 50 μM and 25 μM respectively. Therefore, it appears that simvastatin was effective against *S. aureus* at sub-inhibitory concentrations. As the enhancement of *S. aureus* growth is a novel statin effect, further studies are

warranted to investigate the metabolic consequences of simvastatin treatment on *S. aureus*. *B. cenocepacia* may exhibit reduced susceptibility to simvastatin compared to *S. aureus* and *S. pneumoniae* as this species undergoes mevalonate-independent isoprenoid metabolism. Species possessing a MEP/DOXP pathway may be affected to a lesser extent than those where mevalonate-dependent metabolism is used, as statins most likely do not inhibit the production of intermediate compounds by the mevalonate-independent pathway.

An abundance of information is available regarding the effect of statins on bacterial growth, but to date, there is relatively little information available regarding the effect of statins on individual virulence factors of bacteria. The information currently available suggests that statins only target certain virulence phenotypes. Statins have been found to attenuate both the *in vivo* attachment of *S. pneumoniae* (7) and the epithelial translocation of *P. aeruginosa* (6), but did not impact on the production of quorum sensing compounds and protease by *B. cenocepacia* (59), or toxin production by *S. pneumoniae* (7). Motility and biofilm formation were focused on in this study and the effects of simvastatin on these phenotypes varied in a concentration-dependent manner. Simvastatin reduced attachment of all 3 test strains in an *in vitro* model, but only *S. aureus* was affected by sub-inhibitory concentrations. Furthermore, simvastatin had disparate effects on bacterial motility – it did not influence the motility of *B. cenocepacia*, but it reduced spreading motility of *S. aureus* at sub-inhibitory concentrations. Attachment and motility of *S. aureus* are both associated with the production of teichoic acid (43, 60). As teichoic acid and peptidoglycan production is dependent on isoprenoid metabolism (30, 31), it could be hypothesised that simvastatin-mediated inhibition of *S. aureus* HMGR may inhibit virulence phenotypes by reducing isoprenoid biosynthesis, thus disrupting the

structural integrity of the bacterial cell wall. The disruption of teichoic acid production may also contribute to the inhibitory effect of simvastatin on *S. pneumoniae* biofilm. However, given the substantial inhibitory effect of simvastatin on the growth of *S. pneumoniae*, it appears that the inhibition of streptococcal biofilm formation by simvastatin may occur as a result of growth inhibition rather than attenuation of biofilm-related factors. The reduced effectiveness of simvastatin against *B. cenocepacia* biofilm and motility suggests that the production of mevalonate by this species does not appear to be associated with the production of flagella and pili, and may not influence motility-associated quorum sensing. Unlike the MvaA of *S. aureus*, BCAM0531 does not appear to be associated with terpenoid backbone metabolism. Therefore, it can be hypothesised that simvastatin does not have an effect on the cell wall integrity of *B. cenocepacia*.

One limitation of the use of statins as antimicrobial compounds is that the statin effects in this study were not observed at current physiologically relevant statin concentrations. The maximum plasma concentration of simvastatin in individuals who take a daily dosage is 20-34 ng/ml (61) which equates to roughly 48-81 nM, about 1000 times lower than the lowest effective concentration observed in this study. However, if statins were to be used to treat respiratory infections, an inhalation-based delivery route would need to be established, which could potentially increase localised statin concentrations. In addition, there is potential for the use of statins in combination therapy with other antimicrobial compounds. It was observed that simvastatin potentiated the effects of the aminoglycoside antibiotic gentamicin against *B. cenocepacia*. These results were extremely promising, as the individual effects of simvastatin and gentamicin were only exerted at high concentrations, but the combined effects of gentamicin and simvastatin reduced

bacterial growth at a physiologically relevant gentamicin concentration (50 μg/ml) – this was comparable with sputum levels achieved in a pilot study using nebulised gentamicin (62). Further studies examining the combined antimicrobial potential of statins and antibiotics may help to uncover a method to increase the efficacy of aminoglycosides against antibiotic resistant bacteria. Previous studies involving the effects of a combination of statins and anti-microbial compounds have had mixed success. Farmer and colleagues noted that statins do not potentiate the inhibitory effect of various antibiotics against several Gram negative pathogens (63), but simvastatin has been found to potentiate the effect of penicillin towards *S. pneumoniae* (4). Increased efficacy has also been reported using statins in combination with anti-fungal and anti-malarial drugs (64–66). Further studies examining the combined antimicrobial potential of statins and antibiotics may help to uncover a method to increase the efficacy of aminoglycosides against antibiotic resistant bacteria. Another area for future considerations is the elucidation of the mechanism by which the combined antimicrobial effect of statins and antibiotics is exerted. Aminoglycosides exert their anti-microbial potential by inhibiting protein synthesis (67), and the increased potency of simvastatin plus gentamicin may be due to the combined inhibitory effects on lipid metabolism and protein synthesis. Further studies examining the combined antimicrobial potential of statins and antibiotics may help to uncover a method to increase the efficacy of aminoglycosides against antibiotic-resistant bacteria.

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Chapter 3

Phenotypic and regulatory analysis of the effects of statins on *Pseudomonas aeruginosa*

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Title: Statins inhibit *in vitro* virulence phenotypes of *Pseudomonas aeruginosa*

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Abstract

Cholesterol-lowering statin drugs have been shown to affect the growth of some microbial pathogens. However, little is known about the effect of statins on bacterial virulence factor production/expression. The objective in this study was to investigate whether statins could modulate virulence factor behaviour in the human nosocomial and cystic fibrosis-associated pathogen *Pseudomonas aeruginosa*. Here it was demonstrated that although statins did not have a significant effect on the growth of *P. aeruginosa*, they were capable of modulating 2 key *P. aeruginosa* virulence factors – swarming motility and biofilm formation. Swimming and twitching motility, chemotaxis, type 3 toxin expression and quorum sensing were also tested in the presence of statins, and were found to be unaffected. Moreover, transcriptome analysis revealed that statin treatment did not significantly impact gene transcription of *P. aeruginosa*. However, phenotypic screening of a *P. aeruginosa* mutant bank identified the PvrR regulator and fimbrial biogenesis genes as playing a role in the attenuation of attachment by simvastatin. Furthermore, global proteomic analysis revealed that the reduction/modulation of swarming by simvastatin appeared to be mediated through proteins involved in transport and RNA and protein synthesis.

Introduction

In Chapter 2, it was demonstrated that the cholesterol-lowering statin drug simvastatin was capable of modulating growth and virulence behaviour of respiratory tract infection-associated bacterial pathogens. The effect that sub-inhibitory concentrations of statins have on virulence suggests that statins can influence processes other than metabolism, as these effects appear to be growth-independent. The bacteria studied in Chapter 2 possess homologues of the enzyme that statins inhibit, 3-hydroxy-3-methylglutaryl-CoenzymeA reductase (HMGR). A point for consideration is whether statins can have the same influence on growth and virulence of bacteria which do not possess HMGR homologues. Many bacterial species do not possess HMGR, but instead synthesise isoprenoids via the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway. An example of a respiratory pathogen which lacks mevalonate-dependent isoprenoid biosynthesis is *Pseudomonas aeruginosa*.

P. aeruginosa is a significant opportunistic pathogen, which causes chronic infections in 80 % of people with the genetic disease cystic fibrosis (CF) (1), and also causes pneumonia and corneal and wound infections. *P. aeruginosa* is an ideal model organism as it has a range of well-characterised virulence factors which can be studied using phenotypic and molecular assays. These virulence factors are essential in determining the outcome and persistence of *P. aeruginosa* infection. When *P. aeruginosa* first encounters the host, it attacks by way of an acute infection, causing cytotoxicity via type 3 secreted toxins. *P. aeruginosa* produces 4 type 3 toxins – the bi-functional ExoS and ExoT which have ADP-ribosyltransferase and GTPase-activating domains (2–5); ExoY, which acts as an adenylate cyclase (6); and ExoU, which has phospholipase activity (7). In addition, *P. aeruginosa* displays 2

types of flagella-associated motility, swimming (8) and swarming (9). *P. aeruginosa* motility is also dependent on other factors including, but not limited to, lipopolysaccharide (LPS) synthesis (10) and surfactant production (11).

Cell density-dependent cell-cell communication in *P. aeruginosa* is mediated through the quorum sensing (QS) signalling system. When a sufficiently large bacterial population has been established, the perception of QS signals by the bacteria crosses an essential threshold, leading to the co-ordinated regulation of gene expression and resulting behaviour associated with the chronic phase of infection. *P. aeruginosa* QS signals fall into 2 broad classes: acylated homoserine lactones (AHLs) and quinolones. AHL-mediated quorum sensing is regulated via the LasI/R and RhlI/R sensor-regulator 2 component systems (12–17), while the *Pseudomonas* quinolone signal (PQS) is produced by the *pqsABCDE*, *pqsH* and *phnAB* genes (18, 19) and can cross-talk with RhlI/R quorum sensing (20). As a result of QS, bacteria begin attaching to host cell tissue and flagella-associated motility is inhibited (21–24). Attachment is linked to a third type of *P. aeruginosa* motility – twitching motility – which is mediated through Type IV pili production (25), and is regulated by the LasI/R QS system (24). The chronic phase of infection is truly established when attached bacteria mature into a complete biofilm, by attaching irreversibly to the epithelial cell surface, descending into a sessile state and becoming surrounded by an extracellular matrix composed of polysaccharides, DNA and other components (24). *P. aeruginosa* can also produce a structure called a "microcolony" where bacteria attach to themselves and sputum components in response to limiting O_2 conditions (26). Biofilm maturation is regulated by RhlI/R QS (24). Once *P. aeruginosa* is in this state, it becomes extremely resilient when confronted with antibiotic treatment and the immune response. Efforts to eradicate chronic *P.*

aeruginosa infections are further abrogated by antibiotic resistance mechanisms possessed by the bacteria.

The objective in this study was to examine the effects of statins on a CF-associated bacterial species which did not possess a HMGR homologue. Three statins were used in this study in order to investigate whether the effects observed in Chapter 2 were specific to simvastatin. The growth of *P. aeruginosa* was assessed in the presence of statins, and phenotypic analysis was carried out to examine if statins could influence *P. aeruginosa* virulence behaviour. As the mechanism by which statins could be influencing *P. aeruginosa* was unknown, transcriptomic, proteomic and mutant screening approaches were employed in an attempt to uncover how statins could be affecting *P. aeruginosa* virulence.

Materials and methods

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 1. *P. aeruginosa* strains were grown in Luria Bertani broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar) unless otherwise stated.

Statins

Simvastatin (Sigma-Aldrich, U. K.) and mevastatin (Calbiochem, Germany) were resuspended in DMSO (Sigma-Aldrich) at concentrations of 20 mg/ml. Lovastatin (Sigma-Aldrich) was resuspended in DMSO at 10 mg/ml. All statins and DMSO vehicle controls were filter sterilised and stored at 4 ºC. To prepare hydrolysed forms of statins, lactone ring hydrolysis was carried out by firstly dissolving statins in 96 % ethanol at the following concentrations: simvastatin: 40 mg/ml; mevastatin: 25 mg/ml; lovastatin: 25 mg/ml. 1.5 volumes of 0.1 N NaOH was added to dissolved statin, and this mixture was heated at 50 ºC for 2 hours. The pH of the mixture was adjusted to 7 using HCl, and the volume was adjusted to 1 ml using the ethanol/NaOH solvent.

Table 1: Bacterial strains used in this study.

Strain	Description	Reference
Pseudomonas aeruginosa		
PAO1	Wild type	(27)
PA14	Wild type	(28)
PAO1 *exoS::lacZ*	*exoS* reporter strain	(29)
PA14 *pqsA::lacZ*	*pqsA* reporter strain	(30)
CF194	Clinical isolate from Irish CF patient	(31)
TY5010	Clinical isolate from Turkish non-CF patient	(32)
PA14 *ΔpvrR*	Transposon mutant in *pvrR*	(28)
PA14 *ΔwspF*	Transposon mutant in *wspF*	(28)
PA14 *ΔcupA1*	Transposon mutant in *cupA1*	(28)
PA14 *ΔcupA2*	Transposon mutant in *cupA2*	(28)
PA14 *ΔcupA3*	Transposon mutant in *cupA3*	(28)
PA14 *ΔcupD1*	Transposon mutant in *cupD1*	(28)
PA14 *ΔcupD2*	Transposon mutant in *cupD2*	(28)
PA14 *ΔcupD4*	Transposon mutant in *cupD4*	(28)
PA14 *ΔcupD5*	Transposon mutant in *cupD5*	(28)
PA14 *ΔlptD*	Transposon mutant in *lptD*	(28)
PA14 *Δpnp*	Transposon mutant in *pnp*	(28)
PA14 *ΔdnaK*	Transposon mutant in *dnaK*	(28)
Other strains		
Chromobacterium violaceum CV026	Indicator for homoserine lactone production	(33)

Measurement of bacterial growth

Bacterial growth was measured according to the protocol described in Welsh *et al.,* 2009 (34). Briefly, bacteria were cultured shaking at 37 ºC for 16-18 hours. Cultures were centrifuged at 2330 x g for 10 minutes. Bacterial pellets were washed twice with phosphate buffered saline (PBS; Sigma-Aldrich) and resuspended in Muller-Hinton broth (Oxoid) and the absorbance at 600 nm (OD_{600nm}) was measured using a Beckmann-Coulter spectrometer. Bacteria were inoculated into Muller-Hinton broth (Oxoid) containing 1 mM, 100 µM, 10 µM, 1 µM, 100 nM and 10 nM of statins, or an equivalent DMSO vehicle control, at an OD_{600nm} of 0.0005. 200 µl volumes of bacteria were inoculated into a 96 well microtitre plate, which was incubated static at 37 ºC for 24 hours, following which bacterial growth was measured by quantifying OD_{600nm} using a SpectraMax Plus 384 96 well plate reader (Molecular Devices).

Motility assays

Motility of *P. aeruginosa* was tested using 3 different agars. Swarming agar was composed of 6 g/L Eiken agar (Eiken Chemical), 8 g/L Eiken broth (Eiken Chemical), and 0.5 % (w/v) glucose (Sigma-Aldrich). Swimming and twitching agars consisted of 5 g/L peptone (Merck), 3 g/L yeast extract (Difco), 2 % (v/v) glycerol (Sigma-Aldrich), and 0.3 % (w/v) and 1 % (w/v) agar respectively. Plates were dried at room temperature overnight, and were inoculated with a single colony of *P. aeruginosa* from a freshly cultured plate. All plates were incubated in an upright position at 37 ºC – swarming for 12-18 hours, swimming for 24 hours, and twitching for 45 hours. Swarming and swimming assays were photographed directly following incubation using an Epson v750 scanner. For twitching assays, agar was

removed from plates, and attached bacteria were stained using Coomassie Blue. Excess dye was removed using a destain solution (10 % (v/v) methanol, 10 % (v/v) acetic acid in H_2O), following which plates were photographed.

Measurement of attachment

For analysis of early attachment, *P. aeruginosa* was cultured overnight in LB broth at 37 ºC, and inoculated into 100 ml conical flasks containing 20 ml LB broth supplemented with simvastatin or a DMSO vehicle control (0.209 % (v/v)) at OD_{600nm} 0.05. After 3 hours of growth shaking at 37 ºC, bacteria were inoculated into 24 well plates containing 1 ml of LB broth supplemented with simvastatin or vehicle at OD_{600nm} 0.25, and incubated static at 37 ºC for 2 hours. Media was removed, and wells were washed 3 times with LB broth. Attached bacteria were stained with 0.1 % (w/v) crystal violet for 10 minutes at room temperature, and excess dye was removed by washing wells 5 times with water. Remaining dye was resuspended in 96 % ethanol, and the OD_{570nm} of resuspended crystal violet was measured on a SpectraMax Plus 384 96 well plate reader. For analysis of mutant library strains, bacteria were inoculated at OD_{600nm} 0.25 into 96 well plates containing 135 µl of LB.

For determination of the effect of statins on attached bacteria, *P. aeruginosa* was inoculated into LB broth in a 96 well plate at OD_{600nm} 1 at a volume of 200 µl per well, and incubated static at 37 ºC for 8 hours. Medium containing unattached bacteria was removed, and 200 µl LB broth containing either statin or DMSO was added to each well. This was incubated static at 37 ºC for 12 hours, following which medium was removed and crystal violet staining was carried out as previously described.

Analysis of tight microcolony formation

Tight microcolony formation of *P. aeruginosa* was measured according to Sriramulu *et al.,* 2005 (35). Briefly, bacteria were cultured in LB broth overnight, and the OD_{600nm} was measured. Bacteria were inoculated into 24 well plates where each well contained 1 ml of Artificial Sputum Media (ASM) (5 g/L mucin from porcine stomach muscosa (Sigma-Aldrich, U.K.), 4 g/L salmon sperm DNA (Fluka), 5.9 mg/L diethylene triamine pentaacetic acid (DTPA), 5 g/L NaCl, 2.2 g/L KCl, 5 g/L amino acids and 0.5 % (v/v) egg yolk emulsion (Oxoid), pH 7) at OD_{600nm} 0.05., supplemented with statins or vehicle where appropriate. Plates were incubated shaking at 37 ºC for 72 hours, and photographed using an Epson v750 scanner.

Measurement of extracellular DNA production

Extracellular DNA production by *P. aeruginosa* was measured according to the protocol described in Allesen-Holm *et al.,* 2006 (36). Briefly, bacteria were cultured overnight in AB medium (2 g/L $(NH_4)_2SO_4$, 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 3 g/L NaCl, 0.1 M $CaCl_2$, 0.1 M $MgCl_2$, 3 mM $FeCl_3$) supplemented with 0.5 % (w/v) glucose. The OD_{600nm} of overnight cultures was measured, and bacteria were inoculated at OD_{600nm} 0.001 into 96 well plates containing AB medium supplemented with 0.5 % (w/v) glucose and 50 μM propidium iodide (Fluka). Plates were incubated static at 37 ºC for 24 hours. The OD_{600nm} (growth) and OD_{480nm} (DNA) of each well were measured using a SpectraMax Plus 384 96 well plate reader. Extracellular DNA production was expressed as the ratio of OD_{480nm} to OD_{600nm}.

Analysis of *β*-galactosidase production by *lacZ* promoter fusions

P. aeruginosa exoS-lacZ and *pqsA-lacZ* reporter fusion strains (detailed in Table 1) were cultured shaking for 16-20 hours at 37 ºC. The OD_{600nm} of overnight cultures were measured, and bacteria were inoculated into sterile 100 ml conical flasks containing LB broth supplemented with statins or DMSO where appropriate at OD_{600nm} 0.05. LB containing 10 mM nitrilotriacetic acid (NTA) was used as a positive control to induce *exoS* promoter expression. Cultures were incubated shaking at 37 ºC until they reached OD_{600nm} 1.5 (for *exoS*) or 1.2 (*pqsA*). *β*-galactosidase activity was measured as described by Miller (37). Briefly, 1 ml of culture was removed and centrifuged at 16100 x g for 1 minute and pelleted bacteria were resuspended in 1 ml of LacZ buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 2.7 ml/L β-mercaptoethanol; pH 7). 100 µl of LacZ buffer was added to 500 µl of cells, following which 20 µl 0.05 % (w/v) SDS and 20 µl $CHCl_3$ were added to cells. This was mixed and incubated at 30 ºC for 10 minutes. 100 µl of ONPG (4 mg/ml; freshly prepared in LacZ buffer) was added to cells, which were incubated until they turned a yellow colour. 260 µl of Na_2CO_3 was added to stop the reaction, and cells were centrifuged at 16100 x g for 1 minute. The OD_{420nm} and OD_{550nm} values of supernatant were measured, and the Miller units (representing *β*-galactosidase activity) were calculated using the following formula:

Miller Units = ((OD_{420nm} - (1.75 x OD_{550nm}))/(Volume of cells used (ml) x Time (mins) x OD_{600nm}) x 1000

Quorum sensing indicator strain assays

A single colony of the indicator strain *Chromobacterium violaceum* CV026 was used to make a linear streak on an LB plate which was supplemented with 100 μM of statin or a DMSO vehicle control. PAO1 and PA14 were cross-streaked at both a 90 º angle and parallel to CV026 and plates were incubated at 30 ºC in an upright position for 24 hours. Plates were photographed using an Epson v750 scanner.

RNA microarray analysis

P. aeruginosa PA14 was inoculated into LB broth, containing either 100 μM simvastatin or an equivalent volume of DMSO, at a starting inoculum of OD_{600nm} of 0.01. Cultures were incubated at 37 ºC shaking at 180 rpm until bacteria reached an OD_{600nm} of 0.8 ± 0.5, following which cells were harvested for RNA isolation. RNA protect was used according to manufacturer's recommendations (QIAGEN, Germany). Total RNA was isolated from each cell pellet using a QIAGEN RNeasy® Mini kit. Residual genomic DNA was eliminated using Ambion Turbo DNA-*free*™ kit treatment according to manufacturer's recommendations. RNA quantity was assessed using ND-1000 Spectrophotometer (NanoDrop Technologies, USA). 10 μg of DNA-free RNA was sent to DNAVision, Belgium where it was converted to cDNA, and hybridised to an Affymetrix™ GeneChip® specific for *P. aeruginosa*. Analysis of gene expression was carried out using GeneSpring GX software (Agilent Technologies, USA). Data were normalised using the Robust Multi-array Average (RMA) method and subsequently compared using an unpaired T-Test. Genes were considered differentially regulated if the relative change was ≥ 1.5 and the *P* value was < 0.05.

Quantitative RT-PCR (qRT-PCR) analysis of gene expression

PA14 was cultured with either 100 μM simvastatin or DMSO, and RNA was isolated as previously described. 500 ng of DNA-free RNA was converted to cDNA, using AMV Reverse Transcriptase (Promega) according to manufacturer's specifications. qRT-PCR analysis was carried out using the Roche Universal ProbeLibrary system, using 5 μl of cDNA in each reaction. Details of primers and probes used are listed in Table 2.

Table 2: Primers and probes used in qRT-PCR analysis. The annealing temperature of each primer set is 60 ºC.

Gene	Product Length (bp)	Sequence	Probe No.
PA14_06700	103	Forward: acgaagtgctggaacaggtc	22
		Reverse: atggcgttggcggtatag	
PA14_06710	124	Forward: accgctacaacctgtggttc	149
		Reverse: cagtaggcttcctgcatcg	
PA14_06720	60	Forward: gacccacgaagtgatcgaa	162
		Reverse: ctgaattccatgtgcagtacg	
PA14_06770	75	Forward: cggctaccagaacctgctc	107
		Reverse: gggtagtcgaagcgcaac	
PA14_06790	70	Forward: gagtgctctactggcacatgg	150
		Reverse: cattgcaggacgtagaccag	
ProC	79	Forward: cttcgaagcactggtggag	20
		Reverse: ttattggccaagctgttcg	

Analysis of bacterial chemotaxis

Chemotaxis was analysed using a modified version of the protocol described in Moulton & Montie, 1979 (38). Briefly, bacteria were cultured overnight at shaking in LB broth at 37 ºC. 1 ml of culture was pelleted and resuspended in mineral salt medium (MSM) (7 g/L K_2HPO_4, 3 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 0.05 g/L $MgSO_4.dH_2O$, 0.025 g/L $FeCl_3.6H_2O$). The OD_{600nm} of the culture was measured, and bacteria were inoculated into 1 ml of MSM supplemented with 100 µM simvastatin, or an equivalent amount of DMSO at a density of 1 x 10^7 cfu/ml. One hundred microlitres of this was aliquoted into a clear 96 well plate (Sarstedt). Microcapillary pipettes with a 1-5 µL capacity (Sigma-Aldrich) were filled with MSM containing 10 mM of arginine, serine or glycine (all Sigma-Aldrich), and were placed in wells containing bacteria. Plates were incubated static at 37 ºC for 1 hour. The content of each capillary was eluted onto sterile Whatman paper, and diluted with 0.5 ml LB broth. Samples were then diluted ten-fold, and plated on LB agar plates in duplicate. Plates were incubated overnight at 37 ºC, following which colonies were counted.

Protein isolation

PA14 was inoculated onto 0.6 % Eiken agar plates (prepared as previously described) containing either 100 µM simvastatin or an equivalent volume of DMSO. Plates were incubated at 37 ºC for 16 hours. The outermost 1 cm of bacteria was removed from the plate using a sterile cotton swab and was transferred into a sterile tube containing 5 ml of wash buffer (50 mM Trizma Base, 100 mM phenylmethanesulfonylfluoride (PMSF) (both Sigma-Aldrich). Cells were washed

by centrifugation in a Sorvall SS-34 rotor in a Sorvall RC-5B centrifuge at 12000 X g for 20 minutes. The pellet was resuspended in 5 ml of wash buffer and washed twice more under the same conditions as above. After 3 washes, cells were resuspended in wash buffer and were subjected to 3 cycles of incubation at -70 ºC for 10 minutes, followed by 37 ºC for 5 minutes. Cells were then lysed by sonication (amplitude 10,000 micron, On/30 seconds, Off/30 seconds for 12 minutes). Cytosolic proteins were removed by centrifugation (13000 x g, 20 minutes at 4 ºC). Proteins were quantified using a BCA assay kit according to manufacturer's recommendations (Thermo Scientific) and precipitated using acetone.

2-dimensional gel electrophoresis (2-DGE)

Samples were resuspended in rehydration buffer (5 M urea, 2 M thiourea, 380 mg/L amberlite, 100 mg/L CHAPS, 100 mg/L SB 3-10, 10.8 mg/L DTT, 1.43 mg/L TCEP and 5 µl/L IPG buffer) and quantified using a Bio-rad assay (Bio-Rad). Proteins were rehydrated at a rate of 1 mg/ml. Two hundred micrograms of protein was loaded by in-gel hydration on 11 cm pH 3-7 immobilised pH gradient (IPG) strips (Bio-rad). Isoelectric focusing was carried out using the following protocol:

Voltage	Gradient	µAmp	Time	Unit
250 V	Rapid	50	1:00	hh:mm
500 V	Rapid	50	1:00	hh:mm
8000 V	Gradual	50	1:30	hh:mm
8000 V	Rapid	50	35000	Volt hour
500 V	Rapid	50	24:00	hh:mm

IPG strips were equilibrated and electrophoresis in the second dimension was carried out at 500 V and 40 mA until samples reached the end of the gel.

Gels were fixed in 50 % (v/v) methanol, 5 % (v/v) acetic acid for 20 minutes. They were then stained using 0.1 % silver nitrate for 20 minutes, and developed in 0.04 % (v/v) formalin 2 % (w/v) sodium carbonate. Protein spots were excised and washed in 25 mM ammonium bicarbonate 50 % (v/v) acetonitrile for 30 minutes, followed by 25 mM ammonium bicarbonate for 30 minutes, twice. They were then dehydrated in 100 % (v/v) acetonitrile and dried using a SpeedVac (Savant Instruments, USA) for 5 minutes at room temperature. Samples were then washed with 7 % (v/v) hydrogen peroxide and 100 % (v/v) acetonitrile and stored at – 20 ºC.

Tandem mass spectrometry

Samples were dried and desalted using Zip Tip C_{18} (Millipore) and peptides were then dissolved in 0.1 % (v/v) formic acid. All experiments were done on a LTQ-Orbitrap Elite (Thermo Scientific) coupled with an Easy nLC II system (Thermo Scientific). The mass spectrometer was operated in positive ionization mode with a capillary voltage and a source temperature set at 1.5 kV and 275 °C, respectively. Three µL of sample was injected onto an enrichment column (C18 PepMap100, Thermo Scientific). Separation was achieved with an analytical column needle (NTCC-360/100-5-153, NikkyoTechnos, Japan). The mobile phase consisted of H_2O/0.1 % (v/v) formic acid (buffer A) and 0.1 % (v/v) acetonitrile/formic acid (buffer B). Peptides were eluted at a flow rate of 300 nL/min from 2 to 55 % B over 15 min. The samples were analysed using the CID (collision induced dissociation) method. The first scan (MS spectra) was recorded in the Orbitrap analyzer

(R=60,000) with the mass range *m/z* 400-2000. Then, the 20 most intense ions were selected for MS^2 experiments. All measurements in the Orbitrap analyzer were performed with on-the-fly internal recalibration (lock mass) at *m/z* 445.12002 (polydimethylcyclosiloxane).

Database searches

Raw data files were processed using Proteome Discoverer 1.3 software (Thermo Scientific). Peak lists were searched using the MASCOT search engine (Matrix Science) against the database *Pseudomonas aeruginosa* PA14 containing 5,892 protein sequences (http://www.pseudomonas.com/downloads/sequences). Database searches were performed with the following parameters: 1 missed trypsin cleavage sites allowed; variable modifications: carbamidomethylation of cysteine, and oxidation of methionine.

Statistical analysis

Statistical significance was calculated using a paired T-test unless otherwise stated.

Results

Statins do not affect the growth of *P. aeruginosa*

It has previously been reported that growth of the *P. aeruginosa* reference strain ATCC 27853 was inhibited by 2 statins (34). In this study, rosuvastatin and atorvastatin had a bacteriostatic effect against ATCC 27853 at respective concentrations of 208 mM and 447.5 mM. The effect of statins on the lab reference *P. aeruginosa* strains PAO1 and PA14 was consequently tested in the same manner. PAO1 and PA14 were cultured in the presence of a 10-fold dilution series of statins (from 1 mM to 10 nM), as well as an appropriate DMSO vehicle control. Lovastatin, mevastatin and simvastatin were tested in this study. Addition of DMSO to the growth medium was found to reduce growth of both strains. However, no reduction in growth was observed between vehicle- and statin-treated cells, at any concentration of statins (Figure 1).

The impact of statins on the growth of *P. aeruginosa* ATCC 27853 was also tested, under the same statin concentrations and growth conditions as PAO1 and PA14. Statins did not influence the growth of this strain – the growth levels obtained against each statin and the vehicle control were comparable to those achieved for PAO1 and PA14. It was therefore concluded that statins do not have a significant effect on *P. aeruginosa* growth.

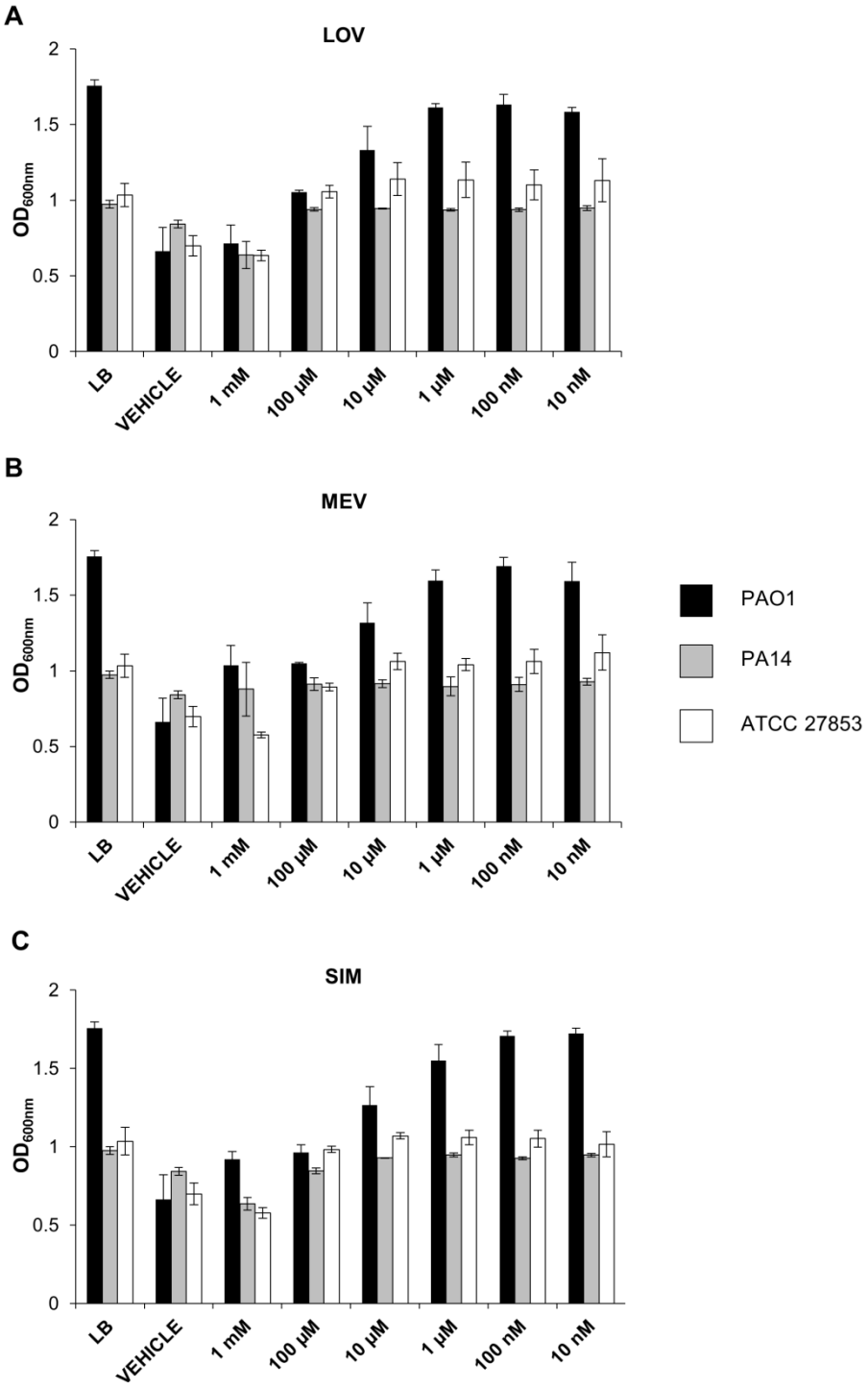


Figure 1: Effect of statins on growth of *P. aeruginosa*. The *P. aeruginosa* strains PAO1, PA14 and ATCC 27853 were cultured in Muller-Hinton broth containing a 10-fold dilution series of **(A)** lovastatin **(B)** mevastatin or **(C)** simvastatin, as well as DMSO (vehicle) (2.09 % (v/v)) and media only (LB) controls, for 24 hours. OD_{600nm} was measured to determine bacterial growth. Data are representative of 3 biological replicates.

The Type III secretion system of *P. aeruginosa* is not affected by statins

It has been demonstrated that virulence behaviour of *P. aeruginosa* can be influenced by sub-inhibitory concentrations of drugs such as colistin (30). It was questioned if statins could have this effect on *P. aeruginosa*, despite their lack of influence on the growth of this species, and this hypothesis was tested by examining the effects of statins on Type III secretion (TTSS) of *P. aeruginosa*, which is crucial for the acute phase of infection (39). The transcriptional expression of *exoS* (PA3841) was examined using a PAO1*exoS::lacZ* reporter strain, cultured in LB supplemented with 10 mM NTA in order to induce the TTSS. The expression of *exoS* was analysed using beta-galactosidase assays in the presence of 100 μM, 10 μM and 1 μM concentrations of simvastatin, lovastatin and mevastatin, which were compared to controls of LB NTA, and a DMSO vehicle control (Figure 2). It was noted that, unlike growth, the vehicle had no influence on *exoS* expression. Furthermore, no statin concentration tested in this study altered the expression of *exoS* compared to the vehicle control, or TTSS-inducing conditions. It was thus determined that statins do not impact the expression of the ExoS toxin by *P. aeruginosa*.

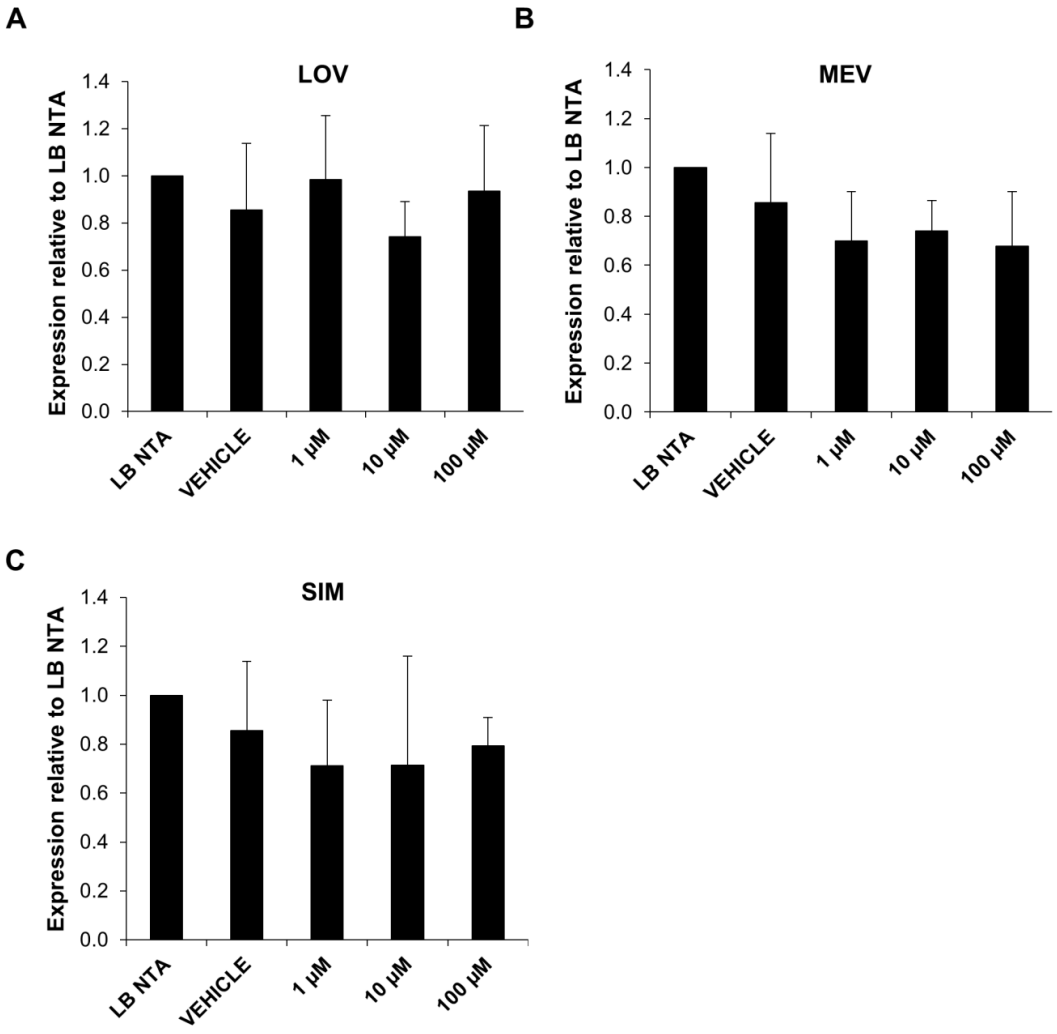


Figure 2: Effect of statins on Type III secretion. *P. aeruginosa exoS::lacZ* was cultured in the presence of 100 µM, 10 µM and 1 µM **(A)** lovastatin, **(B)** mevastatin or **(C)** simvastatin in addition to DMSO and inducing conditions (LB NTA). Expression of *exoS* was analysed at OD_{600nm} using Miller assay and was compared to the expression observed under inducing conditions. No increase or decrease in *exoS* expression was observed in statin- or DMSO-treated cells.

Statins influence swarming motility of *P. aeruginosa*

In order to further examine whether statins had an influence on the acute infection virulence behaviour of *P. aeruginosa*, their potency was tested against bacterial motility. This is not only an important factor in the acute phase of infection, but is also implicated in the switch between the acute and chronic phases of infection. *P. aeruginosa* displays 3 *in vitro* motility phenotypes. Swarming and swimming motility are linked to flagellar motion of the bacterium (as well as several other factors), while twitching motility is associated with the production of Type IV pili. The effect of statins on the motility of *P. aeruginosa* was assessed using lovastatin, mevastatin, and simvastatin. Swarming motility was reduced by 100 μM of all 3 statins (Figure 3A). As this effect was comparable between PAO1 and PA14, the influence of statins on clinically-relevant *P. aeruginosa* strains was assessed, by testing the effect of 100 μM simvastatin, lovastatin and mevastatin against the motility of 2 clinical isolates. CF194 was originally isolated from an Irish CF patient, while TY5010 was recovered from a Turkish non-CF patient. The swarming motility of both these strains was attenuated by all 3 statins tested (Figure 3B).

Each of the statins tested in this study was used in an "inactive" form, which is characterised by an intact lactone ring to which a decalin structure and a butaryl side chain are attached. However, *in situ,* statins are converted to an active form via hydrolysis of the lactone ring, and it is the hydrolysed statin form which binds to HMGR. Therefore, it was decided to examine whether lactone ring hydrolysis was a determining factor in the effects that statins had on *P. aeruginosa* virulence. The lactone rings of lovastatin, mevastatin and simvastatin were hydrolysed using sodium hydroxide/ethanol treatment, and 100 μM of hydrolysed statin was added to swarming plates. A vehicle control of an equivalent amount of NaOH/EtOH solvent

(0.209 % (v/v)) was used. The hydrolysed forms of lovastatin and mevastatin did not decrease motility, or alter the appearance of projections compared to the solvent control (Figure 3C). However, the hydrolysed form of simvastatin caused decreased swarming compared to the vehicle control, in a similar manner to the reduction caused by the unhydrolysed form. These results were consistent between PAO1 and PA14, and appear to indicate that an intact lactone ring is essential for the effect of lovastatin and mevastatin on virulence, but is not required by simvastatin. This suggests that the butaryl side chain of simvastatin may be responsible for its effects on *P. aeruginsosa* virulence.

The effect of statins on swimming and twitching motility of *P. aeruginosa* were also analysed, using 100 μM simvastatin. Despite the effects observed on swarming motility, statins were not capable of influencing *P. aeruginosa* swimming and twitching motility (Figure 3D and E). Both PAO1 and PA14 behaved in a similar manner in the presence of each statin as they did in the presence of the vehicle. Altogether, these data suggested that statins have a specific effect on *P. aeruginosa* swarming motility, without influencing factors associated with swimming or twitching motility.

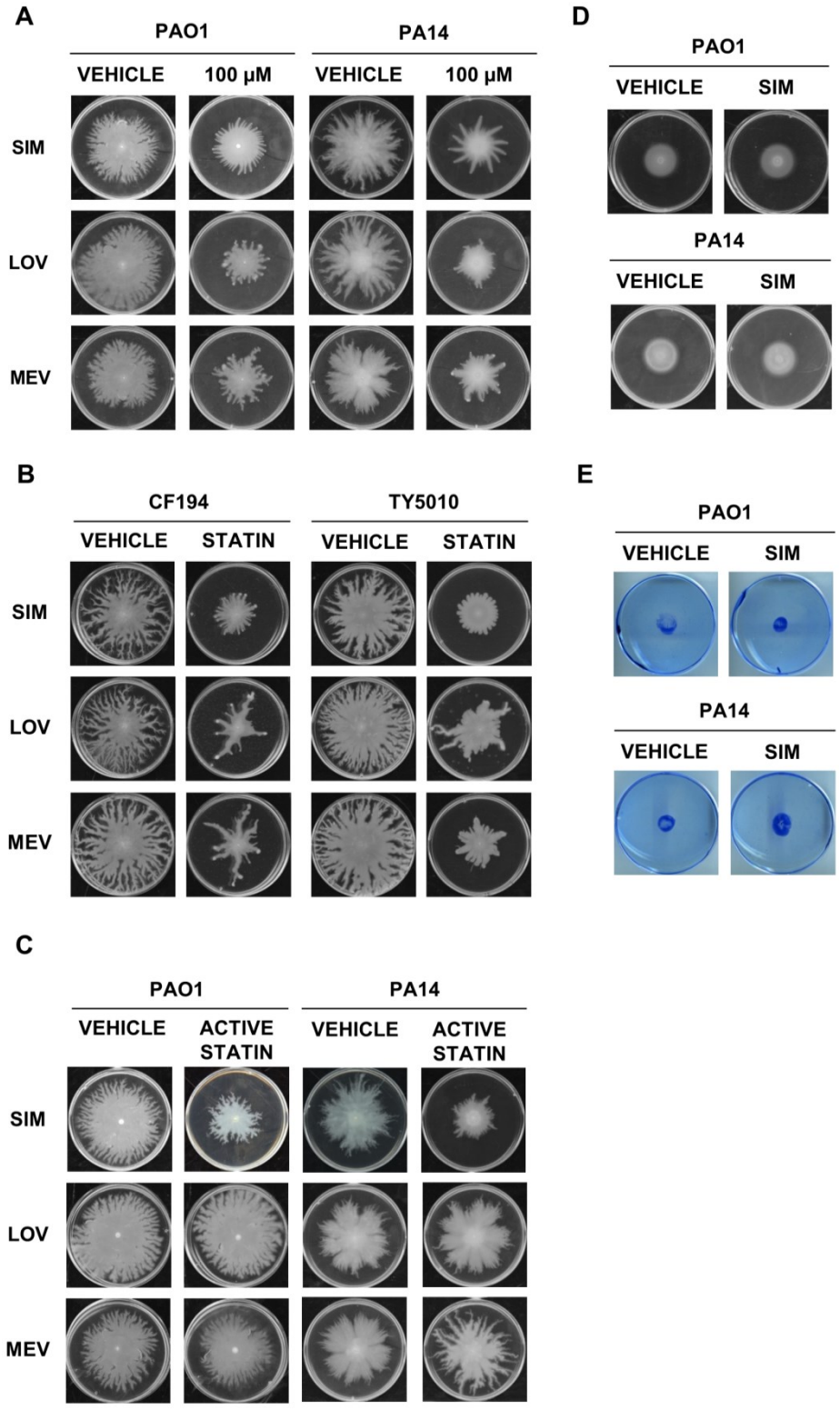


Figure 3: Influence of statins on *P. aeruginosa* swarming motility. Bacteria were cultured on 0.6 % (w/v) Eiken agar. **(A)** Swarming motility of PAO1 and PA14 was reduced by 100 µM simvastatin, mevastatin and lovastatin. **(B)** The clinical isolates CF194 and TY5010 were tested, and swarming of these strains was also attenuated by 100 µM statin

concentrations. **(C)** The lactone ring of simvastatin, lovastatin and mevastatin was hydrolysed by NaOH and heat treatment, to determine whether the activated form of statins yielded the same effect as the inactive form with the intact lactone ring. Hydrolysed lovastatin and mevastatin were no longer capable of attenuating swarming compared to the vehicle; however, 100 μM of hydrolysed simvastatin reduced swarming of PAO1 and PA14 to a comparable extent. **(D)** and **(E)** Swimming and twitching motility of PAO1 and PA14 were measured using 0.3 % and 1 % (w/v) agars respectively. 100 μM simvastatin did not influence either swimming **(D)** or twitching **(E)** motilities of *P. aeruginosa*.

Statins reduce early attachment, but not biofilm maturation

Biofilm formation is a key virulence factor involved in the establishment and persistence of *P. aeruginosa* chronic infections. As an association has been found between reduced swarming motility and attenuated biofilm formation (40), the effect of statins on this phenomenon of was tested. At this point in the study, it was decided to continue using simvastatin only, as it appeared that all the inactive forms of all 3 statins tested were having a similar effect on *P. aeruginosa*.

The first phenotype tested was initial attachment, which is the first stage of biofilm formation and associated with the switch from acute to chronic infections. Bacteria were incubated with 100 μM simvastatin for 2 hours, following which attached bacteria were stained with crystal violet. It was found that 100 μM simvastatin was capable of significantly reducing attachment of PA14 (Figure 4A). A reduction was also observed in response to 10 μM simvastatin, but this was not statistically significant. However, unlike the effect observed on swarming motility, this phenotype was strain-specific. A reduction in attachment was not observed with PAO1 – even 100 μM of simvastatin did not influence the attachment of this strain compared to the DMSO control (Figure 4B). Furthermore, 100 μM simvastatin also failed to reduce the attachment of the clinical isolates CF194 (Figure 4C) and TY5010 (Figure 4D).

Based on these results, the effect of statins on bacteria which had established attachment was examined. PA14 was allowed to attach to a surface for 8 hours, after which time bacteria were treated with 100 μM simvastatin to assess whether statins could disrupt established attachment. After 12 hours of statin treatment, crystal violet staining was utilised to determine the number of bacteria that remained attached. At

this time, simvastatin did not alter the numbers of attached bacteria relative to those treated with vehicle (Figure 4E).

The effect of statins on biofilm maturation was also examined by using assays to measure extracellular DNA (eDNA) production and tight microcolony formation. Both of these phenotypes were tested in the presence of 100 μM simvastatin. Neither eDNA production nor tight microcolony formation was significantly altered in the presence of statins when compared to vehicle-treated bacteria (Figure 4F and 4G). Taken together, the results of this section of the study indicated that statins have a specific effect on the initial stage of *P. aeruginosa* biofilm formation, but are not effective against later phases of this phenomenon.

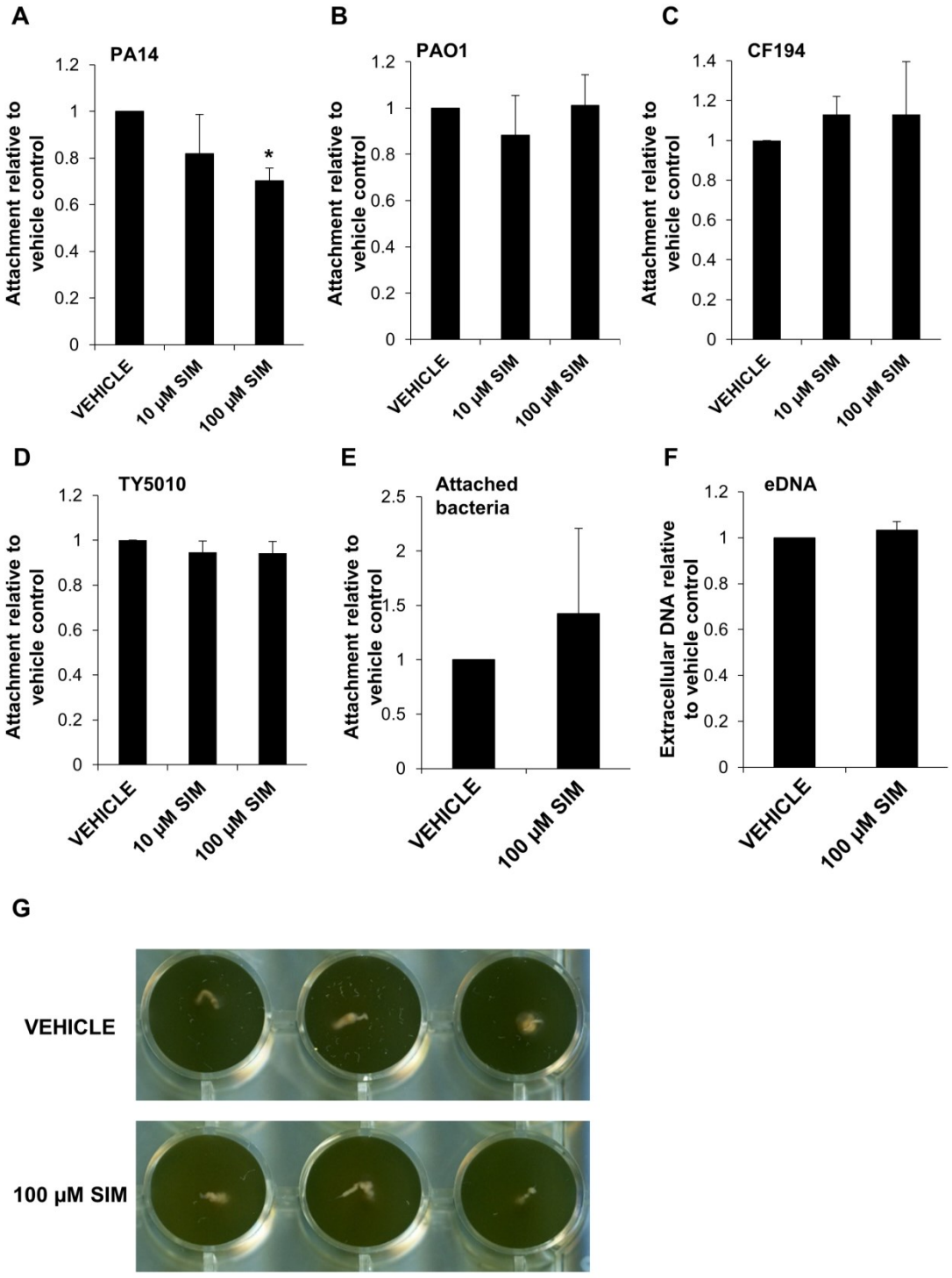


Figure 4: Biofilm-related phenotypes of *P. aeruginosa* in the presence of statins. The effect of statins on bacterial attachment was tested by crystal violet staining after 2 hours of incubation with 100 µM simvastatin. **(A)** Attachment of PA14 was significantly decreased by 100 µM simvastatin, and a non-significant reduction was observed with 10 µM simvastatin. **(B), (C)** and **(D)** Simvastatin did not influence the attachment of PAO1, CF194

and TY5010. **(E)** Crystal violet staining was also used to investigate whether statin treatment of attached bacteria could result in their removal. 100 μM simvastatin did not significantly influence PA14 which had previously attached to a plastic surface. **(F)** The effect of statins on eDNA production by PA14 was examined using propidium iodide staining. There was no change in eDNA levels in 100 μM simvastatin-treated cells compared to DMSO-treated cells. **(G)** PA14 was cultured in artificial sputum media in the presence of 100 μM simvastatin to determine whether statin treatment could affect tight microcolony formation. This phenotype (visible as a solid structure in the centre of the well) was not altered by simvastatin compared to the DMSO control. $*P \leq 0.05$. Data are representative of 3 biological replicates.

P. aeruginosa quorum sensing is not altered by statins

As swarming motility and attachment are linked to quorum sensing, the effect of statins on this phenomenon was examined. *P. aeruginosa* produces several types of AHL quorum sensing molecules, and the *Pseudomonas* quinolone signal (PQS) and its precursor molecule 2-heptyl-4-quinolone (HHQ). PQS-dependent signalling has been found to be responsible for decreased swarming motility (22) and increased biofilm formation (41) and it was hypothesised that a statin-mediated alteration in quorum sensing may be responsible for the inhibition of swarming motility by statins. PQS and HHQ are synthesised via the *pqsABCDE* operon (42) and therefore the effect of statins on the first gene in this operon, *pqsA*, was analysed. A PA14 strain containing a *pqsA-lacZ* reporter construct was cultured in the presence of 100 μM simvastatin and *pqsA* expression was examined using a Miller assay. The expression of *pqsA* in statin-treated bacteria was equivalent to its expression in vehicle-treated bacteria, demonstrating that statins did not have an effect on *pqsA* expression (Figure 6A). It can be hence inferred that statins did not influence the regulation of the *pqsABCDE* operon.

The impact of statins on AHL-mediated QS was investigated by utilising a well-characterised indicator strain, *Chromobacterium violaceum* CV026. *C. violaceum* wildtype produces a purple pigment called violacein in an AHL-dependent manner. However, CV026 possesses a Tn5 insertional mutation in the region responsible for AHL synthesis (a *luxI* homologue), meaning that this strain cannot produce violacein unless it is exposed to exogenous sources of AHLs. Therefore, CV026 can be used as a biosensor to measure AHL activity. The effect of statins on AHLs was tested by cross-streaking CV026 with *P. aeruginosa* PAO1 and PA14, in the presence of 100 μM simvastatin or a DMSO control. Violacein production was observed at equal

levels in statin-treated bacteria and in controls, indicating that statins did not alter AHL production (Figure 6B). It should be noted that CV026 biosensor activity is optimal for AHLs which have C_8 acyl chains (C_8-HSL). However, given that AHL production in *P. aeruginosa* is mediated through the LasI/LasR system (43, 44), it can be inferred that from the observations made in this study that statins do not affect production of AHLs by *P. aeruginosa*.

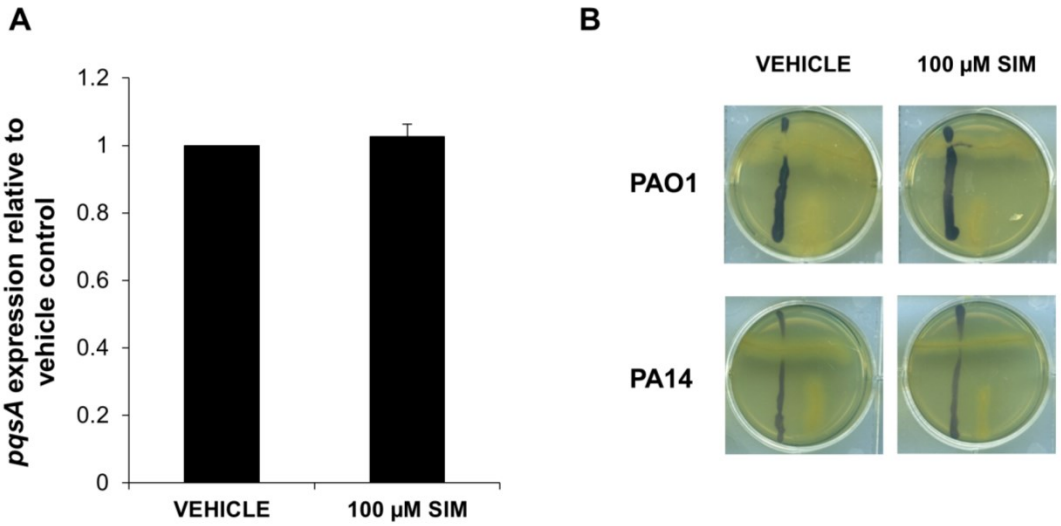


Figure 5: Effect of simvastatin on quorum sensing of *P. aeruginosa*. (**A**) A *pqsA* reporter strain was utilised to examine if statins influenced quinolone production. 100 µM simvastatin did not influence the expression of *pqsA* compared to the DMSO control. **(B)** The effect of statins on AHL production was tested using the indicator strain *Chromobacterium violaceum* CV026. PAO1 and PA14 were cross-streaked with CV026 in the presence of 100 µM simvastatin. There was no change in pigment production by CV026 treated with simvastatin compared to that treated with DMSO, indicating that statin treatment did not influence AHL production.

Transcriptomic analysis of the effect of simvastatin on *P. aeruginosa*

The results of phenotypic analysis indicated that the effect of statins on *P. aeruginosa* was targeted towards 2 particular types of virulence behaviour, swarming motility and early attachment. These virulence factors are united in that they are both regulated by complex yet well-defined genetic pathways. Therefore, the effect of statins on gene expression of *P. aeruginosa* was examined using a global transcriptomic approach. *P. aeruginosa* PA14 was cultured with either 100 μM simvastatin or an equivalent volume of DMSO, and cells were harvested at OD_{600nm} of 0.8 ± 0.05 for microarray analysis. Following statistical analysis under the least stringent conditions, a total of 47 genes with a fold change of ≥ 1.5 were identified (Table 3) (Appendix A5). It was immediately noted that, according to heat map analysis, total gene expression was at a similar level between statin-treated and vehicle-treated cells (Figure 6A). Furthermore, the normalised intensity values of genes in biological replicate 1 of statin-treated cells appeared to cluster differently than those of biological reps 2 and 3, indicating that some disparity was present in gene regulation between the replicates (Figure 6B). This was reflected in the average fold changes of genes, many of which had large standard deviations due to incongruent gene expression between each biological replicate of statin-treated bacteria. Altered genes fell into 7 functional categories, with the majority of altered genes classified as having either metabolic or hypothetical functions (Figure 6C). Other altered functional clusters were chemotaxis, transport, ribosomal, regulation and secretion. No genes typically associated with motility or biofilm were found to be altered.

Five candidate genes were chosen to validate the array - PA0514 (PA14_6700), PA0515 (PA14_06710), PA0516 (PA14_06720), PA0520 (PA14_06770), and

PA0521 (PA14_06790). These genes were chosen as they appear to have a common function in that they are present in a cluster responsible for the production of nitrite reductase (45). Furthermore, these genes are part of 2 operons – PA0514-516 are predicted to be part of the *nirSMCFDLGHJEN* cluster, and PA0520 and PA0521 are part of the predicted *nirQ* operon (46). qRT-PCR analysis of these genes was carried out in statin-treated PA14 cells compared to vehicle-treated bacteria (Figure 6D). No effect was noted on PA0514 and PA0515 – the fold changes of these genes compared to vehicle were 1.19 and 1.14 respectively. The fold changes of these genes in the microarray analysis were -3.28 and -1.54. Meanwhile, PA0516 and PA0520 were increased to a greater extent by simvastatin, with non-statistically significant fold changes of 1.64 and 1.7, compared to relative fold changes of -0.67 and 114.22 in the microarray. PA0521 was increased almost 3 fold by simvastatin, compared to an overall fold increase of 6.56 in microarray data. Given that the fold changes of 4 out 5 genes tested in this experiment differed from those observed in the microarray, and taking the variation in microarray expression levels into consideration, it was concluded that statins do not have a significant or reproducible effect on *P. aeruginosa* gene expression.

Table 3: Microarray analysis of differentially-regulated PA14 genes altered in the presence of simvastatin. Fold change (FC) is the average of 3 biological replicates and STDEV is the standard deviation of the fold change. P-value is based on an unpaired Student's T-Test.

PA No.	Name	FC	STDEV	p-value	Function
PA0025	*aroE*	-0.88	0.20	0.00	Shikimate 5-dehydrogenase
PA0028		0.90	0.70	0.03	Hypothetical protein
PA0047		4.71	8.69	0.01	Hypothetical protein
PA0171		5.76	9.50	0.04	Hypothetical protein
PA0179		-1.61	1.76	0.02	Probable 2 component response regulator/chemotaxis response regulator
PA0271		1.50	1.49	0.01	Hypothetical protein
PA0437	*codA*	2.66	3.13	0.01	Cytosine deaminase/chitinase
PA0514	*nirL*	-3.28	4.19	0.04	Heme biosynthesis
PA0515		-1.54	1.77	0.03	Probable transcriptional regulator
PA0516	*nirF*	-0.67	0.95	0.01	Heme d1 biosynthesis
PA0520	*nirQ*	114.22	198.41	0.02	Regulatory protein
PA0521	*nirO*	6.56	12.11	0.05	Probable cytochrome c oxidase subunit
PA0656		1.99	4.05	0.02	Probable HIT family protein
PA0835	*pta*	-1.18	0.52	0.02	Phosphate acetyltransferase
PA1523	*xdhB*	0.57	0.37	0.01	Xanthine dehydrogenase
PA1664	*orfX*	-2.02	2.96	0.03	Putative protein secretion/export apparatus
PA2567		17.34	29.04	0.00	Hypothetical protein (predicted c-di-GMP phosphodiesterase)
PA2663	*ppyR*	0.73	0.80	0.03	*psl* and pyoverdine operon regulator

PA2788		-2.31	3.63	0.04	Probable chemotaxis transducer
PA3049	*rmf*	10.09	18.13	0.03	Ribosome modulation factor
PA3054		-1.28	2.90	0.05	Hypothetical protein (predicted carboxypeptidase)
PA3179		-2.19	3.00	0.03	Hypothetical protein (predicted pseudouridine synthase activity)
PA3418	*ldh*	2.66	3.85	0.00	leucine dehydrogenase
PA3452	*mqoA*	0.56	0.37	0.04	Malate:quinone oxidoreductase (pyruvate metabolism)
PA3609	*potC*	1.85	1.83	0.03	Polyamine transport protein
PA3615		-0.38	1.21	0.00	Hypothetical protein
PA3641		-50.27	94.05	0.01	Probable amino acid permease
PA3651	*cdsA*	-1.65	1.39	0.03	phosphatidate cytidylyltransferase
PA3741		-1.05	0.09	0.01	Hypothetical protein
PA3820	*secF*	0.46	0.33	0.02	Secretion protein
PA3821	*secD*	1.03	0.74	0.01	Secretion protein
PA3882		2.37	2.86	0.04	Hypothetical protein
PA4349		1.67	0.92	0.02	Hypothetical protein (putative acyl-coA dehydrogenase)
PA4351	*olsA*	1.57	0.23	0.00	1-acyl-sn-glycerol-3-phosphate acyltransferase
PA4357		1.58	0.77	0.04	Conserved hypothetical protein (provisional ferrous iron transport protein)
PA4358		1.36	0.92	0.00	Probable ferrous iron transport protein
PA4432	*rpsI*	0.98	1.02	0.03	30S ribosomal protein S9
PA4673		-0.65	0.19	0.00	Hypothetical protein (predicted GTPase)
PA4811	*fdnH*	-1.39	1.09	0.04	Nitrate-inducible formate dehydrogenase

PA4916		-1.76	1.29	0.05	Hypothetical protein (putative ADP-ribose pyrophosphate)
PA4919	*pncB1*	2.44	1.27	0.02	Nicotinate phosphoribosyltransferase
PA4920	*nadE*	-1.57	0.93	0.01	NH3-dependent NAD synthetase
PA4933		1.23	0.82	0.00	Hypothetical protein
PA4934	*rpsR*	0.62	0.17	0.01	30S ribosomal protein S18
PA4935	*rpsF*	-0.73	0.75	0.03	30S ribosomal protein S6
PA5025	*metY*	-0.51	0.34	0.01	Homocysteine synthase
PA5207		2.15	2.16	0.03	Probable phosphate transporter

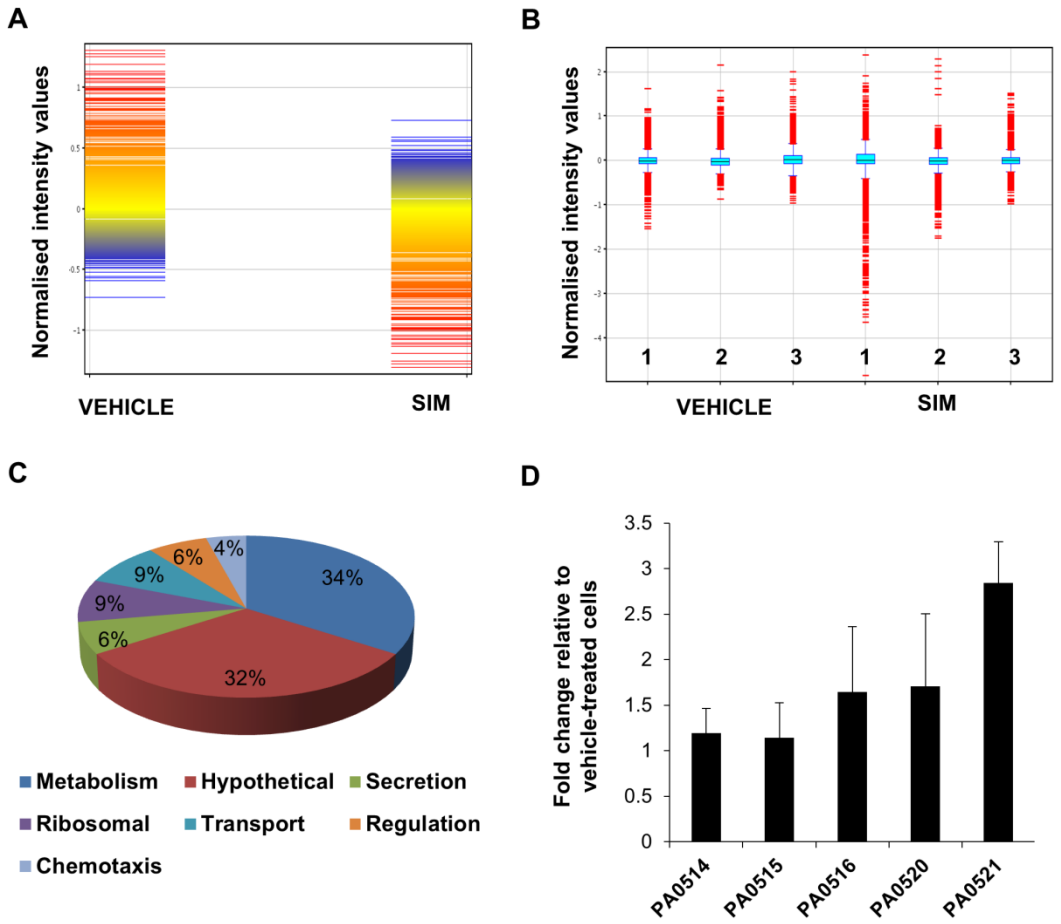


Figure 6: Analysis and validation of microarray data. (A) Heat mapping demonstrated that the overall gene expression levels were comparable between vehicle-treated and statin-treated cells. **(B)** Intensity values of expressed genes were similar in vehicle-treated cells, but 1 biological replicate of statin-treated cells appeared to be out-lying. **(C)** Functional grouping of statin-regulated genes in *P. aeruginosa* demonstrated that the majority of altered transcripts had either hypothetical or metabolic functions. **(D)** Expression of PA0514, PA0515, PA0516, PA0520 and PA0521 in the presence of 100 µM simvastatin. Fold change is calculated as the average ratio of statin-treated bacteria to vehicle-treated bacteria for 3 biological replicates.

Analysis of the effect of statins on *P. aeruginosa* chemotaxis

As the expression of swarming and attachment-associated genes was not altered in microarray analysis, it was hypothesised that the effect of statins on these phenotypes may be mediated through post-translational modification of proteins, alteration of protein-protein interactions or direct interactions between statins and proteins. Chemotaxis is one phenotype which is linked to both motility and attachment (47), and therefore the effect of statins on this phenomenon was examined. Firstly, experimental optimisation was undertaken by investigating the chemo-attractive capability of the amino acids arginine, glycine and serine towards *P. aeruginosa* PA14 in order to find the strongest chemoattractant. It was determined that arginine was more chemoattractive to *P. aeruginosa* than either glycine or serine (Figure 7A).

The impact of statin treatment on this effect was subsequently examined. Bacteria were inoculated into media containing 100 μM simvastatin or an equal volume of DMSO, and were exposed to capillaries containing media supplemented with 10 mM arginine. Bacteria cultured in all conditions were capable of significant chemotaxis towards arginine (p-values were 0.009 for un-treated bacteria, 0.023 for vehicle-treated bacteria and 0.002 for statin-treated bacteria), suggesting that statin-treated bacteria were equally capable of movement towards arginine compared to controls (Figure 7B). Therefore, it appeared that chemotaxis towards arginine was not affected by simvastatin.

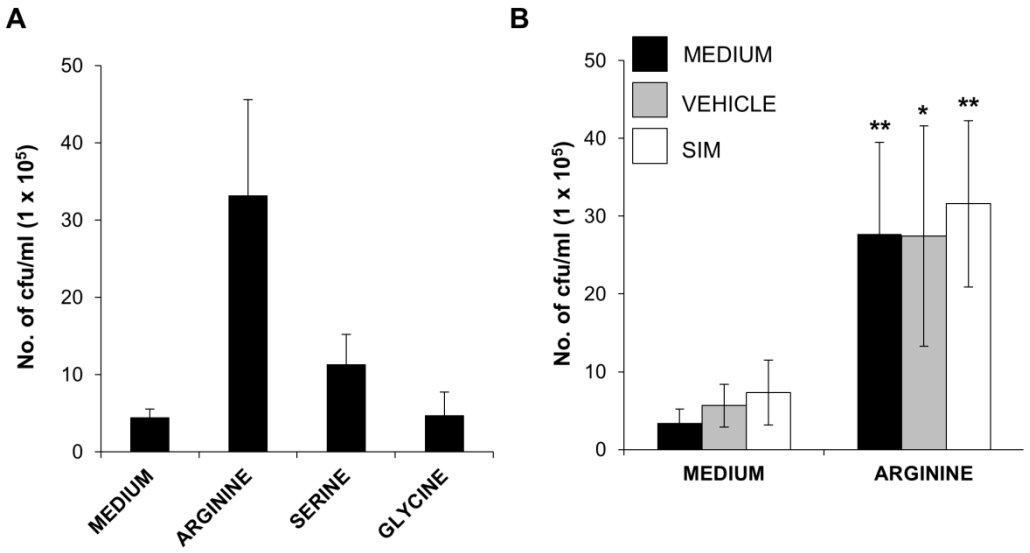


Figure 7: *P. aeruginosa* chemotaxis and statins. (A) Medium containing arginine, glycine and serine was aspirated into capillaries, and the movement of *P. aeruginosa* PA14 towards the amino acids was observed. Arginine proved to have the strongest chemo-attractant effect on PA14. **(B)** PA14 was equally capable of chemotaxis towards arginine under all treatment conditions. $*P \leq 0.05$, $**P \leq 0.001$. Data are representative of 3 biological replicates.

The effect of simvastatin on *P. aeruginosa* attachment is mediated through the PvrR response regulator

P. aeruginosa PA14 possesses over 140 environmental sensors and response regulators (46). In order to determine whether any of these proteins were involved in the attenuation of swarming and attachment by simvastatin, the PA14 transposon library constructed by Liberati *et al.* (28) was employed. Mutants in each of the PA14 sensory and regulatory proteins were screened for swarming and attachment phenotypes in the presence of 100 μM simvastatin. It was not possible to elucidate the role of these proteins in the effect of simvastatin on motility, as many of the mutant strains were found to be non-motile. However, 27 mutant strains screened were found to display restored or increased attachment in the presence of simvastatin. Of these, 1 strain – a mutant in the *pvrR* gene (*ΔpvrR*), which encodes the PvrR regulatory protein – reproducibly demonstrated increased attachment in the presence of simvastatin compared to WT PA14 ($P = 0.02$) (Figure 8A).

PvrR negatively regulates the expression of the *cupA* and *cupD* gene clusters which are involved in fimbriae production (48, 49). Furthermore, PvrR possesses an EAL domain, meaning that it functions catabolically as a cyclic-di-GMP (c-di-GMP) phosphodiesterase (50, 51). The c-di-GMP signalling molecule is known to promote biofilm production and inhibit swarming motility of *P. aeruginosa* (52, 53). In addition, the ectopic expression of PvrR was found to restore virulence to an attenuated mutant of the WspF methylesterase, suggesting that PvrR and WspF may interact (54). Therefore, the effect of simvastatin on the attachment of mutants in the *cupA* and *cupD* operons, as well as on a *wspF* mutant, was examined in order to determine whether these gene products played a role in the attenuation of *P. aeruginosa* attachment by simvastatin (Figure 8B). The attachment of *ΔwspF* and

ΔcupA1 mutants was significantly increased by simvastatin compared to the WT (*P* = 0.017 and 0.019 respectively). In addition, non-significant increases were observed in *ΔcupA2* and *ΔcupD1* compared to WT. This suggested that simvastatin may inhibit fimbriae production in *P. aeruginosa*. WspF is involved in *P. aeruginosa* motility, as the swarming phenotype of a *wspF* mutant strain was found to be attenuated (54). As simvastatin increased attachment in a *ΔwspF* background, it was questioned whether statin treatment could also restore the swarming phenotype of a *wspF* mutant. *ΔwspF* was cultured in the presence of 100 μM simvastatin, but remained non-motile (Figure 8C).

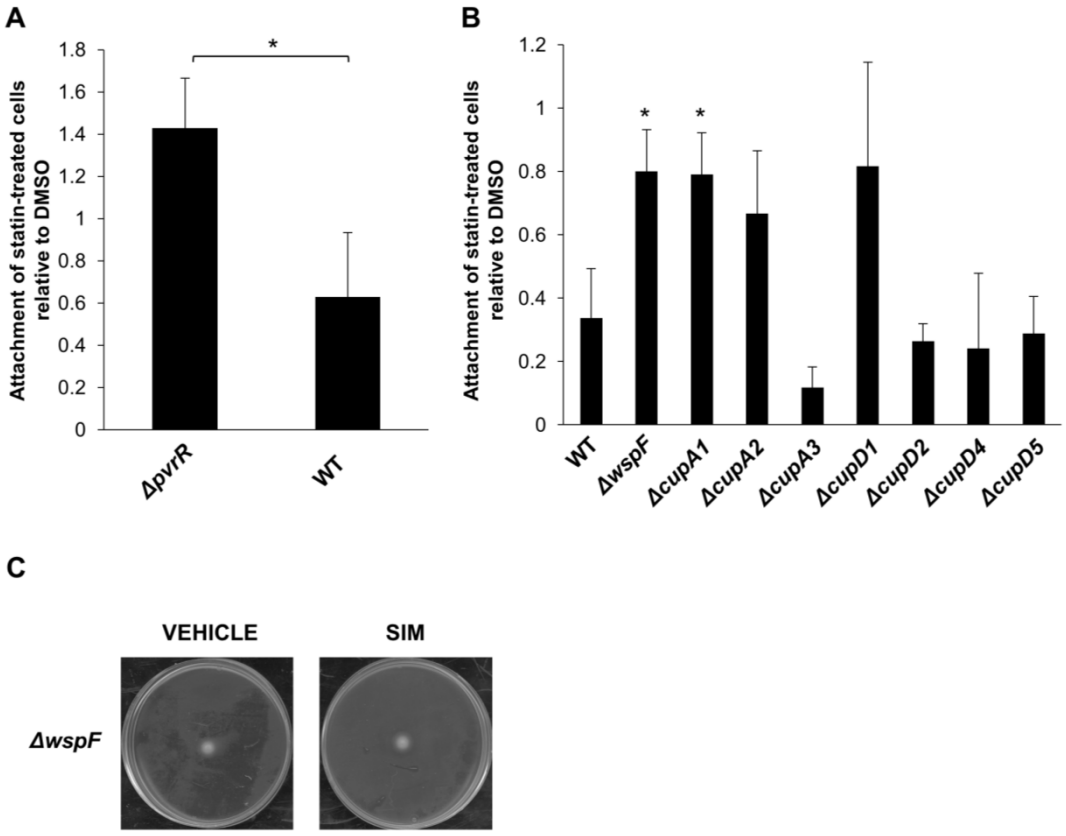


Figure 8: The role of *pvrR*, *wspF* and fimbriae-associated *cup* genes in the effect of simvastatin on *P. aeruginosa*. Bacterial attachment in the presence of 100 μM simvastatin was tested by crystal violet staining after 2 hours of incubation. **(A)** Simvastatin increased attachment in a PvrR mutant. **(B)** Attachment of the PvrR regulatory targets WspF, CupA1, CupA2 and CupD1 was increased by simvastatin. **(C)** Simvastatin did not restore motility to an attenuated *wspF* mutant. *$P \leq 0.05$. Data represent 3 biological replicates.

Regulation of the *P. aeruginosa* proteome by simvastatin

A role for PvrR, CupA1, CupA2 and CupD1 in the impact of simvastatin on *P. aeruginosa* PA14 had previously been identified. The transcriptomic expression of these proteins was not found to be altered in microarray analysis, and it therefore appeared that simvastatin may be influencing *P. aeruginosa* at the proteomic level. This hypothesis was subsequently examined by isolating protein expression at the swarm tip of *P. aeruginosa* PA14 cultured in the presence and absence of simvastatin. Total protein was harvested from the outermost 1 cm of the swarm tip, and samples were separated by 2-DGE. Altered proteins were identified using mass spectrometry (Appendix A6 and A7). This analysis demonstrated that 6 proteins were reproducibly induced by simvastatin treatment (Table 4). Two of these proteins appear to play a role in virulence. LptD is an outer membrane protein which transports LPS to the outer membrane (55), whereas AcnB is a metabolic protein which may repress expression of Exotoxin A (ExoA) (56). Of the remaining proteins, 2 are involved in translation (FusA1 and RpsA) (57, 58), while Pnp is involved in transcription (59). Finally, DnaK acts as a chaperone during chromosomal DNA replication and is involved in the heat shock response (60). Interestingly, 5 of the altered proteins were also found to be post-translationally modified by simvastatin.

In order to verify that the altered proteins were involved in the attenuation of swarming by simvastatin, swarming assays were carried out on PA14 transposon mutant strains in 3 target proteins – Δ*lptd*, Δ*pnp* and Δ*dnaK* (Figure 9). Δ*lptd* was motile to a comparable extent as the wild type, and the motility of this strain was attenuated by simvastatin in a similar manner to that of the wild type. However, Δ*pnp* displayed only limited motility, which was reduced in the presence of

simvastatin. In addition, Δ*dnaK* was non-motile irrespective of simvastatin treatment.

Table 4: Upregulated PA14 swarm front proteins in the presence of simvastatin. Results are based on 2 biological replicates.

Accession	PA No.	Name	Function	Post-translational modification	
				Oxidation	Carbamidomethylation
PA14_07770	PA0595	LptD/ OstA	Organic solvent tolerance protein	+	+
PA14_08820	PA4266	FusA1	Elongation factor G	+	+
PA14_23330	PA3162	RpsA	30S ribosomal protein	-	-
PA14_41470	PA1787	AcnB	Aconitate hydratase	+	+
PA14_62710	PA4740	pnp	Polyadenylase	+	+
PA14_62970	PA4761	DnaK	Molecular chaperone	+	-

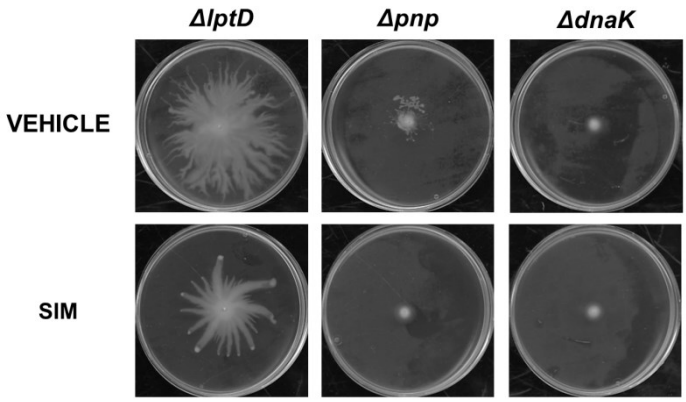


Figure 9: Swarming motility of mutants in altered *P. aeruginosa* proteins. Mutant strains in *lptD*, *pnp* and *dnaK* were analysed in the presence of 100 μM simvastatin or an equivalent amount of vehicle. Simvastatin inhibited swarming motility of *ΔlptD*, and reduced the limited motility of *Δpnp*. *ΔdnaK* was non-motile regardless of treatment. Data are representative of 3 biological replicates.

Discussion

It has previously been demonstrated that growth and virulence behaviour of bacterial species can be altered by simvastatin (Chapter 2). However, all of the species investigated possessed a homologue of HMGR, and at least one other mevalonate pathway protein homologue. It was thus questioned whether statins were effective against these bacteria because of their metabolic capabilities, and sought to investigate further by testing the effects of statins on a bacterial species which does not possess HMGR. *P. aeruginosa* was chosen for this analysis because it is a virulent CF-associated pathogen. Unlike the other bacterial species tested, statins did not influence the growth of *P. aeruginosa*. This may be due to the mevalonate-independent isoprenoid metabolism in this species. Previous studies have reported that both Type 1 and Type 2 statins decreased the growth of *P. aeruginosa* (34, 61). These studies used the strains ATCC 27853 and ATCC 9027. However, it was demonstrated that statins did not impact the growth of ATCC 27853 in this study. One possible explanation for this is that the highest statin concentration used in this study was lower than those used in previous reports. The highest concentration that was tested was 1 mM, whereas Welsh *et al*. reported MICs equating to 447.5 mM (atorvastatin) and 208 mM (rosuvastatin) (34). Although statins did not affect the growth of *P. aeruginosa*, it appears that they have different efficacies against different strains. This hypothesis is support by the effects of simvastatin on *P. aeruginosa* biofilm formation observed in this work. Simvastatin had no effect on the attachment of PAO1 and the clinical isolates CF194 and TY5010, suggesting that its effect on PA14 was mediated through PA14-specific genes or proteins.

Statins did not affect the promoter expression of the TTSS toxin ExoS. This correlates with an observation made by Rosch and colleagues that simvastatin

treatment did not influence the secretion of the *Streptococcus pneumoniae* toxin pneumolysin (62). Statins were able to reduce swarming, but not swimming, twitching or chemotaxis, suggesting that they did not influence the production and activity of flagella or pili, but instead targeted physiological capabilities of the bacteria. Over 200 genes are involved in swarming motility, and these are involved in processes as diverse as RNA and protein synthesis, metabolism and transport (63). It therefore appeared that simvastatin may influence some of these processes. The results obtained in 2-DGE analysis of the *P. aeruginosa* swarm tip appeared to confirm this, as simvastatin altered expression of proteins responsible for RNA and protein synthesis, as well as transport of LPS.

Several biofilm-related phenotypes were tested in the presence of simvastatin, but only 1 of these (early attachment) was attenuated, suggesting that statins impact the beginning of biofilm production, but not biofilm maturation. The alteration of attachment suggested that statins may also be altering *P. aeruginosa* quorum sensing, as biofilm formation is regulated by PQS- and AHL-mediated signalling (24, 64). In particular, LasI/R-mediated signalling typically occurs when bacteria become irreversibly attached to surfaces (24). Neither type of signalling was altered in the presence of simvastatin. Overall, the phenotypic data obtained suggested that the effect of statins on *P. aeruginosa* was specifically directed towards early biofilm formation.

A reproducible statin effect on the *P. aeruginosa* transcriptome was not observed in this study. The genomic contents of PAO1 and PA14 are similar, but the PA14 genome is larger than that of PAO1, due to the presence of 2 pathogenicity islands (65) and PA14 is known to possess almost 500 genes which are not present in PAO1 (66). One technical limitation of this work is that PA14 cDNA was hybridised to a

gene chip specific for PAO1, as a PA14 chip is not currently available. This issue was addressed by examining the effect of simvastatin on PA14 sensor and regulator proteins. It was identified that the PvrR regulatory protein appeared to be involved in the effects of simvastatin on *P. aeruginosa*. Furthermore, CupA1, CupA2 and CupD1 expression also appears to be altered by simvastatin treatment. CupA1 and CupD1 both encode fimbrial subunits, while CupA2 acts as a chaperone protein (49, 67). Both *pvrR* and the *cupD* operon are encoded by the PAPI-1 pathogenicity island (65), meaning that they are absent in PAO1. This may account for the inability of simvastatin to reduce the attachment of this strain. This could also suggest that the clinical isolates CF194 and TY5010 lack a PvrR homologue. The genomes of CF194 and TY5010 have not yet been sequenced, but it is possible that they do not possess orthologues of PAPI-1 and PAPI-2. The WspF methylesterase was also found to be associated with the impact of simvastatin on *P. aeruginosa* attachment. WspF expression is associated with decreased biofilm formation (68), and it therefore appears that simvastatin may increase the expression or post-translational activity of this protein. Furthermore, it appears that simvastatin may attenuate the virulence of *P. aeruginosa* by reducing intercellular c-di-GMP levels, as both PvrR and WspF are associated with decreased c-di-GMP (48, 68).

The *P. aeruginosa* proteome was modified by simvastatin. This analysis confirmed the hypothesis that the effect of simvastatin on *P. aeruginosa* was extremely specific, as only 6 proteins were increased by statin treatment. In addition, this may be the first study to demonstrate post-translational modification of bacterial proteins by statins. It appears that the most promising candidate proteins from this work were LptD and AcnB, due to their roles in virulence. Simvastatin attenuated swarming motility in an *lptD* mutant, suggesting that, although it altered LptD, this protein was

not essential for reduction of motility. However, as LPS is required for *P. aeruginosa* motility (10), it is possible that simvastatin may decrease motility by alteration of LPS synthesis or transport. It was not feasible to examine the swarming phenotype of an *acnB*-deficient strain. Therefore, future studies should be conducted to elucidate the essentiality of AcnB in the repression of swarming by simvastatin, and to examine impact of simvastatin on LPS and ExoA production.

One interesting observation from this study was that the effects observed for the three statins tested – lovastatin, mevastatin and simvastatin – were comparable. These 3 statins are type 1 statins, meaning that they are lipophilic and are composed of a conserved central lactone ring, to which a distinct butaryl side chain and decalin ring are attached (69). The relative structural similarity of the statins tested could give reason to the comparable effects exerted on bacterial virulence. However, *in situ* these statins are hydrolysed by hepatocytes to give an "active" form where the lactone ring is cleaved (70). Therefore, an *in vitro* hydrolysis of the statins was performed to determine whether the active form of the statins still yielded the same inhibitory effects. Activated lovastatin and mevastatin no longer had an inhibitory effect on swarming motility, but activated simvastatin still had a modulatory effect, suggesting that the side chain of simvastatin has some impact in the exertion of its effects. Simvastatin differs from lovastatin and mevastatin by way of 2 methyl groups in the secondary carbon position in its side chain (C-2), and the inhibition of swarming motility by hydrolysed simvastatin suggests that this functional group may play a role in mediating the anti-virulence effects of simvastatin. It is unclear from the current literature whether statins are metabolised or altered by *P. aeruginosa*. In the liver, statins are converted to their active form by esterase activity, or can be metabolised by cytochrome P450. Although *P. aeruginosa* contains esterase and

cytochrome P450 orthologues, the results of this study seem to indicate that statins are not hydrolysed by the bacteria in the same manner that they are by hepatocytes.

Based on the data accumulated in this study, it is evident that *P. aeruginosa* is affected by statins. It may be thus inferred that *P. aeruginosa* has some mechanism whereby it perceives statins and its behaviour is altered accordingly. The results obtained in this study indicate that statins could potentially be used as a novel anti-microbial agent against *P. aeruginosa*, but they would not be suitable for use as a sole anti-microbial agent, due to their extremely specific and limited anti-virulence effects, and the fact that the concentrations used in this study were much higher than the levels typically found in blood. However, as it has been demonstrated that inhalation of the antibiotic tobramycin increased its levels in the lungs (71, 72), there may be potential for statin use either in combination therapy, or as an inhaled drug.

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Chapter 4

The impact of simvastatin on pulmonary effectors of *Pseudomonas aeruginosa* infection

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Title: The impact of simvastatin on pulmonary effectors of *Pseudomonas aeruginosa* infection

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Abstract

The statin family of cholesterol-lowering drugs is known to have pleiotropic properties which include anti-inflammatory and immunomodulatory effects. Statins exert their pleiotropic effects by altering expression of human immune regulators including pro-inflammatory cytokines. Previously it was found that statins modulate virulence phenotypes of the human pathogen *Pseudomonas aeruginosa*, and therefore it was investigated whether simvastatin could alter the host response to this organism in lung epithelial cells. Simvastatin increased the expression of the *P. aeruginosa* target genes KLF2, KLF6, IL-8 and CCL20, demonstrating the novel induction of KLF6 and a novel pro-inflammatory effect of statins on lung cells. Furthermore, both simvastatin and *P. aeruginosa* induced alternative splicing of KLF6. *P. aeruginosa* was also capable of inducing KLF6 alternative splicing in a cystic fibrosis model. The impact of *P. aeruginosa* on KLF6 alternative splicing was found to be mediated through the bacterial ExoS toxin. In addition, the novel effect of simvastatin on wtKLF6 expression was found to be responsible for induction of the KLF6-regulated genes CCL20 and iNOS, which have roles in the immune response. Simvastatin also increased the adhesion of *P. aeruginosa* to host cells, without altering invasion or cytotoxicity. Taken together, these results demonstrated several novel effects of simvastatin on the pulmonary cellular immune response.

Introduction

The key function of the statin family of drugs is inhibition of cholesterol biosynthesis. However, these compounds are also known to have cholesterol-independent pleiotropic effects. One of the many pleiotropic effects of statins is the attenuation of bacterial infections. It has previously been demonstrated that statins improved survival in patients who had sepsis and pneumonia (1–6). This can be attributed in part to the inhibition of anti-inflammatory pathways. For instance, statins can reduce activation of the pro-inflammatory cytokine Interleukin-8 (IL-8) by bacterial and fungal surface antigens in *ex vivo* blood cells (7). Production of another infection-induced chemokine, Chemokine (C-C motif) ligand 20 (CCL20; also known as macrophage-inhibitory protein 3α (MIP-3α)), is also reduced by statin treatment of keratinocytes (8). As well as inhibiting pro-inflammatory components, statins also increase the expression of anti-inflammatory components, such as endothelial nitric oxide synthase (eNOS) (9) and thrombomodulin (10). The anti-inflammatory and immunomodulatory effects of statins are mediated through the alteration of expression of transcription factors such as NFκB (11).

One group of transcriptional regulators that is now emerging as both a key group of immune and inflammatory regulators and a target of statin treatment is the Kruppel-like Factors (KLFs). These are a family of transcription factors characterised by the presence of 3 conserved zinc fingers (12). Two KLF family members, KLF2 and KLF6, have previously been found to be increased in an *in vitro Pseudomonas aeruginosa* infection model (13). *P. aeruginosa* is an important human pathogen that causes serious infections in immunocompromised individuals and is the main pathogen associated with chronic refractory pulmonary infections in cystic fibrosis (CF) patients. The pathogenesis of *P. aeruginosa* can be attributed to a plethora of

virulence associated phenotypes including the production and secretion of toxins via the Type 3 secretion system (TTSS). Previously, the exotoxins ExoS and ExoY secreted by the TTSS were shown to be required for *P. aeruginosa* induction of KLF2 and KLF6 in CF airway epithelial cells (13). KLF2 is mainly characterised as an anti-inflammatory regulator which can control the activation of monocytes and macrophages (14, 15), and has been found to be induced by toxin activity of *Staphylococcus aureus* (16), *Yersinia enterocolitica* (17) and *Clostridium botulinum* (18). Furthermore, statins can also induce the expression of KLF2 (18, 19).

The role of KLF6 in infections is currently less clear than that of KLF2. KLF6 is most widely-recognised as a tumour suppressor protein which inhibits proliferation (20) and induces apoptosis (21). However, KLF6 is known to be alternatively spliced into a full-length transcript (wtKLF6) and 3 truncated variants (SV1, SV2, SV3) (22). In contrast to the tumour-suppressive action of wtKLF6, the splice variants of KLF6, particularly SV1, have been found to have oncogenic properties and have been implicated in several types of cancer including lung, liver, ovarian and prostate (22–25). Given that they do not possess the 3 zinc fingers of wtKLF6 it is believed the splice variants cannot exert their biologic effects through direct binding of DNA but instead must interact with other proteins (26). For example, SV1 has been shown to bind directly to wtKLF6 accelerating its degradation and thus antagonising its tumour suppressor function (27).

While to date, it has not been characterised, it is likely that KLF6 does play a role in bacterial infections, as it is upregulated by *S. aureus* (16) and *P. aeruginosa* (13), and can promote the apoptosis of cells infected with respiratory syncytial virus (28) and influenza A (29). Furthermore, some regulatory targets of KLF6 are known to have roles in the immune response to infections. KLF6 directly activates promoter

expression of ASAH1 (acid ceramidase) (30) and iNOS (inducible nitric oxide synthase; NOS2) (31), 2 proteins that regulate the production of signalling molecules with roles in the inflammatory response. ASAH1 negatively regulates the production of ceramide, which is upregulated as part of the host response to *P. aeruginosa* (32), leading to increased inflammation (33). iNOS is responsible for the production of nitric oxide (NO), a signalling molecule which is responsible for increased inflammation, lung damage and bacterial killing during infections (34, 35). In addition, KLF6 also induces the expression of the pro-inflammatory chemokine CCL20 (MIP-3α). It has been proposed that this effect is indirect, mediated through the KLF6-dependent inhibition of another transcription factor called Peroxisome Proliferator-Activated Receptor γ (PPARγ) in kidney cells (36).

In this study, it was sought to investigate the effect of simvastatin on the expression of *P. aeruginosa*-responsive immune modulators, including KLF6 splice variants, in a lung epithelial cell model. In addition to KLF2 and KLF6, *P. aeruginosa* has been shown to alter the expression of several modulators of the host immune response including IL-8 (37), CCL20 (38) and TLR5 (Toll-like Receptor 5) (39), which activates NFκB-dependent inflammation in response to bacterial cell surface component flagellin (40, 41). It was demonstrated that simvastatin induces pro-inflammatory modulators of the immune response and that both *P. aeruginosa* and simvastatin induce KLF6 splice variants in A549 epithelial cells, with the wtKLF6 being the dominant variant. Thus, it was observed that simvastatin could alter the genetic and physiological immune response in lung cells and KLF6 was identified as a novel target of statins and an important transcriptional regulator in the lung.

Materials and methods

Mammalian cells and bacterial strains

All cell lines and bacterial strains used in this study are detailed in Table 1. A549 cells were grown in minimal Eagle's medium (MEM) (Sigma-Aldrich, U. K.) supplemented with 10 % foetal bovine serum (FBS), 50 units/ml pen-strep and 2 mM L-glutamine. Phoenix packaging cells were utilised for the production of recombinant retroviruses and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10 % FBS, 50 units/ml pen-strep and 2 mM L-glutamine. IB3-1 cells were cultured on plastic coated with bovine serum albumin (BSA)-collagen-fibronectin using LHC-8 medium supplemented with 10% FBS and 50 units/ml of penicillin-streptomycin. Infection assays were carried out using co-culture medium (LHC-8 or MEM minus pen-strep), and cells that were used in the determination of cytotoxicity were cultured in serum-free medium. All medium components were obtained from Gibco unless stated otherwise. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

All *P. aeruginosa* strains were cultured in Luria-Bertani broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) and were subcultured for infection assays in co-culture medium. A complete list of cell lines and strains used in this study is detailed in Table 1.

Generation of stable cell lines

wtKLF6 and SV1-specific cDNA fragments were cloned into the pSUPER retroviral si-RNA vector (Oligoengine) between the *Hind*III and *Bgl*II sites (described in Narla

et al., 2005a and Narla *et al.,* 2005b (22, 42)). Phoenix cells were co-transfected with 4 µg of each pSUPER-wtKLF6, pSUPER-luc (vector control) and a pAMPHO viral vector (Clontech) using the X-tremeGENE reagent (Roche). Transfection was performed as per the manufacturer's recommendations. Fresh medium was added to cells at 8 hours post transfection, and virus was collected 12 hours after this. Virus was collected by removing medium from cells, and passing it through a 0.45 µm filter. Following the first collection, virus was collected twice more at successive 12 hour timepoints. All collected viruses were stored at 4 ºC.

A549 cells were seeded in 10 cm diameter plates at a density of 8000 cells/cm^2, and allowed to grow for 16-20 hours. Medium was aspirated, and 6 ml of virus containing 10 µg/ml polybrene (Sigma) was added to each dish. Cells and virus were co-incubated for 12 hours at 37 ºC and 5 % CO_2 for 12 hours, after which medium was aspirated and 2 successive infections were carried out in the same manner. Following this, medium was aspirated and replaced with fresh medium containing 0.5 µg/ml puromycin (Sigma). Medium was replaced every 2-3 days and cells were cultured until 80-90 % confluency was achieved, following which they were trypsinised and passaged as previously described. All pSUPER-cells were maintained in medium supplemented with 0.5 µg/ml puromycin throughout the course of each experiment.

Table 1: Cell lines and bacterial strains used in this study

	Type	Reference
Mammalian cell line		
A549	Wild type squamous alveolar epithelia	ATCC
A549 pSUPER-wtKLF6	si-RNA-mediated knockdown of wtKLF6	This study
A549 pSUPER-Retro Luc	Vector Control	This study
IB3-1	Bronchial epithelial cell line derived from a CF patient with CFTR *ΔF508*	ATCC
Phoenix	HEK 293T transfected with gag, pol and env constructs for retrovirus production	(43)
Bacterial strain		
P. aeruginosa PAO1	Wild type	(44)
P. aeruginosa ΔexoS	Mutant in *exoS*	(45)
P. aeruginosa ΔexoY	Mutant in *exoY*	(45)
P. aeruginosa ΔexoSΔexoY	Double mutant in *exoS* and *exoY*	(45)
P. aeruginosa PAO1 RP289	Isogenic wild type	(45)

Statin treatment and infection of cells

Simvastatin (Sigma-Aldrich) was resuspended in DMSO at a concentration of 20 mg/ml, filter sterilised and stored at 4 °C. A549 cells were washed twice with PBS, trypsinised, and seeded into plastic vessels at a density of 8 x 10^3cells/cm^2. Cells were then incubated until they achieved 80 % confluency, following which they were treated with either 10 μM simvastatin or an equivalent volume of DMSO. Cells were treated with simvastatin for 24 hours.

Bacteria were cultured shaking at 37 °C at 16-18 hours in LB broth, following which they were sub-cultured in infection medium shaking at 37 °C for 3 hours. Subsequently, the culture was centrifuged (2330 x g; 10 minutes), and washed twice with PBS, following which the bacterial densities were adjusted so as to infect cells at a multiplicity of infection (MOI) of 50:1.

RNA isolation and quantitative real-time PCR (qRT-PCR)

RNA isolation was carried out using an RNeasy mini kit (QIAGEN, Germany) for A549 cells or an RNeasy Plus mini kit (QIAGEN) for IB3-1 cells according to manufacturer's specifications, and was quantified using a ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Genomic DNA was removed using Turbo DNA-*free*™ (Ambion), and samples were confirmed to be free of DNA by PCR. RNA was converted to cDNA using oligod(T) and AMV reverse transcriptase (both Promega). qRT-PCR was carried out using the Universal ProbeLibrary system (Roche) according to manufacturer's specifications, and

samples were normalised to hypoxanthine phosphoribosyltransferase (HPRT). A full list of primers used in this study is detailed in Table 2.

Table 2: Primers used for qRT-PCR analysis of host gene expression.

Gene	Product Length (bp)	Sequence	Probe No.*
HPRT	102	Forward: tgaccttgatttattttgcatacc	73
		Reverse: cgagcaagacgttcagtcct	
KLF2	67	Forward: cctcccaaactgtgactggt	115
		Reverse: ctctgtagccacgctgtgc	
wtKLF6	70	Forward: aaagctcccacttgaaagca	2
		Reverse: ccttcccatgagcatctgtaa	
SV1	109	Forward: ggcacttccgaaagcaca	24
		Reverse: cctcagaggtgcctcttcat	
SV2	96	Forward: gggaaccttctcaactgtgg	24
		Reverse: aaggcttttctcctggcttc	
SV3	96	Forward: acgcacacaggtgtttttcc	32
		Reverse: ccttttagcctacaggatccac	
IL-8	77	Forward: gccaggatccacaagtcct	98
		Reverse: tggtggctaatacttttttccact	
CCL20	77	Forward: tggcttttctggaatggaat	117
		Reverse: tgtgcaagtgaaacctccaa	
ASAH1	86	Forward: ttgacatttggggatctggt	10
		Reverse: attcaacacccacgctgaa	
iNOS	68	Forward: ttccttacgaggcgaagaag	3
		Reverse: tcagagcgctgacatctcc	
PPARγ	78	Forward: accagctgaatccagagtcc	81
		Reverse: gcgggaaggactttatgtatga	

* The annealing temperature of all primer sets is 60 ºC.

Determination of cytotoxicity

Cytotoxicity of statin treatment and/or *Pseudomonas aeruginosa* PAO1 infection was measured using a LDH Cytotoxicity Detection Kit (Roche) according to manufacturer's instructions. Infection and statin-treatment of A549 cells were set up as previously described, except that in this instance serum-free medium was used, as FBS has been found to cause high background in this assay. The OD_{492nm} of samples was measured at 1.5, 3 and 6 hours post infection using a SpectraMax Plus 384 96 well plate reader (Molecular Devices).

Measurement of bacterial adhesion and invasion

Adhesion and invasion of bacteria were quantified as described by Burns *et al.,* 1996 (46). Briefly, A549 cells were treated with simvastatin and infected with *P. aeruginosa* PAO1 as previously described. For determination of invasion, after 1 hour of infection ceftazimide (1 mg/ml) and gentamicin (2 mg/ml) (both Sigma-Aldrich) were added for a further 2 hours, following which cells were washed twice with PBS and lysed using 0.1 % Triton X-100. Intercellular bacteria were enumerated by serial dilutions and plate counts. To measure bacterial adherence, extracellular bacteria were removed from epithelial cells, which were lysed to provide a total and intercellular count following 3 hours of infection. The adherent count was determined by subtracting the number of intercellular and extracellular bacteria from the total bacterial count.

Statistical analysis

A paired T-test was used to determine statistical significance where appropriate.

Results

Simvastatin influences the gene expression of *P. aeruginosa*-responsive host immune modulators

In order to investigate the effect of statins on the expression of infection-responsive and immunomodulatory genes, 5 genes previously shown to be induced during *P. aeruginosa* infection were selected for analysis: KLF2, KLF6, IL-8, CCL20 and TLR5 (13, 37–39). The expression of these candidate genes was analysed in A549 squamous epithelial lung cells which were treated with 10 μM simvastatin or an equivalent volume of DMSO vehicle control for 24 hours, following which they were infected with *P. aeruginosa* PAO1 at an MOI of 50:1 for 3 hours. Gene expression was analysed using qRT-PCR. Simvastatin has been found to induce KLF2 in endothelial cells & human peripheral blood monocytes (18) and the results obtained in this study complied with these findings. KLF2 was significantly increased in statin-treated A549 cells compared to DMSO-treated cells ($P = 0.012$) (Figure 1A). Interestingly, *P. aeruginosa* also significantly induced KLF2 expression ($P = 0.033$) but to a much lesser degree than simvastatin alone and there was no additive effect in statin-treated infected cells ($P = 0.008$). Previous studies have shown that statins were ineffective against KLF6 expression (18), however here a significant induction of wtKLF6 by simvastatin treatment was demonstrated in A549 lung epithelial cells ($P = 0.005$) (Figure 1B). *P. aeruginosa* also significantly induced KLF6 ($P = 0.015$), but to a greater extent than simvastatin (fold change), and this effect was sustained in simvastatin-treated infected cells ($P = 0.0019$). Thus, there was also no additive effect of simvastatin and *P. aeruginosa* on wtKLF6 expression.

As expected, *P. aeruginosa* infection significantly induced IL-8 expression; transcript levels in infected cells were over 100 times higher than control cells (Figure 1C). Interestingly, in contrast to published reports (7) it was observed that simvastatin increased the expression of IL-8 by 14.75-fold compared to vehicle treated epithelial cells. However, when cells were pre-treated with simvastatin and subsequently infected with *P. aeruginosa* the level of IL-8 expression was not significantly different to untreated infected cells. Therefore, again there was no additive effect of simvastatin and *P. aeruginosa* on IL-8 expression.

CCL20 expression was also significantly increased by *P. aeruginosa* compared to uninfected cells ($P = 0.03$) and it was increased to a lesser extent (14.5-fold; $P = 0.02$) by simvastatin alone compared to vehicle-treated cells (Figure 1D). Interestingly however, in simvastatin-treated infected cells, a significant increase in CCL20 expression was observed ($P = 0.02$). This effect was greater than CCL20 induction by simvastatin ($P = 0.02$) or *P. aeruginosa* ($P = 0.054$) individually, suggesting that CCL20 expression was synergistically induced by a combination of these factors.

In contrast to IL-8 and CCL20, TLR5 expression was not significantly altered by simvastatin (Figure 1E). The expression of this gene was significantly increased by *P. aeruginosa* ($P = 0.015$) but interestingly, under combined statin treatment and infection expression levels were comparable to statin-treated uninfected cells suggesting that simvastatin may reduce *P. aeruginosa*-mediated induction of TLR5.

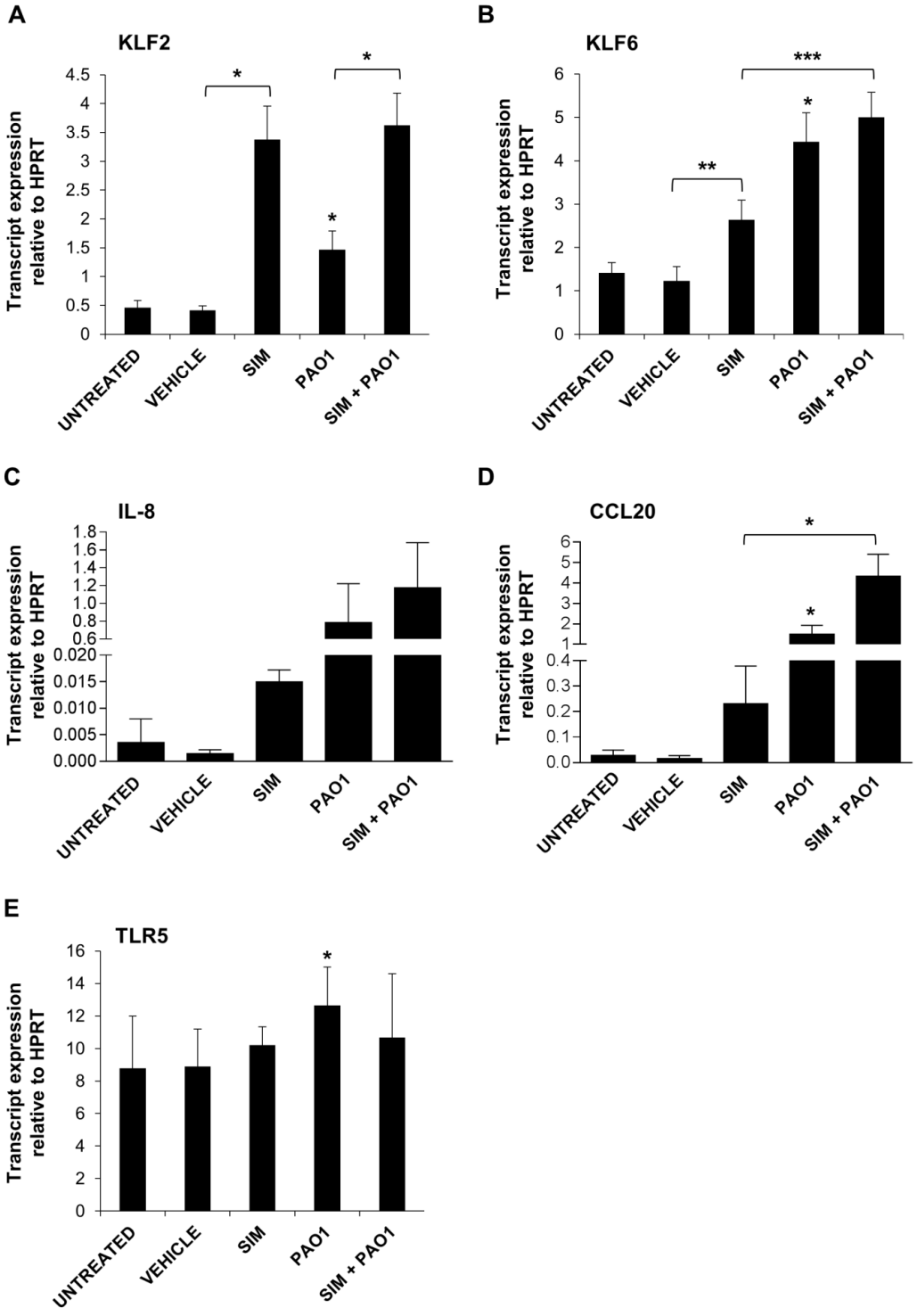


Figure 1: Effect of statin treatment on the host response to *P. aeruginosa* infection. A549 cells were treated with 10 μM simvastatin for 24 hours, and were subsequently infected with *P. aeruginosa* PAO1 for 3 hours at MOI 50:1. The effect of simvastatin, PAO1 and simvastatin + PAO1 was examined on **(A)** KLF2 **(B)** KLF6 **(C)** IL-8 **(D)** CCL20 and **(E)** TLR5. **P*, ≤ 0.05, ***P*, ≤ 0.01, ****P*, ≤ 0.001.

Novel induction of KLF6 alternative splicing by *P. aeruginosa* and simvastatin

While the results obtained here confirm *P. aeruginosa* induction of wtKLF6 expression (13), the expression of KLF6 splice variants in the context of bacterial infections has never been characterised. Therefore, this study sought to examine whether *P. aeruginosa* could influence the alternative splicing of KLF6 in lung epithelial cells. For this analysis, A549 cells were infected with *P. aeruginosa* PAO1 for 3 hours, at an MOI of 50:1, and the expression of KLF6 splice variants was analysed by qRT-PCR.

In uninfected cells, a basal level of expression was observed for each splice variant; however, wtKLF6 was clearly the dominant variant (Figure 2A). *P. aeruginosa* induced the expression of all 3 KLF6 splice variants but to varying degrees. Of the 3 variants, SV3 was increased to the greatest extent ($P = 0.005$), and SV2 was also significantly induced ($P = 0.013$). SV1 displayed the lowest level of induction of the 3 variants and this did not reach significance. Interestingly, wtKLF6 was induced to the greatest magnitude by *P. aeruginosa* ($P = 0.015$). Thus, wtKLF6 was the dominant variant in A549 cancer lung epithelial cells under basal and *P. aeruginosa* infected conditions.

Given the prominence of *P. aeruginosa* as an opportunistic pathogen in CF patients, it was subsequently examined whether *P. aeruginosa* could induce KLF6 alternative splicing in CFTR-deficient cells. For this analysis, IB3-1 cells were utilised. IB3-1 cells are bronchial epithelial cells which possess the most common CFTR mutation, Δ508, which is present in approximately 70 % of CF patients (47). IB3-1 cells were infected with *P. aeruginosa* PAO1 for 3 hours and expression of the KLF6 splice variants was examined using qRT-PCR. Interestingly, it was observed that the

pattern of basal splice variant expression differed between IB3-1 and A549 cells. In IB3-1 cells (Figure 2B), wtKLF6 was expressed at a slightly lower level than in A549 cells. Furthermore, SV3 expression was extremely low in IB3-1 cells, compared to both its expression in A549 cells, and the expression of the other transcripts. In contrast, SV1 and SV2 were present at higher levels in IB3-1 cells than in A549 cells, and also appeared to be more abundantly expressed than wtKLF6. *P. aeruginosa* induced all splice variants in CF cells, but the pattern of expression varied compared to that of A549 cells. *P. aeruginosa*-induced wtKLF6 expression was lower in IB3-1 cells than in A549 cells, although the fold increase of wtKLF6 was comparable between both cell types. Similarly, SV1 expression was increased by *P. aeruginosa* in CF cells in a comparable manner to A549 cells, but unlike wtKLF6, its corresponding transcript levels were higher in CF cells. SV2 was significantly increased by *P. aeruginosa* in IB3-1 cells ($P = 0.02$), but the extent of this increase was lower than in A549 cells. SV3 was also significantly induced by *P. aeruginosa* in CF cells ($P = 0.03$), but both the fold change and transcript expression were lower here than in non-CF cells. It therefore appears that CFTR deficiency may influence the expression levels and *P. aeruginosa*-mediated induction of KLF6 splice variants.

The mechanism behind the induction of KLF6 alternative splicing by *P. aeruginosa* was subsequently investigated. KLF2 and KLF6 were previously found to be induced by the ExoS and ExoY toxins from the *P. aeruginosa* Type 3 secretion system (TTSS) (13), and therefore this study examined whether these toxins were also responsible for induction of KLF6 alternative splicing. A549 cells were infected with *P. aeruginosa* PAO1 single mutants in *exoS* (*ΔexoS*) and *exoY* (*ΔexoY*), a double *exoSexoY* mutant (*ΔexoSΔexoY*), and an isogenic PAO1 WT strain (RP289),

for 3 hours. qRT-PCR was used to test the expression of wtKLF6, SV1, SV2 and SV3 in infected cells relative to uninfected cells. As expected, WT PAO1 significantly increased the expression of all variants: wtKLF6 (P = 0.005), SV1 (P = 0.008), SV2 (P = 0.0007) and SV3 (P = 0.01) (Figure 2C). In cells infected with *ΔexoS*, no significant change was observed in any of the transcripts tested. This was also the case for cells that were infected with *ΔexoSΔexoY*. However, when cells were infected with *ΔexoY*, expression of all variants was induced to a comparable level to those of cells infected with the WT strain. These data indicated that ExoS was responsible for the *P. aeruginosa*-mediated induction of KLF6 and its splice variants, and demonstrated a lack of essentiality of ExoY in this mechanism.

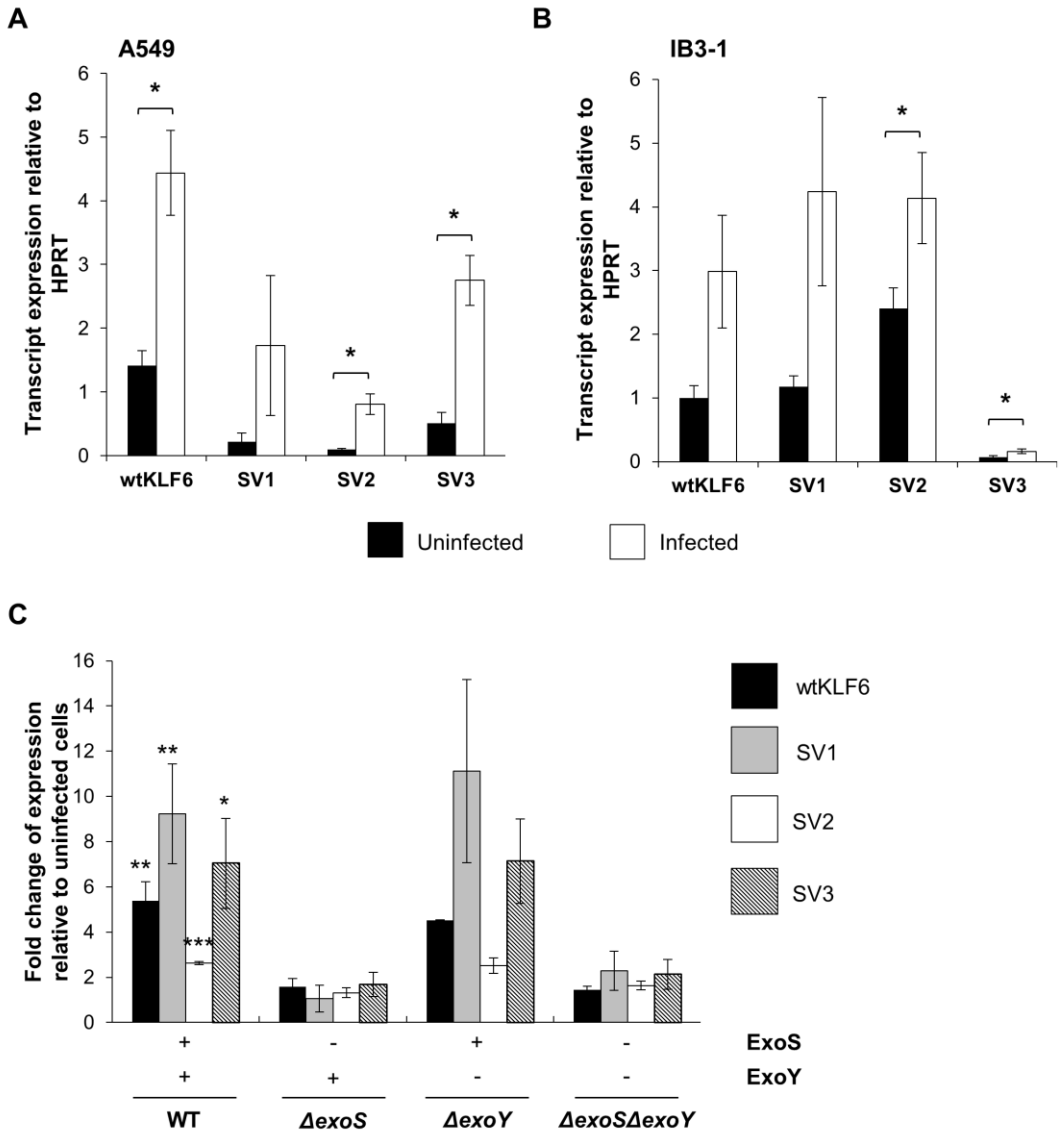


Figure 2: The effect of *P. aeruginosa* on KLF6 alternative splicing. Cells were infected with *P. aeruginosa* PAO1 for 3 hours at MOI 50:1 and expression of wtKLF6, SV1, SV2 and SV3 was examined using qRT-PCR. **(A)** *P. aeruginosa* PAO1 induced KLF6 alternative splicing in A549 lung cells. **(B)** The expression of KLF6 splice variants was examined in CFTR-deficient cells (IB3-1). All variants were upregulated in response to PAO1, but the fold changes of SV2 and SV3 were lower than those of cells with a non-CF phenotype. **(C)** The *P. aeruginosa* ExoS toxin is required for the induction of KLF6 splice variants. **P*, ≤ 0.05, ** *P*, ≤ 0.001, *** *P*, ≤ 0.0001. Data represent 3 biological replicates.

As both statins and *P. aeruginosa* had significant effects on the RNA level of expression of wtKLF6, and *P. aeruginosa* could induce KLF6 alternative splicing, the expression of KLF6 splice variants in response to simvastatin alone and in combination with *P. aeruginosa* infection in lung epithelial cells was subsequently examined. As before, A549 cells were pre-treated with 10 μM simvastatin for 24 hours and subsequently infected with *P. aeruginosa* PAO1 for 3 hours and splice variant expression was analysed using qRT-PCR. Simvastatin increased the expression of SV1 (Figure 3A), SV2 (Figure 3B) and SV3 (Figure 3C), with SV3 levels reaching significance ($P = 0.01$). As with wtKLF6, *P. aeruginosa* had a greater effect on the expression of all 3 splice variants than simvastatin and there was a slight but not significant additive effect on SV1 and SV3 in simvastatin pre-treated infected cells. Of particular note was that the transcript levels of wtKLF6 were higher than those of SV1, SV2 and SV3 under all conditions, suggesting that wtKLF6 was the dominantly expressed variant in A549 cells and in response to simvastatin and *P. aeruginosa* infection.

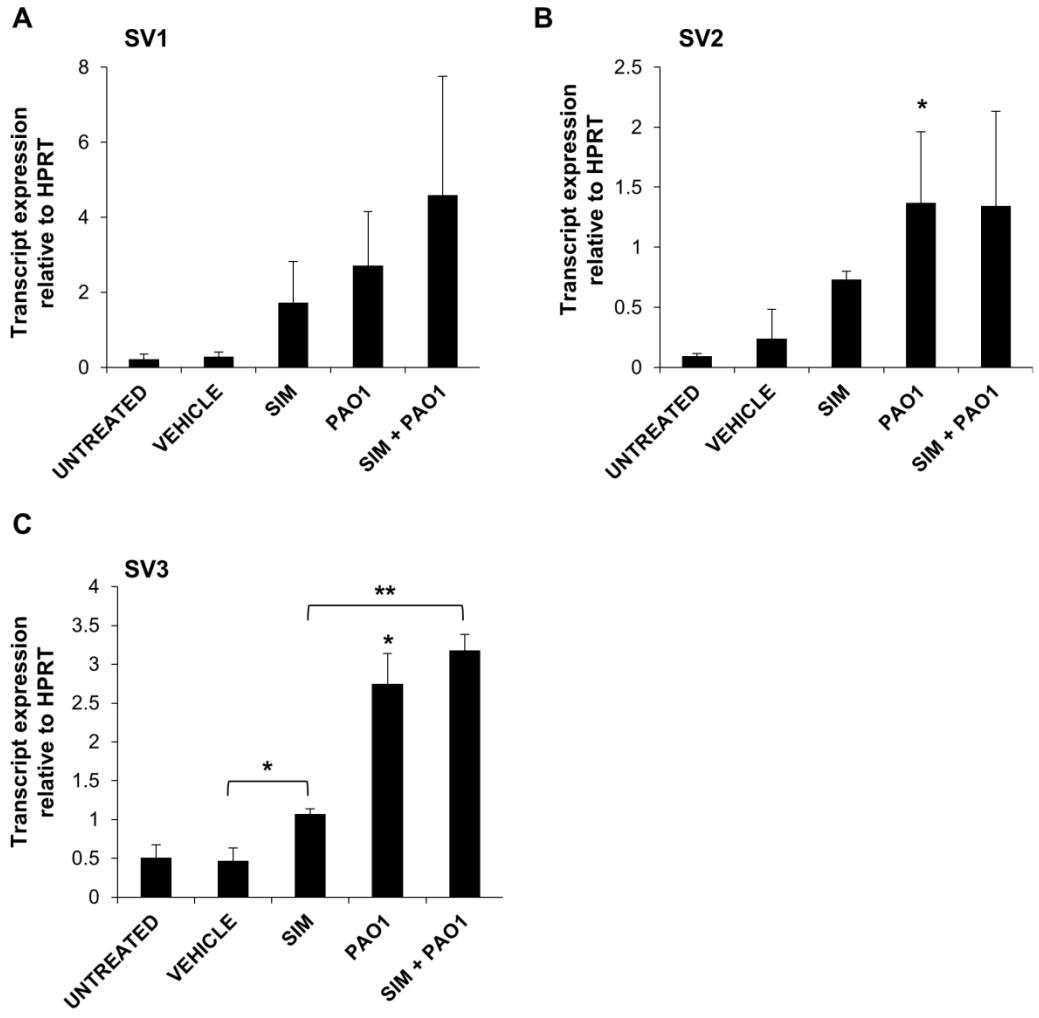


Figure 3: Induction of KLF6 splice variants by simvastatin. A549 cells were treated with 10 μM simvastatin for 24 hours, following which they were infected with *P. aeruginosa* PAO1 for 3 hours at MOI 50:1. The expression of **(A)** SV1, **(B)** SV2 and **(C)** SV3 was examined using qRT-PCR. **P*, ≤ 0.05, ***P*, ≤ 0.01.

Elucidating the downstream effect of statin-mediated KLF6 induction

In order to further examine the influence and impact of the induction of KLF6 splice variants by *P. aeruginosa* and statins, stable knockdown cell lines were generated for wtKLF6 and SV1 (respectively designated si-wtKLF6 and si-SV1). A549 cells were transfected with pSUPER retroviral vectors containing fragments of wtKLF6 and SV1 cDNA, and positive clones were selected using puromycin. To verify that si-cells were displaying reduced expression of wtKLF6 and SV1, they were harvested for RNA isolation, and the expression of wtKLF6 and SV1 was examined by qRT-PCR. It was demonstrated that in cells containing pSUPER-wtKLF6, wtKLF6 expression was significantly reduced by 47.2 % compared to the cells containing the vector control (designated si-luc) (P = 0.008) (Figure 4A). However, in cells containing pSUPER-SV1, SV1 was expressed at a comparable level to cells containing the vector control (Figure 4A), demonstrating that the si-SV1 vector was not functioning as expected.

To examine the role of wtKLF6 and SV1 in *P. aeruginosa* infection, si-wtKLF6, si-SV1 and vector control cell lines were infected with *P. aeruginosa* PAO1 for 3 hours at MOI 50:1 (Figure 4B). wtKLF6 and SV1 were induced to a similar extent by *P. aeruginosa* infection in their respective si-cell lines as in the si-luc cells. This analysis correlated with the previously-observed technical issues with the pSUPER-SV1 vector, but it also indicated that the effect of *P. aeruginosa* on wtKLF6 expression was able to override the inhibitory effects of the pSUPER-wtKLF6 vector. Therefore, the si-wtKLF6 cell line was found to be unsuitable for the analysis of the impact of wtKLF6 in *P. aeruginosa* infections.

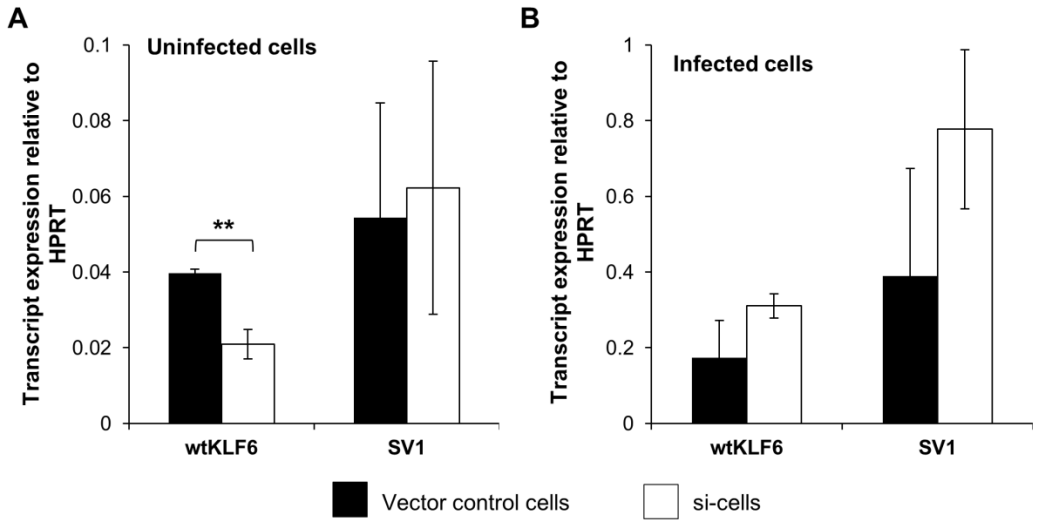


Figure 4: Validation of stable knockdown cell lines. (A) wtKLF6 mRNA expression was reduced by 47.2 % in si-wtKLF6 cells compared to vector control cells ($P = 0.008$) but SV1 expression was not altered between si-SV1 cells and vector control cells. **(B)** Both wtKLF6 and SV1 were increased by *P. aeruginosa* infection in their respective knockdown cell lines.

To investigate the consequences of KLF6 knockdown in lung cells, expression analysis was carried out on 4 KLF6 regulatory target genes: ASAH1, CCL20, iNOS and PPARγ. In si-wtKLF6 cells, the expression of 3 target genes was attenuated (Figure 5A); ASAH1, CCL20 and iNOS expression was decreased by an average of 30.3 % (non-significant), 51.5 % ($P = 0.012$) and, 72.9 % ($P = 0.047$) respectively in si-wtKLF6 cells compared to vector control cells. However, the expression of PPARγ was comparable between si-wtKLF6 and si-luc cells, suggesting that the expression of PPARγ in the model used in this study was not dependent upon wtKLF6. This also suggests that KLF6 regulation of CCL20 was not dependent on PPARγ, although it may be possible that the level of wtKLF6 reduction obtained in si-cells may not be enough to inhibit PPARγ expression.

Thus, wtKLF6 plays a key, multifaceted regulatory role in lung epithelia and the effect of simvastatin on wtKLF6 and its regulatory targets in si-wtKLF6 lung cells was subsequently examined. This analysis was carried out using qRT-PCR of transcripts isolated from pSUPER-wtKLF6 cells and vector control cells which had been treated with 10 μM simvastatin for 24 hours. Firstly, transcript expression of wtKLF6 in pSUPER-wtKLF6 cells was examined to determine whether this model was suitable for use with statin treatment, given the technical issues which arose when they were subjected to infection experiments. After 24 hours of treatment, wtKLF6 was significantly increased by simvastatin in both vector control ($P = 0.015$) and pSUPER-wtKLF6 ($P = 0.004$) cell lines. However, despite this increase, wtKLF6 was still decreased by 33 % in si-wtKLF6 cells compared to vector control cells in the presence of simvastatin ($P = 0.02$) compared to a 46 % decrease between vehicle-treated si-wtKLF6 and vector control cells (Figure 5B). Based on these

results, it was concluded that wtKLF6 was sufficiently reduced in knockdown cells to allow the examination of statin-related phenotypes.

The effect of simvastatin treatment was subsequently examined on KLF6 regulatory target genes, in order to investigate the downstream effects of statin-mediated KLF6 induction. A simvastatin-mediated increase in the expression of ASAH1 in vector control and si-wtKLF6 cells was observed (Figure 5C). While ASAH1 was decreased in DMSO (vehicle)-treated si-wtKLF6 cells, its expression in statin-treated si-wtKLF6 cells was equivalent to that of statin treated si-luc cells, signifying that the induction of ASAH1 by simvastatin did not appear to be mediated through wtKLF6.

The wtKLF6-dependent decrease in CCL20 that was observed in untreated si-cells was maintained in vehicle-treated si-cells ($P = 0.04$) (Figure 5D). However, in the presence of simvastatin CCL20 expression was decreased in vector control cells, implying that the selective marker itself may have been interfering with its expression. Nevertheless, in si-wtKLF6 cells, the expression of CCL20 was equivalent between simvastatin-treated and DMSO-treated cells, suggesting that the novel CCL20 induction observed in statin-treated wild type A549 cells may be wtKLF6-dependent.

The third KLF6 target gene examined was iNOS. Interestingly, in contrast to CCL20, simvastatin treatment reduced iNOS expression in vector control cells (Figure 5E). However, in si-wtKLF6 cells, this reduction in iNOS expression by simvastatin was not observed, and expression was at an equivalent level to that of vector control cells. This latter suggests that simvastatin may inhibit iNOS expression in a KLF6-dependent mechanism.

PPARγ (Figure 5F), was significantly reduced in si-wtKLF6 cells compared to vector control cells in the presence of the vehicle ($P = 0.02$). Simvastatin treatment increased the expression of PPARγ in both cell types to an equal but non-significant extent, regardless of the presence or absence of knockdown. Therefore, PPARγ appears to be a statin target in lung cells, albeit a target which is regulated independently of KLF6.

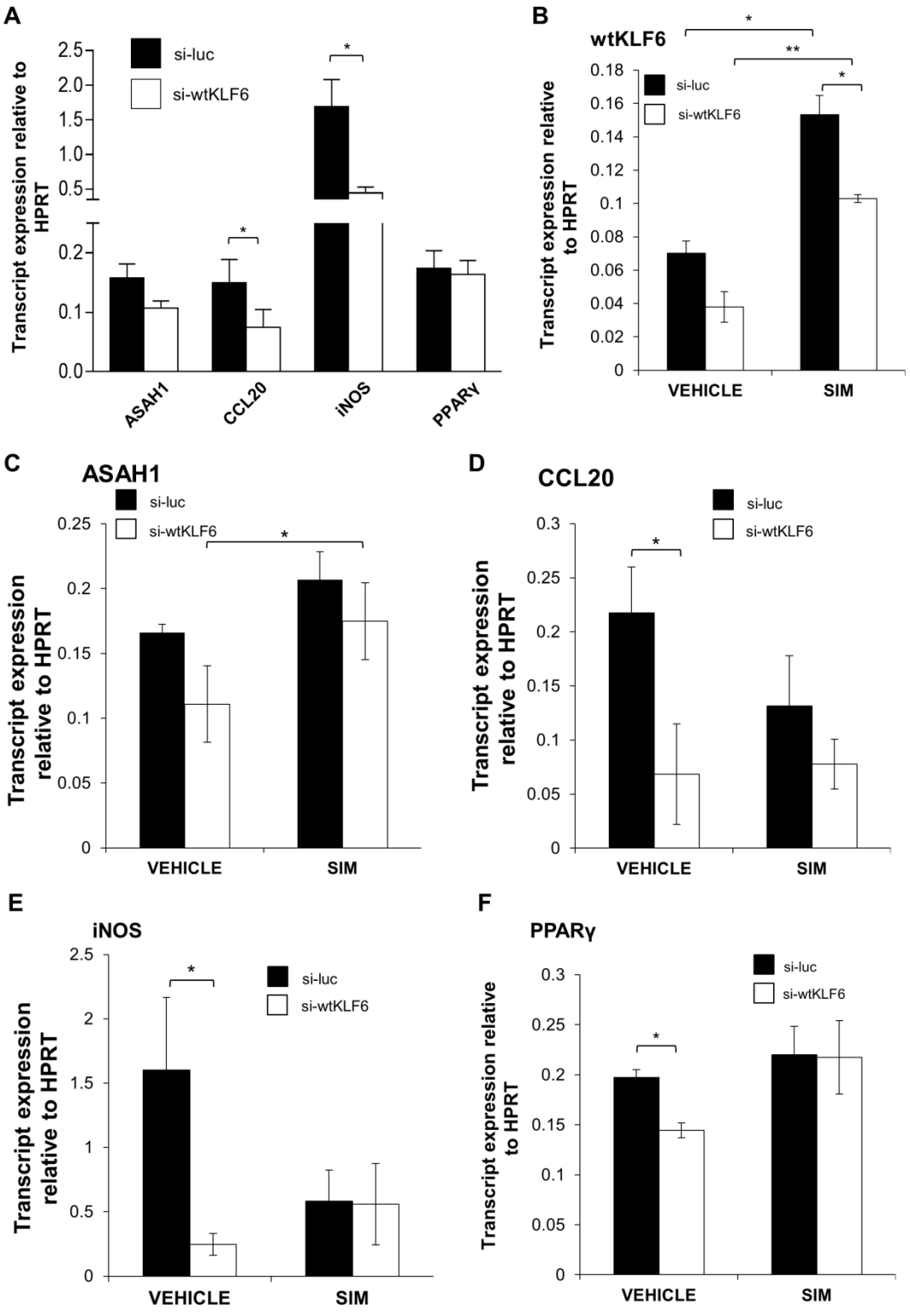


Figure 5: wtKLF6 knockdown and simvastatin effects in the lung. (A) Expression of the wtKLF6 target genes ASAH1, CCL20, iNOS and PPARγ was examined by qRT-PCR in si-wtKLF6 cells compared to si-luc cells. ASAH1, CCL20 and iNOS were inhibited in si-cells compared to vector control cells. **(B)** Simvastatin treatment did not override the effect of the

wtKLF6 knockdown vector. **(C)** wtKLF6 does not play a role in simvastatin-mediated ASAH1 expression. However, the effects of simvastatin on CCL20 **(D)** and iNOS **(E)** appeared to be regulated via wtKLF6. **(F)** PPARγ was also slightly increased by simvastatin in lung cells. **P*, ≤ 0.05, ***P,* ≤ 0.01.

The effect of simvastatin on cellular cytotoxicity and *P. aeruginosa* adhesion and invasion

Having established that simvastatin treatment can alter the induction of host immune response genes, and having previously demonstrated the alteration of *in vitro P. aeruginosa* motility and early biofilm formation by statins (48) (Chapter 2), the effect of statins on *P. aeruginosa*-related cytotoxicity, adhesion and invasion of A549 epithelial cells was investigated. The cytotoxic effect of *P. aeruginosa* is exerted via its TTSS toxins, in particular ExoS (49), which in turn is required for *P. aeruginosa*-mediated induction of KLF6 (13), suggesting that KLF6 induction may lead to *P. aeruginosa*-initiated cell death. Furthermore, the induction of IL-8 and CCL20 has previously been linked to bacterial adhesion and invasion (50–52) and therefore statins may have a regulatory impact on these phenotypes. To examine the effect of statins on these physiological outcomes, A549 cells were treated with 10 μM simvastatin for 24 hours and subsequently infected with *P. aeruginosa* PAO1.

Cytotoxicity was quantified by measuring lactate dehydrogenase (LDH) release from cells as a marker of tissue breakdown during statin treatment and *P. aeruginosa* infection (Figure 6A). LDH release was measured at 3 hours post infection (HPI), as this was the interval at which gene expression was measured, but LDH release was also evaluated at an early interval (1.5 HPI) and a late interval (6 HPI) to determine whether statins could influence the kinetics of *P. aeruginosa* infection as well as host response. At 1.5 HPI, there was no significant effect on LDH release in any samples compared to untreated cells. At 3 HPI, very minimal effects on cytotoxicity were observed. Simvastatin-treated cells exhibited slightly increased but non-significant LDH release compared to vehicle-treated cells, which also displayed a very modest level of cytotoxicity. *P. aeruginosa* had a significant cytotoxic effect ($P = 0.03$)

compared to untreated cells, and statin treatment and *P. aeruginosa* combined caused a moderate, non-significant increase in LDH release compared to statins and *P. aeruginosa* individually.

Interestingly, at 6 hours post infection, simvastatin alone had a low but significant cytotoxic effect compared to untreated and DMSO treated cells respectively (P = 0.018, P = 0.04). While *P. aeruginosa* was significantly cytotoxic compared to untreated cells (P = 0.027) and statin-treated cells (P = 0.044), in simvastatin-treated cells *P. aeruginosa* infection was significantly more cytotoxic compared to untreated cells (P = 0.0003), vehicle-treated cells (P = 0.0006) and simvastatin-treated, uninfected cells (P = 0.0005). This increase in cytotoxicity between infected cells and statin-treated infected cells suggested that the presence of simvastatin may increase cell damage caused by *P. aeruginosa*.

The role of wtKLF6 in inhibiting cell proliferation and inducing apoptosis has been extensively established (53); it was thus investigated whether the wtKLF6 played a role in the cytotoxic effects exerted by simvastatin on lung epithelial cells. si-wtKLF6 cells and vector control cells were treated with either 10 μM simvastatin or DMSO vehicle for 24 hours in serum-free medium, and cytotoxicity was quantified by measuring LDH release. In comparison to untreated cells, simvastatin caused a significant but comparable increase in LDH release in both vector control (P = 0.04) and si-wtKLF6 (P = 0.005) cells (Figure 6B). This demonstrated that wtKLF6 does not play a role in statin-mediated cytotoxicity and cell death, thus suggesting that wtKLF6-induced CCL20 and iNOS do not regulate these processes.

The effect of simvastatin on bacterial adhesion and invasion was also examined in A549 cells treated with 10 μM simvastatin for 24 hours and subsequently infected

with *P. aeruginosa* PAO1 for 3 hours. Simvastatin increased the adhesion of PAO1 to A549 cells compared to those treated with vehicle, but, in contrast, statin treatment did not alter invasion of *P. aeruginosa* compared to vehicle-treated cells (Figure 6C).

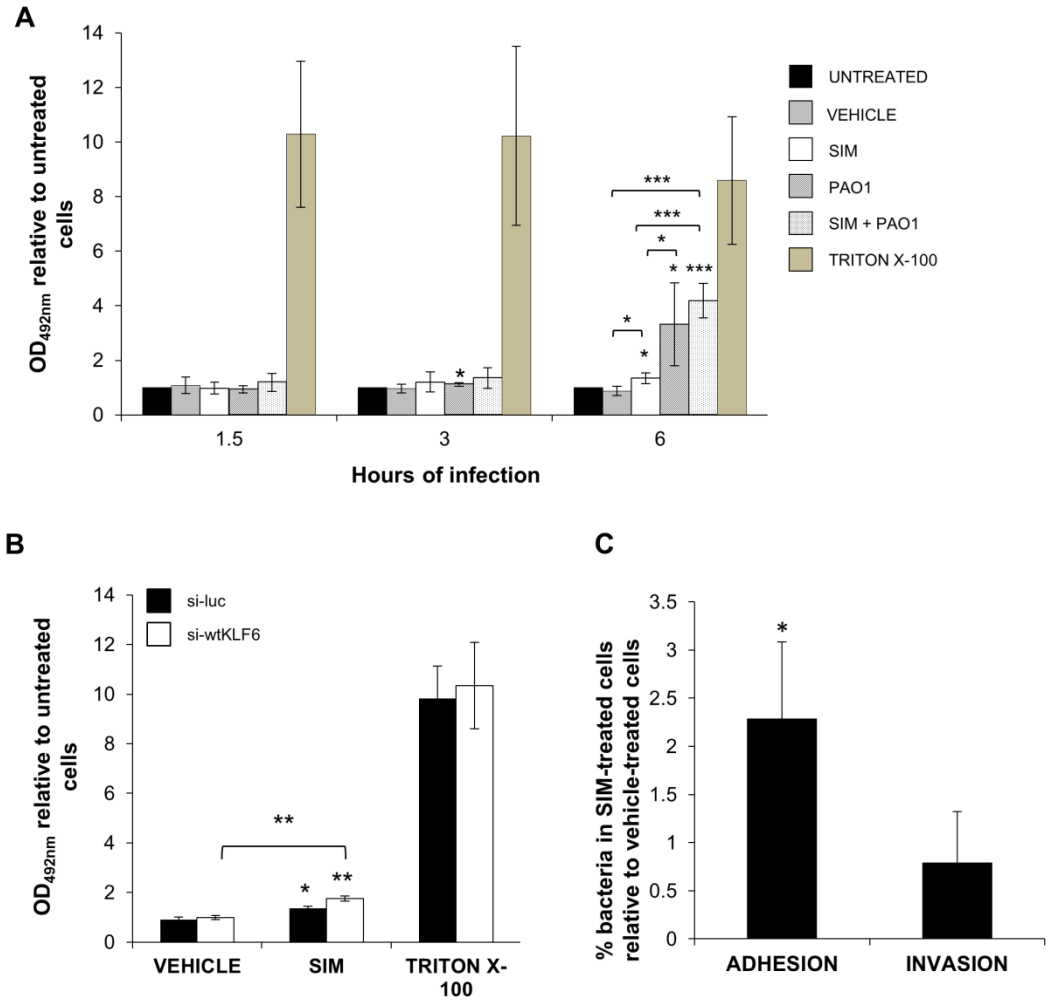


Figure 6: Cellular responses to simvastatin treatment and infection. Cells were treated with 10 μM simvastatin for 24 hours **(A, B, C)**, following which they were infected with *P. aeruginosa* PAO1 at MOI 50:1 **(A, C)**. **(A)** Simvastatin did not attenuate *P. aeruginosa*-mediated LDH release at 1.5, 3 or 6 hours post infection. **(B)** wtKLF6 was not implicated in simvastatin-mediated cytotoxic effects on lung cells. **(C)** Simvastatin treatment increased bacterial adhesion to cells, but did not influence invasion at 3 hours of infection. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Discussion

In this study, it was demonstrated that simvastatin can modulate the expression of genes involved in the host response to *P. aeruginosa* infections in lung epithelial cells. Previous studies have shown that attenuation of pro-inflammatory cytokine production is a key pleiotropic effect of statins in several tissue types (reviewed in (53) and (54)), including bronchial epithelial cells (55). However, Kiener and colleagues showed that statins could increase monocytic pro-inflammatory cytokine and chemokine production in a mevalonate-dependent manner, thus sensitising them to challenge by inflammatory agents (56). The latter of these studies correlates with the observation in this study whereby simvastatin treatment induced IL-8 and CCL20 expression and that this was sustained and synergistically increased respectively upon infection with *P. aeruginosa*. The mechanism for this synergistic effect on CCL20 expression is unknown but it could be hypothesised that it may be partly due to the increase in bacterial adhesion in the presence of simvastatin, as CCL20 expression in bacterial infections is linked to this phenotype (52). However, *P. aeruginosa* adherence has also been linked to the induction of IL-8 (52), which was not synergistically induced in this study. Therefore, other factors are likely to be involved in the additive induction of CCL20 by *P. aeruginosa* and simvastatin. Furthermore, *P.* aeruginosa-mediated IL-8 and CCL20 production has been shown to be TLR5-dependent (39, 52), but TLR5 does not appear to be involved in the simvastatin induction of these factors, as TLR5 expression was not altered by statin treatment. In this study, the novel alteration of ASAH1 and iNOS expression in response to simvastatin treatment was also observed. The induction of ASAH1 by simvastatin could potentially be beneficial, as it may decrease ceramide-mediated inflammation in people with CF and other inflammatory disorders.

The anti-inflammatory regulator KLF2 was also induced by simvastatin. These results correlate with previous reports of statin induction of KLF2 and it was demonstrated that simvastatin had a significantly greater affinity for KLF2 induction than *P. aeruginosa*. This is possibly because statins have been shown to directly induce KLF2 expression by binding to a MEF2 transcription factor site in the *klf2* promoter region (18), while the induction of KLF2 by *P. aeruginosa* is proposed to be indirectly regulated by Type 3 toxin-mediated inhibition of host protein (RhoA) activity (13, 57, 58). In contrast to KLF2, KLF6 has not previously been shown to be induced by statins. Here it was demonstrated for the first time that simvastatin did induce KLF6 in airway epithelial cells. Furthermore, as *in silico* prediction of binding sites in the KLF6 promoter region demonstrates that a putative MEF2A binding site is located 228 bp upstream of the *klf6* start site (data not shown), it may be proposed that statins may induce *klf6* transcription in a similar manner to *klf2*. However, this does not account for the induction of KLF6 alternative splicing by statins. Production of KLF6 splice variants is regulated by a signalling cascade which involves the Rho GTPase Ras and the downstream Akt and phosphatidylinositol 3-kinase (PI3-K) signalling proteins (24). Previous studies have demonstrated that statins can modulate the phosphorylation and activation of the Akt signalling molecule in the Ras pathway (59, 60), which may account for splice variant production by simvastatin. However, this may be balanced with the characteristic statin-mediated inhibition of Ras prenylation first described by Leonard *et al*. (61).

The induction of KLF6 alternative splicing by *P. aeruginosa* was also identified for the first time. Previously it was reported that KLF6 is induced by *P. aeruginosa* TTSS toxins (13), and here it was demonstrated that the induction of KLF6 splice

variants by *P. aeruginosa* is dependent upon the ExoS toxin. The induction of KLF6 splice variants by *P. aeruginosa* could thus be mediated through 2 TTSS-dependent mechanisms. Firstly, *P. aeruginosa* may induce KLF6 alternative splicing by modulating Ras signalling, as ExoS has been found to regulate activation of Ras (62). Secondly, the *P. aeruginosa* TTSS also induces the production of reactive oxygen species (ROS) during infection (63) and these compounds can induce KLF6 alternative splicing (64). It was interesting to note that wtKLF6 was the dominant KLF6 variant in A549 lung epithelial cells both at basal level and in response to *P. aeruginosa* infection, in spite of them being a cancer cell line. Previously, increased SV1 has been linked to various cancers including that of the lung. Nevertheless these results clearly demonstrate a role for wtKLF6 in modulating the host response to infection.

Interestingly, KLF6 alternative splicing may also play a role in CF, as wtKLF6 and its 3 variants were all induced by *P. aeruginosa* in CFTR-deficient bronchial epithelial cells (IB3-1 cells). However, it was particularly interesting that SV2 and SV3 levels were significantly lower in IB3-1 cells compared to A549 cells harbouring normal CFTR. The production of KLF6 splice variants is known to be regulated by the splice factors ASF/SF2 and SRp40 (22, 24). The expression of SRp40 and another splice factor – SRp20 – is known to be increased in macrophages by *P. aeruginosa* PQS (65). Therefore, the increased induction of these splice factors may pose a potential mechanism for the induction of KLF6 alternative splicing, although these data suggest that this is mediated through ExoS-regulated Ras modulation rather than by secreted compounds. Furthermore, although SRp40 expression levels have been found not to vary between CF and non-CF cells (66), the ability of this protein to regulate alternative splicing is regulated by phosphorylation

(67), a process that can be differentially regulated between CF patients and healthy individuals (68). These results suggests that CFTR deficiency may alter the regulation of splice variant production, and indeed the alternative splicing of another infection-related protein – TLR4 – is known to be altered when CFTR is non-functional (69). In addition, wild type (but not mutated) CFTR can act as a recognition molecule for *P. aeruginosa* (70), and altered binding of *P. aeruginosa* to CF cells may also alter ExoS production and thus influence Ras/Akt-mediated splice variant production.

wtKLF6 was found to regulate the statin effects on CCL20 and iNOS but not ASAH1 in lung epithelial cells. Interestingly, the data in this study showed that wtKLF6 did not appear to regulate the expression of PPARγ in lung cells as it did in kidney cells (36), suggesting that this interaction and subsequent CCL20 induction may be tissue-specific. The regulation of statin-mediated CCL20 and iNOS induction by wtKLF6 affirms the role of this protein in the immune response, and suggests that wtKLF6 has a role as a regulator of immune signalling. However, wtKLF6-dependent ASAH1 induction suggests that, like statins, wtKLF6 may activate both pro- and anti-inflammatory responses, and may thus play a role in maintaining a balanced inflammatory response. Furthermore, KLF6 splice variants may also antagonise the pro-inflammatory effects of wtKLF6; SV1 has been found to have a negative regulatory effect on the pro-inflammatory cytokine TNFα, whereas wtKLF6 may induce this protein (64).

Statins have been proposed as novel therapeutics in the fight against infections (5, 6) and this work demonstrates some potential effects simvastatin could have if used as an anti-microbial agent in respiratory infections. The manipulation of pro-inflammatory cytokines and KLFs by simvastatin may lead to improved survival and

bacterial clearance mediated by IL-8 and CCL20, whereas statin-induced KLF2 may serve as a regulator to downplay other elements of the inflammatory response. The dominance of wtKLF6 compared to the other splice variants suggests that a general anti-proliferative effect is conferred on simvastatin-treated and *P. aeruginosa* infected cells. Further studies are needed to elucidate the mechanistic role of KLF6 splice variants in the *P. aeruginosa*-host response, and to determine whether statins influence the expression of downstream splice variant targets.

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Chapter 5

General Discussion

Novel developments generated in this work

The overall objective of this work was to examine the interaction between statins and bacterial pathogens individually and subsequently study their combined impact on the host response. In this work, novel statin effects were identified against the growth and virulence of cystic fibrosis (CF)-associated pathogens. Simvastatin was found to inhibit the growth of *Burkholderia cenocepacia* for the first time, and lower simvastatin concentrations than previously reported (1) were demonstrated to be effective against *Streptococcus pneumoniae*. This study also identified the novel statin-mediated reduction of both the *in vitro* motility of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and the *in vitro* biofilm formation of all 4 species tested. In addition, simvastatin was found to increase the anti-microbial potential of gentamicin against *B. cenocepacia.* Furthermore, several novel targets of simvastatin were identified in *P. aeruginosa.* The PvrR and WspF regulators and several Cup fimbrial proteins were found to play a role in the attenuation of biofilm by simvastatin, while 6 proteins were upregulated by statin treatment in swarming cells.

This study also identified novel statin effects in eukaryotic cells. Simvastatin was found to induce the expression of the pro-inflammatory cytokines IL-8 and CCL20 in lung epithelial cells. In addition, this study not only demonstrated a novel induction of KLF6 by simvastatin, but also identified the induction of KLF6 splice variants by statins and *P. aeruginosa* for the first time. Furthermore, it was elucidated that *P. aeruginosa* regulated KLF6 alternative splicing by way of the ExoS toxin. A role for the statin-mediated induction of wtKLF6 was identified, as this protein was found to regulate CCL20 and iNOS expression in response to statin treatment. Simvastatin pre-treatment was also found to have a novel effect on adhesion of *P. aeruginosa* to host cells.

Overall significance of results

Throughout the course of this work, statins have been demonstrated to have multi-faceted effects in the context of infection and immunity. Four key areas where statins target bacteria were identified: growth, motility, biofilm and host interactions (Figure 1). According to the data obtained in this study, statins appear to inhibit bacterial growth in a HMGR-dependent manner. Previous studies have demonstrated that bacteria which lacked a HMGR were inhibited by statin treatment (2, 3). However, these studies used statin concentrations which were much greater than those used in this work. It was elected to use comparably lower statin concentrations in this study in an attempt to observe inhibitory concentrations that were as close as possible to *in situ* plasma concentrations.

The inhibition of motility and biofilm by statins occurred at both growth-inhibitory and sub-inhibitory statin concentrations. This may be the first study to demonstrate reduced bacterial motility in the presence of statins. In addition, the reduction of attachment observed here correlates with a previously-reported reduction of *Candida albicans* biofilm formation by sub-inhibitory simvastatin concentrations (4). It was also observed that statins can also alter the host response to bacterial infections, at a concentration that inhibited motility and biofilm but not growth. Interestingly, simvastatin treatment increased adhesion of *P. aeruginosa* to host cells, despite decreasing *in vitro* attachment. This may be a result of increased actin depolymerisation caused by statin treatment, as actin depolymerisation is associated with increased attachment to host cells in *E. coli* infections (5). It was found that simvastatin did not alter bacterial invasion, and these results correlated with those reported by Shibata *et al*. (6).

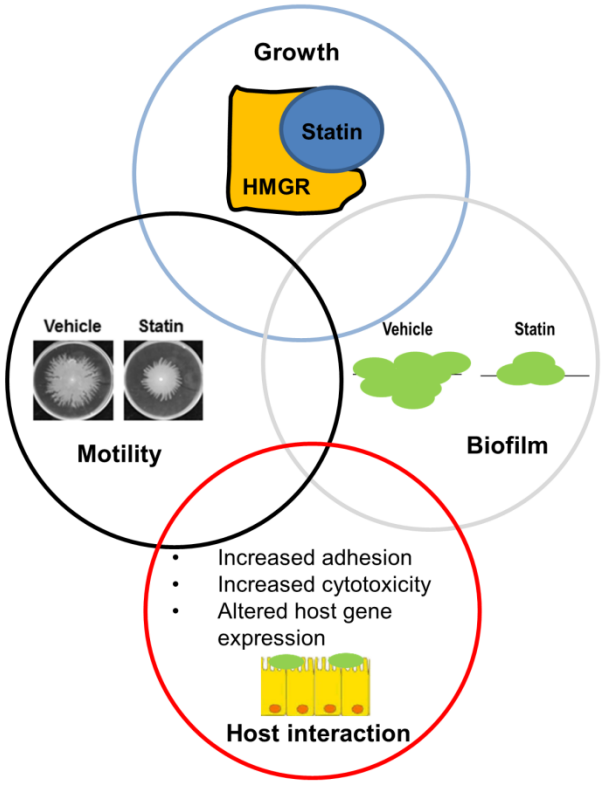


Figure 1: Methods of anti-bacterial effects of statins. This study identified 4 key areas of statin interaction with bacteria. Statins decreased bacterial growth in an apparently HMGR-dependent mechanism. Motility and biofilm-related phenotypes were attenuated by statins at both inhibitory and sub-inhibitory concentrations. Finally, statins influenced the host response to bacterial infections at sub-inhibitory concentrations that could modulate virulence.

It was demonstrated that statins attenuate the swarming motility and early attachment of *P. aeruginosa.* These phenotypes are associated with the switch from the motile sessile stage of acute infection to the beginning of biofilm formation, which is associated with chronic infection. Understanding the mechanism by which statins influence *P. aeruginosa* motility and biofilm formation is important for elucidating the overall value of statins as anti-microbial agents and how they would best be administered. Certain *P. aeruginosa* pathways that may be targeted by statins have been identified in this study, and a possible model for the interplay between statins and these pathways is proposed in Figure 2.

It was observed that simvastatin increased attachment in a *pvrR* deletion mutant. PvrR negatively regulates biofilm formation by inhibiting expression of the *cupA* and *cupD* fimbrial gene clusters and degrading cyclic-di-GMP (c-di-GMP) (7–9). It was therefore proposed that simvastatin may increase PvrR activity, thus decreasing c-di-GMP and the expression of the PvrR regulatory targets CupA1, CupA2 and CupD1. This would consequently decrease fimbriae expression, thus decreasing bacterial attachment. However, a potential role was not identified for PvrS, the cognate sensor of PvrR, in the impact of simvastatin. In addition, the RcsB regulator can induce biofilm dispersal in a PvrR-dependent manner (8), but it was observed that RcsB did not appear to be involved in the statin effects on attachment.

A role for the methylesterase WspF in the effect of simvastatin on *P. aeruginosa* biofilm formation was also identified, as simvastatin increased attachment of a WspF mutant strain. WspF decreases intercellular c-di-GMP levels and exopolysaccharide production (10, 11), suggesting that modulation of these compounds by simvastatin may result in decreased attachment. However, low c-di-GMP levels are associated with increased swarming motility (12), meaning that reduction of c-di-GMP by PvrR

and WspF are most likely not associated with the statin-mediated reduction in swarming. Neither PvrS/R, RscB nor WspF appeared to be involved in the attenuation of swarming motility by simvastatin. It therefore appears that statins may modulate swarming in a c-di-GMP-independent manner. However, it is possible that the proposed reduction in fimbriae by simvastatin could contribute to decreased swarming motility, as microarray analysis and screening of transposon mutant banks have demonstrated that fimbrial proteins may be involved in swarming motility (13–15).

In order to elucidate the mechanism behind the attenuation of swarming by simvastatin, proteins were isolated from the swarm tip of statin-treated bacteria. This analysis revealed that simvastatin increased the expression of proteins involved in transcription, translation, LPS transport and metabolism. The LptD transport protein was increased by simvastatin. This protein is responsible for the transport of LPS to the outer membrane. As structurally correct LPS is required for motility (16), it was hypothesised that simvastatin may attenuate swarming motility by aberrantly regulating transport of LPS to the outer membrane by potentially increasing expression of or post-translationally modifying LptD.

Another statin target of *P. aeruginosa* was the aconitase AcnB. Aconitase is part of the citric acid cycle, where it converts citrate to isocitrate. This enzyme requires iron to function, and is inactive under conditions of limiting iron (17). Conversely, increased iron concentrations cause the inhibition of swarming motility (18). To date, it is unknown whether statins can influence *P. aeruginosa* iron uptake and metabolism. However, microarray analysis has demonstrated that simvastatin induced the expression of genes associated with iron transport in *S. pneumoniae* (1). Therefore, further study may be warranted on the effects of statins on *P. aeruginosa*

iron metabolism. Furthermore, AcnB has been linked to reduced Exotoxin A (ToxA) production (17), and although the effects of simvastatin on ToxA were not examined in this study, this may present another virulence pathway which is altered by statins.

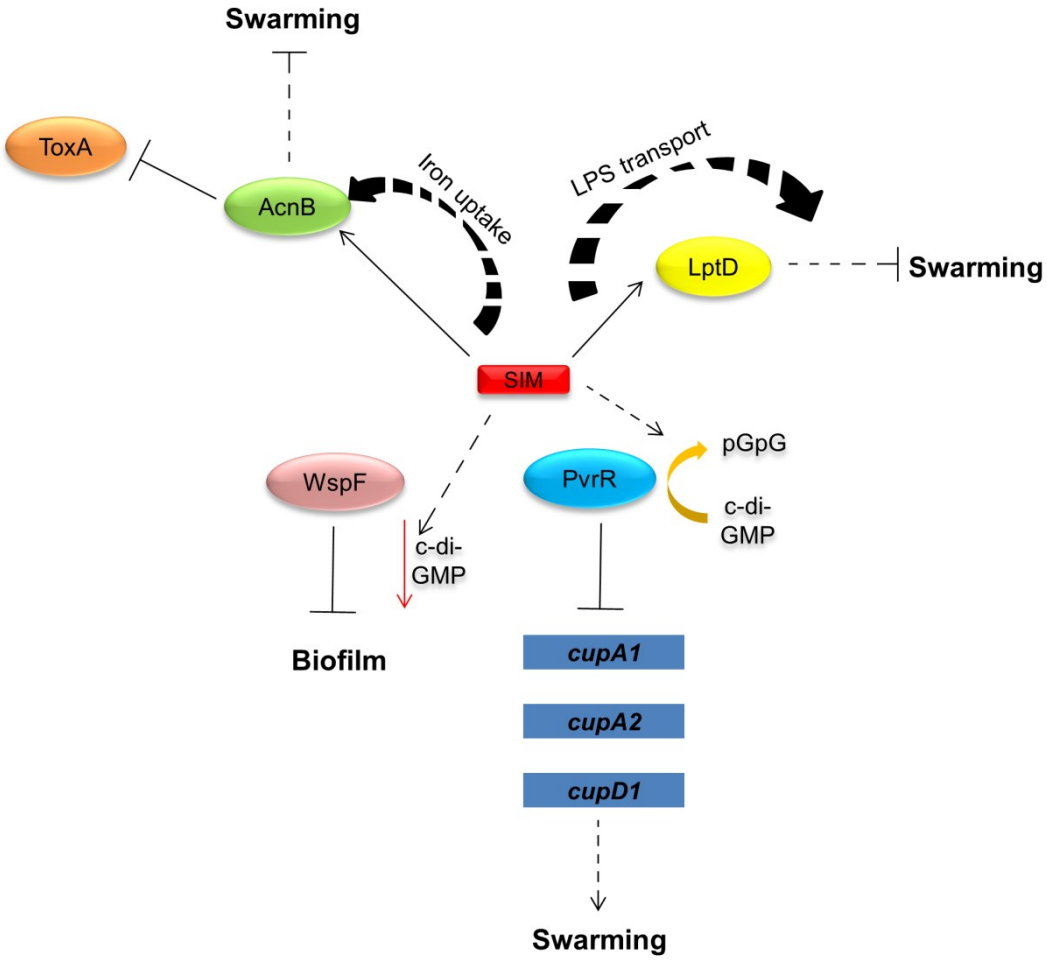


Figure 2: Hypothesis examining the effects of simvastatin on *P. aeruginosa*. Simvastatin appears to have several key modes of action against *P. aeruginosa*. It may alter the activity of PvrR and WspF, thus reducing c-di-GMP and biofilm formation. In addition, simvastatin increased the expression of LptD and AcnB, which may alter LPS transport to the outer membrane, and exotoxin A production, respectively. Broken arrows represent hypotheses which require further elucidation.

Three key simvastatin effects were observed on lung epithelial cells: altered expression of *P. aeruginosa* target genes, induction of KLF6 alternative splicing and increased adhesion of *P. aeruginosa*. A mechanistic model detailing total statin effects is proposed in Figure 3. *In vitro* attachment was decreased by simvastatin, but it was observed that simvastatin increased adhesion of *P. aeruginosa* to lung epithelial cells. This contrasts with a previous observation that simvastatin reduced adhesion of *S. pneumoniae* to human brain microvascular epithelial cells (1). One hypothesis for statin-mediated increased attachment to cells is that *P. aeruginosa* uses actin to bind to host cells (19). Therefore, statin-mediated actin depolymerisation of human cells may therefore contribute to the increased attachment observed in this study. Another issue is that 100 μM simvastatin was required for a significant reduction in *in vitro* attachment, and the 10 μM concentration used in this study may not have been sufficient to reduce attachment to cells.

A positive correlation has previously been observed between increased attachment and IL-8 expression (19), and therefore it is possible that statin-induced adhesion may contribute to the sustained IL-8 expression in infected cells. However, the mechanism of IL-8 induction by simvastatin is unclear. Previous studies have reported that statins reduced IL-8 expression (20). Kiener *et al.* reported that statin-increased IL-8 in monocytes was reversed by the addition of mevalonate, but did not propose a cytokine-based regulatory mechanism (21). Furthermore, the data in this study demonstrated that the expression of CCL20 by simvastatin was mediated through wtKLF6. Both IL-8 and CCL20 are induced by the activation of TLR5 by *P. aeruginosa* flagellin (22) but TLR5 expression was not altered in this study, suggesting that IL-8 and CCL20 were induced by an alternative mechanism.

It is possible that ExoS may induce expression of KLF2 and KLF6 alternative splicing by targeting Rho GTPase signalling. KLF2 is induced when RhoA is inhibited (23), and ExoS inhibits RhoA activity (24). Furthermore, production of KLF6 splice variants is associated with Ras GTPase signalling, in particular the phosphorylation of phosphatidylinositol 3-kinase (PI3-K) and Akt (25). ExoS can inactivate Ras (26), and reduces downstream Ras signalling by inhibiting phosphorylation of these proteins (27). Inhibition of Ras signalling results in dominant production of wtKLF6, and a lower level of splice variant production (25). Therefore, it appears that the inhibition of Ras signalling by *P. aeruginosa* ExoS may be responsible for the dominant production of wtKLF6 in infected cells. The inhibition of Rho GTPase activity by statins is well-documented (28). However, statins can also inhibit PI3-K and Akt phosphorylation (29, 30), meaning that, like ExoS, simvastatin may also cause dominant expression of wtKLF6 in this manner. Furthermore, statins can directly induce KLF2 expression by binding to a MEF2A consensus sequence in its promoter (31), and transcription of KLF6 and its splice variants may be induced in the same manner. There was not a cumulative effect on KLF2 and KLF6 expression when statins and *P. aeruginosa* were combined, and this correlates with *in vitro* data demonstrating that statins did not influence expression of *exoS*.

The novel induction of KLF6 and its variants by simvastatin treatment was demonstrated in this study. Previously, statins were not found to influence KLF6 in vascular endothelial cells (31), suggesting that statins may have a tissue-specific effect on KLF6 expression. The induction of wtKLF6 by statins was found to be responsible for the induction of CCL20 and repression of iNOS by simvastatin, suggesting that wtKLF6 has a role in the immune response. This concept correlates

with a recent report which suggested that wtKLF6 positively regulated TNFα expression (32). The induction of CCL20 by simvastatin may increase bacterial killing and clearance of infection as this compound has anti-microbial properties (33). Simvastatin and *P. aeruginosa* had a synergistic effect on CCL20 induction, and this could be a possible result of wtKLF6-dependent CCL20 activation by simvastatin and the induction of CCL20 by *P. aeruginosa* attaching to cells (22). Statin-induced KLF6 expression attenuated iNOS in this study, but it has previously been reported that KLF6 can activate iNOS transcription in fibroblasts and T cells (34). This again suggests that the observations in this work may occur as a result of tissue-specific statin effects. It is possible that the attenuation of iNOS by simvastatin may occur in order to suppress particular components of the immune response; a similar hypothesis has been proposed for the induction of KLF2 by bacterial toxins (23). Moreover, nitric oxide reduces attachment of *P. aeruginosa* to cells (35); therefore, it is possible that the statin-mediated repression of iNOS observed in this study may contribute to the increased attachment of *P. aeruginosa* to lung cells in the presence of statins.

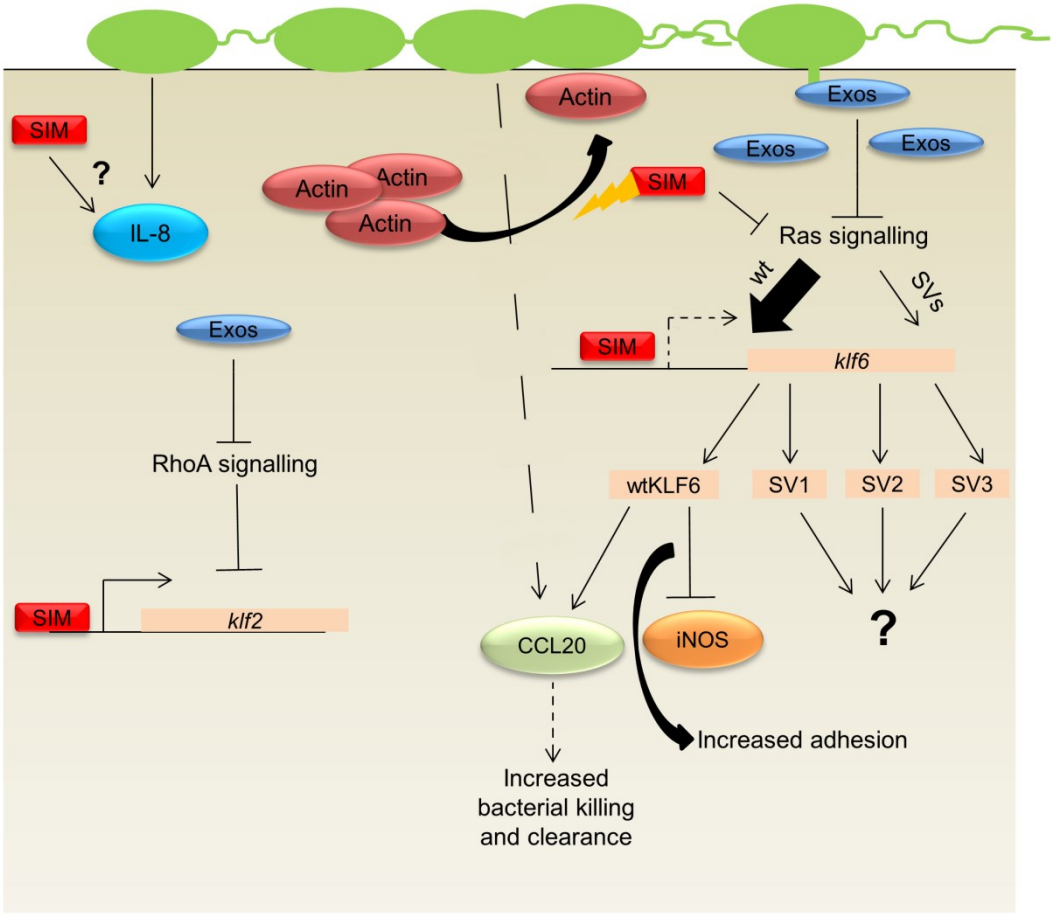


Figure 3: Proposed model of statin activity against *P. aeruginosa* infections. The *P. aeruginosa* ExoS toxin induces KLF2 and KLF6 splice variant expression, and this may mediated through inhibitory effects on RhoA and Ras signalling, respectively. Inhibition of Ras signalling by simvastatin and *P. aeruginosa* may result in dominant expression of wtKLF6. Simvastatin can induce KLF2 expression by binding to a MEF2 promoter site, and it is possible that transcription of *klf6* may also be induced in this manner by simvastatin. Furthermore, simvastatin may increase bacterial adhesion by altering the actin cytoskeleton. The mechanism by which simvastatin alters IL-8 expression is unclear. However, it was hypothesised that the additive effect observed on CCL20 may be a result of increased bacterial attachment, and induction via wtKLF6. iNOS was reduced by wtKLF6 in statin-treated cells, and this may pose another mechanism whereby bacterial adhesion is induced. Broken arrows indicate mechanisms which require further elucidation.

Clinical significance of this study

Susceptibility to statins – the bacterial mevalonate pathway

In this study, it was demonstrated that statins had an impact on the growth of bacteria with HMGR homologues, but did not influence the growth of *P. aeruginosa*, which lacks HMGR. However, *B. cenocepacia* does not possess a canonical mevalonate pathway, and it was hypothesised that the HMGR of this species is not involved in isoprenoid metabolism. Based on the data acquired in this study, it was proposed that the level of efficacy of statins against bacterial pathogens depends on the presence of not only HMGR, but also the mechanism of terpenoid backbone biosynthesis used by the bacteria. The basis of this hypothesis is that, according to this study, statins appeared most effective against *S. aureus* and *S. pneumoniae*, both of which possess the 8 canonical mevalonate pathway and isoprenoid biosynthesis genes. Higher statin concentrations (100 μM) were required to attenuate the virulence (biofilm formation) of *B. cenocepacia*, which possesses *mvaA* and *uppS* only, although the growth of this species was reduced at similar statin concentrations to those that affected *S. aureus*. It was hypothesised that statins had a reduced impact against *B. cenocepacia* (and *P. aeruginosa*) due to the absence of a mevalonate pathway. In conclusion, if statins were to be used as antimicrobial agents, they may be most effective against bacterial species that undergo isoprenoid biosynthesis using the mevalonate pathway, but they could still be used against non-mevalonate pathway bacteria as part of combination therapy.

Statins, bile and cholesterol: a potential benefit for CF patients?

Cholesterol is the end product of the mevalonate pathway, but this compound has downstream applications of its own. Notably, following its formation in the liver, cholesterol can be oxidised to form the 2 primary bile acids cholic acid and chenodeoxycholic acid (36). Approximately half of all cholesterol synthesised daily – 500 mg – is metabolised into the primary bile acids (37). Bile acids are typically absorbed by the small intestine where their role is to emulsify and solubilise ingested lipids. However, bile acids have been found to be aspirated into the lungs of individuals with both respiratory disease and gastro-oesophageal reflux (GER), a condition where the contents of the stomach leak into the oesophagus (38–42). GER is a common complication in people with CF – the incidence of GER in CF patients is anywhere between 35 % to 80 %, and it is thought that up to 80 % of CF patients may have bile acids present in their lungs (38).

Bile production in the context of CF is significant for 2 reasons. Firstly, bile was found to induce a chronic phenotype in CF-associated pathogens, by reducing motility and inducing biofilm formation (43). Secondly, the inhibition of mevalonate production by statins is known to decrease the formation and secretion of bile acids and salts (44, 45). The inhibition of cholesterol and bile salt production by statins may prove beneficial to CF patients with GER. If statins are taken via their conventional oral delivery route, the consequent inhibition of bile acid production may attenuate the aspiration of bile into the CF lung. This in turn may reduce the bile-regulated adaptation of bacteria from acute to chronic phase infections, especially given that this study demonstrated the statin-mediated inhibition of behaviour associated with the switch from acute to the chronic biofilm formation phase.

Pro-inflammatory effects of statins

Statins are classically regarded as inhibitors of infection, and have previously been found to reduce expression of IL-8 and CCL20 (20, 46), 2 pro-inflammatory cytokines that were induced by simvastatin in this study. The results observed in this study are therefore novel and require further study. It is possible that the induction of inflammation by statins in this study may be a tissue-specific effect, and therefore the regulatory mechanism of statin-mediated inflammation in lung cells needs to be examined. Furthermore, the effect of statins on CF-affected lung cells also needs to be studied. IL-8 production is aberrantly increased in CF-patients (47), and therefore the impact of statins on IL-8 production in a CF background needs to be elucidated. The reduction of iNOS may also prove problematic, as NO production is decreased in CF patients (48). However, the induction of KLF2 in statin-treated infected cells is promising, as this protein confers anti-inflammatory effects on lung and endothelial tissue and macrophages (46, 49) and sustained expression of KLF2 may help to reduce the initial induction of inflammation by statins and *P. aeruginosa*. Furthermore, KLF2 expression is decreased in CF patients (50) and therefore the induction of KLF2 by statins may help to ameliorate CF-associated inflammation. A clinical trial has been proposed to examine the potential for the use of statins as therapeutic agents in CF (51), and the results of such studies may help to uncover the *in situ* effects of statins on pulmonary inflammation.

Implications of the induction of KLF6 splice variants

It was demonstrated that *P. aeruginosa* and statins were individually capable of inducing the expression of splice variants in lung cells. The expression of these

variants has typically been associated with cancer (52); however, they have also been found to be induced as a result of alcohol-induced oxidative stress (32), implying that KLF6 splice variants may be induced in non-cancer scenarios although to date their expression during microbial infection has not been elucidated. This study suggests that wtKLF6 is the dominant variant induced by both simvastatin and *P. aeruginosa*. This suggests that the net effect conferred on the cells will be apoptotic. ExoS induces apoptosis of infected cells (24), and it is possible that ExoS-mediated wtKLF6 production may act as a key mechanism of cell death during infection. However, statins may also influence apoptosis of infected cells, as a slightly increased level of cytotoxicity was observed when statins and *P. aeruginosa* were combined, compared to in untreated infected cells. This is in accordance with previous reports which demonstrated that statins can induce apoptosis of lung cells (53). However, this effect appears to occur independently of wtKLF6, as cytotoxicity was comparable between statin-treated si-wtKLF6 cells and vector control cells.

The results of this study demonstrate wtKLF6 to be a regulator of iNOS and CCL20 in lung cells, both of which contribute to the innate immune response. This suggests that wtKLF6, like other members of the KLF family, may potentially have a greater role in regulation of immunity than previously thought. The role of the induction of KLF6 splice variants is unclear. As SV1 and SV2 have antagonistic functions towards wtKLF6 in cancer cells, it is possible that these compounds may act in an anti-inflammatory manner. An anti-inflammatory capacity of SV1 has been suggested by Urtasun *et al*. who reported that SV1 could reduce expression of TNFα (32).

Future Perspectives

Potential further studies of RTI-associated pathogens

Simvastatin reduced the growth of species which possess the *mvaA* gene, but do not impact the growth of *P. aeruginosa* (which lacks *mvaA*). This could be indicative that statins exert their inhibitory effect on strains which possess HMGR via inhibition of the mevalonate pathway. Therefore, examination of the effect of statins on the growth and virulence of an *mvaA* mutant strain needs to be carried out in order to verify this hypothesis. The most significant information could arguably be gained from the generation of a *B. cenocepacia* HMGR mutant strain. As a clear role has not yet been defined for this enzyme in *B. cenocepacia*, characterisation of the phenotypic behaviour of an *mvaA*-deficient strain in this species could potentially define the role of this gene in *B. cenocepacia*. Synergy between simvastatin and the antibiotic gentamicin was also identified against this species. This suggests that simvastatin could be used in combination therapy to treat antimicrobial infections. Further studies could focus on screening combinations of different antibiotics and varying simvastatin concentrations, to determine if they produce optimal bacteriocidal effects. In particular, the potential for the use of simvastatin in combination therapy against *P. aeruginosa* needs to be elucidated, as statins did not affect the growth of this species.

Another point to consider is the effect of statins on fungi and viruses. Statins have been found to inhibit the growth of the several yeast species, including *Candida* and *Cryptococcus* species (54) and members of the class *Zygomycetes* (55). Sub-inhibitiory simvastatin concentrations can reduce biofilm formation of *Candida albicans*, and this effect is attenuated by ergosterol, thus implying that the anti-

biofilm effect of simvastatin is dependent on the mevalonate pathway (4). Statins also impact viral infections – the infectious potential of influenza A, hepatitis C and cytomegalovirus (CMV) is reduced by statins (56–58). Taking these studies into consideration, it appears that the effects that were observed in this work may not be just limited to RTI-associated bacteria, but may also reduce the efficacy of fungal and viral RTIs.

Further examination of the interaction between simvastatin and *P. aeruginosa*

In this study, PvrR and WspF were found to be involved in the effect of simvastatin on the attachment of *P. aeruginosa*. Intracellular levels of the signalling molecule c-di-GMP are increased in mutants of these proteins (7, 11). Therefore, simvastatin may attenuate the biofilm formation of *P. aeruginosa* by decreasing c-di-GMP levels in a PvrR- and WspF-dependent manner. This hypothesis could be elaborated upon by quantifying the intracellular c-di-GMP levels in statin-treated cells using high-performance liquid chromatography.

The reproducible alteration of 6 *P. aeruginosa* proteins by simvastatin was also observed. However, only 3 of these proteins were examined further. Therefore, in order to fully elucidate the roles of AcnB, FusA1 and RpsA in the attenuation of swarming by simvastatin should be examined. This could be achieved by construction of single deletion mutants in these proteins, and examining their motility phenotype in the presence and absence of simvastatin. Furthermore, as LptD and AcnB have roles in regulating LPS and ExoA respectively (17, 59), the impact of simvastatin on production of LPS and ExoA should be elucidated. In addition, the impact of simvastatin on iron metabolism should be elucidated, given that AcnB is

induced under high iron concentrations. Finally, it appeared that 5 of the simvastatin-altered proteins were post-translationally modified by either oxidation or carbamidomethylation. It is unclear how simvastatin treatment caused these modifications, and the mechanism behind these modifications could prove insightful as it could allow for further elucidation of pleiotropic statin effects on proteins.

Development of statin analogues

Elucidation of the bacterial response to statin treatment could allow for the development of statins analogues. These compounds could be tested against bacteria, with a view to developing a compound which could significantly inhibit bacterial growth and virulence at a physiologically relevant concentration and that would also be safe for human consumption. This approach has previously been taken with the *P. aeruginosa* signalling molecules PQS and HHQ – synthetic analogues of these compounds were developed and tested against several bacterial and fungal species (60, 61). In accordance with the results of this study, the 3 key criteria required for potential synthetic statin analogues would be inhibition of bacterial growth and behaviour, efficacy at physiologically relevant concentrations and minimal side effects. A potential benefit of hypothetical statin analogues is that they may have a reduced risk of side effects compared to statins. One key limitation of statins is that they pose several health risks, including liver damage and rhabdomyolysis, although these are extremely rare. However, a significant minority of the general population is intolerant to statins and develop myalgia and myopathy. The use of statin analogues could potentially circumvent this problem.

Elucidation of the role of KLF6 splice variants in infection

During the course of this work, a study of the downstream effects of *P. aeruginosa*-induced KLF6 alternative splicing had been planned. However, the correct knockdown of SV1 in a si-RNA cell line could not be verified. In addition, infection of a si-wtKLF6 cell line with *P. aeruginosa* caused upregulation of wtKLF6 to such an extent that the effects of the knockdown vector were abrogated. Therefore, the downstream effects of the induction of KLF6 alternative splicing by *P. aeruginosa* could not be examined. Future work should focus on the elucidation of these effects and the mechanism behind them. This analysis could be carried out using a *P. aeruginosa* strain which produces lower amounts of ExoS than PAO1 or by transiently transfecting si-cells with a vector expressing the ExoS protein. By using either of these models, the downstream effects of ExoS-induced wtKLF6 and SV1 could be studied on host gene expression and physiology.

Concluding Remarks

This study identified several potential benefits of the prospective use of statins as anti-microbial agents. The results obtained here provide a case for the potential of statin use to treat CF-associated bacterial infections. In addition, the bacteria tested in this study can also cause other types of infections, including those of the urinary tract, cornea and wounds. Therefore, it is possible that statins may have multi-species anti-microbial effects against many different types of localised infections. Furthermore, the multi-species effects of simvastatin against bacterial attachment would indicate that they may be effective against polymicrobial biofilms. Statins could also be potentially used in combination therapy with other anti-microbial agents to treat bacterial infections.

This study also highlighted several limitations of using statins as anti-microbial compounds. One such limitation is that statins do not appear to have an effect on previously established biofilms, meaning that if statins were to be used to fight infections, they would probably be most effective as a prophylactic agent. However, simvastatin pre-treatment increased bacterial adhesion to lung cells, meaning that further study is required to determine whether the *in vitro* reduction of attachment by statins can be replicated *in vivo* and eventually in patients. Another potential limitation would be the delivery route of statins. These drugs are conventionally administered orally in tablet form, and typically have a low bioavailability (as low as 5 % for lovastatin and simvastatin) and low plasma concentrations (10-20 ng/ml for lovastatin and 20-34 ng/ml for simvastatin) (62). Other antimicrobials, notably the aminoglycoside antibiotic tobramycin, have been found to be effective against respiratory infections when aerosolised and delivered via inhalation (63, 64). This delivery route also increases the local concentration of the antibiotic. Therefore, in

order to achieve the concentrations required to inhibit bacterial growth and virulence that were observed in this study, aerosolisation of statins may have to be considered, and any potential risk factors must be identified.

Despite their limitations, statins have shown some promise as novel anti-microbial therapies, which are greatly needed. In September 2013, the Centres for Disease Control and Prevention (CDC) issued a sobering report which estimated that in the U.S.A. alone, antibiotic-resistant microorganisms cause approximately 2 million infections per year, and at least 23,000 people in the USA now die every year as a direct result of antibiotic-resistant infections (65). According to this report, 3 of the bacterial species studied in this work – *P. aeruginosa*, *S. aureus* and *S. pneumoniae* – are collectively responsible for over 30,000 deaths and over 4.1 million infections per year. Therefore, statins may present an opportunity to aid the eradication of antibiotic-resistant infections. However, the scope of the antimicrobial and anti-virulence capacities of statins observed in this study is limited. Therefore, more research is needed, particularly in the areas of efficacy and safety, to determine whether statins are a suitable means of treating bacterial infections.

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Appendix

A1. Effect of gentamicin on the growth of *S. aureus* and *B. cenocepacia*

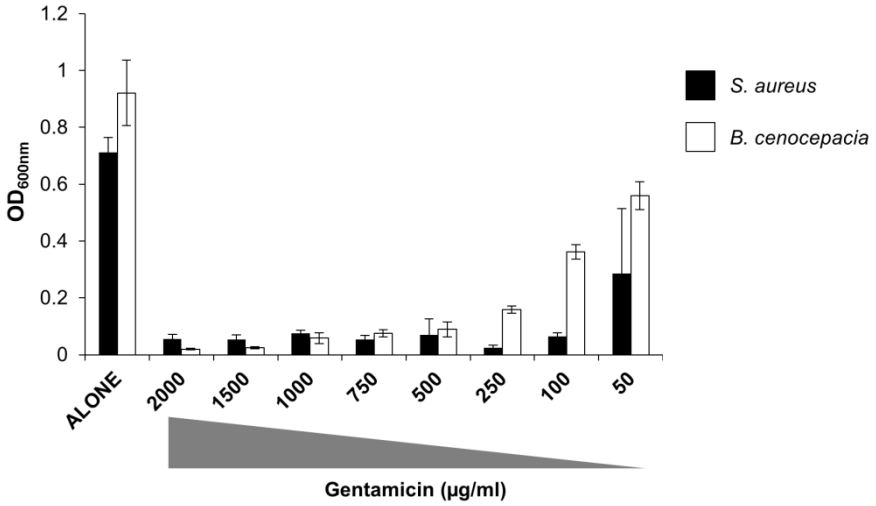


Figure A1. Inhibition of the growth of *S. aureus* and *B. cenocepacia* by the aminoglycoside antibiotic gentamicin. *S. aureus* NCDO949 and *B. cenocepacia* J2315 were cultured in the presence of Muller-Hinton broth containing a concentration gradient of gentamicin and incubated at 37 ºC for 24 hours, following which growth was measured by determination of OD_{600nm}. Preliminary analysis (1 biological replicate with 3 technical replicates) demonstrated that *S. aureus* was more susceptible to lower gentamicin concentrations than *B. cenocepacia*.

A2. Effect of lovastatin and mevastatin on attached bacteria and extracellular DNA production

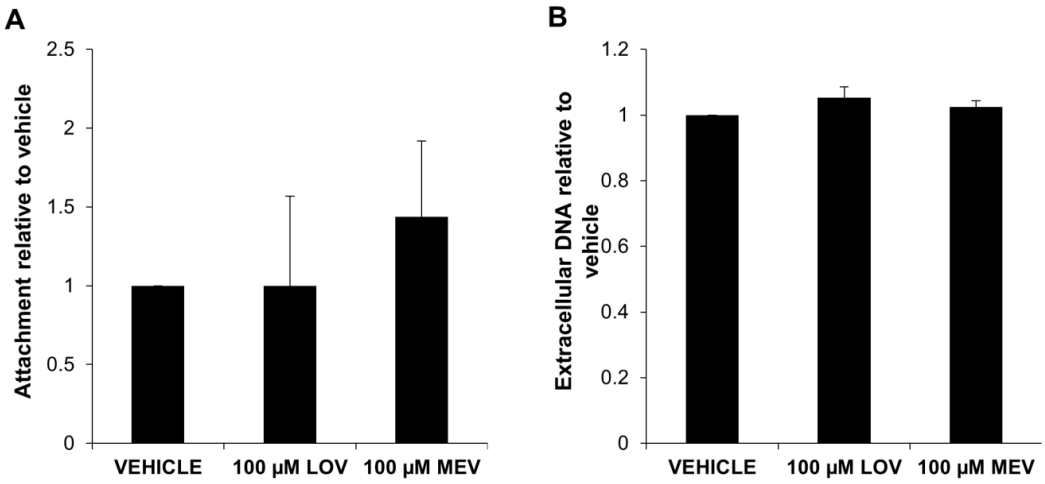


Figure A2: Biofilm phenotypes of PA14 in the presence of lovastatin and mevastatin. **(A)** 100 µM of lovastatin and mevastatin did not influence detachment of previously attached PA14 cells. **(B)** Extracellular DNA production by PA14 was not affected by either lovastatin or mevastatin. Data are presented as the average of 3 biological replicates.

A3. Effect of lovastatin and mevastatin on *pqsA* expression

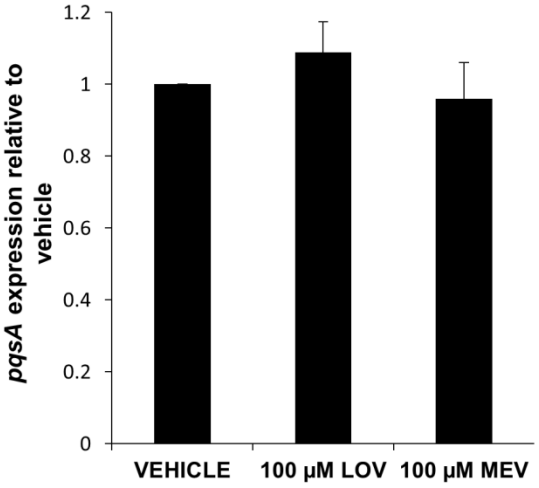


Figure A3: *pqsA* expression in the presence of lovastatin and mevastatin. 100 µM of either statin did not affect expression of *pqsA*. This experiment was performed in triplicate.

A4. Effect of hydrolysed simvastatin on *P. aeruginosa* quorum sensing

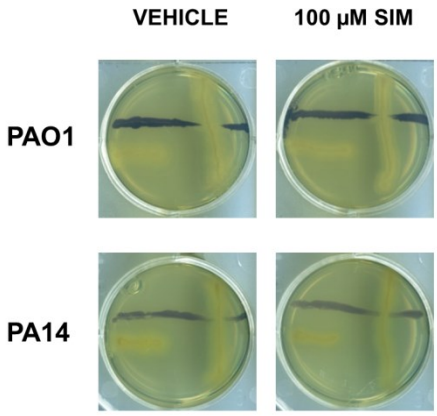


Figure A4: Production of homoserine lactones (HSLs) in the presence of hydrolysed simvastatin. The active form of simvastatin did not have any impact on HSL production of PAO1 and PA14. Images are representative of those taken for 3 independent experiments.

A5

Table A5: Microarray data demonstrating the effect of simvastatin on PA14. The average fold change (AVG FC) and standard deviation (STDEV) was calculated from the fold change (FC) of each biological replicate. P-value is based on an unpaired Student's T-test.

PA No.	Symbol	FC Rep 1	FC Rep 2	FC Rep 3	AVG FC	STDEV	p-value
PA0025	*aroE*	-0.67	-0.91	-1.07	-0.88	0.20	0.00
PA0028		1.39	1.21	0.10	0.90	0.70	0.03
PA0047		14.74	-0.48	-0.13	4.71	8.69	0.01
PA0171		16.71	0.92	-0.34	5.76	9.50	0.04
PA0179		-3.64	-0.71	-0.50	-1.61	1.76	0.02
PA0271		3.19	0.39	0.93	1.50	1.49	0.01
PA0437	*codA*	5.71	2.83	-0.55	2.66	3.13	0.01
PA0514	*nirL*	-7.91	-2.18	0.25	-3.28	4.19	0.04
PA0515		-3.48	-1.13	-0.02	-1.54	1.77	0.03
PA0516	*nirF*	-1.77	-0.14	-0.11	-0.67	0.95	0.01
PA0520	*nirQ*	343.32	-0.34	-0.33	114.22	198.41	0.02
PA0521	*nirO*	20.54	-0.41	-0.44	6.56	12.11	0.05
PA0656		6.66	-0.40	-0.30	1.99	4.05	0.02
PA0835	*pta*	-1.17	-1.71	-0.67	-1.18	0.52	0.02

PA1523	*xdhB*	0.98	0.48	0.26	0.57	0.37	0.01
PA1664	*orfX*	-5.44	-0.27	-0.36	-2.02	2.96	0.03
PA2567		50.87	0.85	0.30	17.34	29.04	0.00
PA2663	*ppyR*	1.62	0.49	0.08	0.73	0.80	0.03
PA2788		-6.50	-0.27	-0.15	-2.31	3.63	0.04
PA3049	*rmf*	31.02	-0.45	-0.32	10.09	18.13	0.03
PA3054		-4.58	0.82	-0.07	-1.28	2.90	0.05
PA3179		-5.47	-1.50	0.40	-2.19	3.00	0.03
PA3418	*ldh*	7.07	0.96	-0.05	2.66	3.85	0.00
PA3452	*mqoA*	0.92	0.56	0.19	0.56	0.37	0.04
PA3609	*potC*	3.92	1.18	0.44	1.85	1.83	0.03
PA3615		-1.74	0.55	0.05	-0.38	1.21	0.00
PA3641		-158.77	7.98	-0.02	-50.27	94.05	0.01
PA3651	*cdsA*	-2.61	-2.28	-0.06	-1.65	1.39	0.03
PA3741		-1.14	-0.98	-1.02	-1.05	0.09	0.01
PA3820	*secF*	0.75	0.54	0.10	0.46	0.33	0.02
PA3821	*secD*	1.83	0.89	0.38	1.03	0.74	0.01
PA3882		5.59	1.39	0.12	2.37	2.86	0.04
PA4349		2.73	1.13	1.15	1.67	0.92	0.02

PA4351	*olsA*	1.77	1.64	1.32	1.57	0.23	0.00
PA4357		2.10	0.70	1.94	1.58	0.77	0.04
PA4358		2.43	0.90	0.76	1.36	0.92	0.00
PA4432	*rpsI*	2.16	0.32	0.46	0.98	1.02	0.03
PA4673		-0.84	-0.63	-0.46	-0.65	0.19	0.00
PA4811	*fdnH*	-2.64	-0.64	-0.90	-1.39	1.09	0.04
PA4916		-1.74	-0.48	-3.06	-1.76	1.29	0.05
PA4919	*pncB1*	3.06	3.29	0.98	2.44	1.27	0.02
PA4920	*nadE*	-2.05	-2.17	-0.50	-1.57	0.93	0.01
PA4933		1.18	2.08	0.43	1.23	0.82	0.00
PA4934	*rpsR*	0.68	0.75	0.42	0.62	0.17	0.01
PA4935	*rpsF*	-1.59	-0.34	-0.27	-0.73	0.75	0.03
PA5025	*metY*	-0.71	-0.11	-0.69	-0.51	0.34	0.01
PA5207		4.54	1.55	0.36	2.15	2.16	0.03

A6

Table A6: Biological replicate 1 of altered swarm tip proteins in the presence of simvastatin or DMSO. Proteins were identified using tandem mass spectrometry. Oxidation (Ox) and carbamidomethylation (Carb) of proteins were also identified.

			Post-translational modification	
Accession	**Sample**	**Description**	**Ox.**	**Carb.**
PA14_02220	Statin	Putative chemotaxis transducer	+	-
PA14_07760	DMSO	Peptidyl-prolyl cis-trans isomerase SurA	-	-
PA14_07770	Statin	Organic solvent tolerance protein OstA precursor	+	+
PA14_07910	DMSO	Ribulose-phosphate 3-epimerase	+	-
PA14_08680	DMSO	Elongation factor Tu	+	+
PA14_08710	Statin	Transcription antitermination protein NusG	+	-
PA14_08820	Statin	Elongation factor G	+	+
PA14_11130	DMSO	Short chain dehydrogenase	-	-
PA14_16630	DMSO	Putative outer membrane protein, OmpA	-	-
PA14_16950	Statin	Tetrahydrodipicolinate succinylase	+	-
PA14_17100	Statin	Ribosome recycling factor	+	-
PA14_17150	Statin	Putative outer membrane antigen	+	-
PA14_17270	DMSO	Acetyl-CoA carboxylase carboxyltransferase subunit alpha	-	-
PA14_23330	Statin	30S ribosomal protein S1	-	-
PA14_23370	DMSO	Putative UDP-N-acetylglucosamine 2-epimerase	-	+
PA14_24270	Statin	Aminopeptidase N	+	+
PA14_29240	DMSO	Putative hydrolase	-	-
PA14_30370	DMSO	Hypothetical protein	-	-
PA14_33040	DMSO	Glycine cleavage system protein T2	-	+

PA14_34600	DMSO	Putative glyceraldehyde-3-phosphate dehydrogenase	-	-
PA14_38470	DMSO	Gamma-carboxygeranoyl-CoA hydratase	-	-
PA14_41470	Statin	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	+	+
PA14_41570	DMSO	Major porin and structural outer membrane porin OprF precursor	-	-
PA14_41670	Statin	Phosphoenolpyruvate synthase	+	+
PA14_43940	DMSO	Succinyl-CoA synthetase subunit alpha	-	+
PA14_44120	DMSO	Putative 3-hydroxyisobutyrate dehydrogenase	-	-
PA14_51390	DMSO	3-oxoacyl-(acyl carrier protein) synthase III	+	-
PA14_53250	DMSO	Chitin-binding protein CbpD precursor	+	+
PA14_57740	DMSO	Hypothetical protein	+	+
PA14_58180	DMSO	Aspartyl/glutamyl-tRNA amidotransferase subunit A	-	-
PA14_60330	DMSO	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	+	+
PA14_62710	Statin	Polynucleotide phosphorylase/polyadenylase	+	+
PA14_62970	Statin	Molecular chaperone DnaK	+	-
PA14_63020	DMSO	Ferric uptake regulation protein	+	-
PA14_64440	DMSO	Hypothetical protein	+	+
PA14_66770	DMSO	ATP-dependent protease peptidase subunit	+	-
PA14_67930	DMSO	Imidazoleglycerol-phosphate dehydratase	+	+
PA14_71620	DMSO	Phosphoribosylaminoimidazole carboxylase, catalytic subunit	+	-
PA14_73250	DMSO	F0F1 ATP synthase subunit gamma	+	+

A7

Table A7: Biological replicate 2 of altered swarm tip proteins in the presence of simvastatin. Proteins were identified using tandem mass spectrometry. Oxidation (Ox) and carbamidomethylation (Carb) of proteins were also identified.

			Post-translational modification	
Accession	**Sample**	**Function**	**Ox.**	**Carb.**
PA14_06290	Statin	Malate synthase G	-	-
PA14_07770	Statin	Organic solvent tolerance protein OstA precursor	-	-
PA14_08820	Statin	Elongation factor G	+	+
PA14_09000	Statin	50S ribosomal protein L6	+	-
PA14_11690	Statin	Inorganic pyrophosphatase	-	+
PA14_13130	Statin	Hypothetical protein	-	-
PA14_18690	Statin	Putative peroxidase	+	-
PA14_23330	Statin	30S ribosomal protein S1	-	-
PA14_25830	Statin	Hypothetical protein	-	-
PA14_25880	Statin	Electron transfer flavoprotein alpha-subunit	-	-
PA14_34460	Statin	Hypothetical protein	-	-
PA14_41470	Statin	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	+	+
PA14_43850	Statin	Heat shock protein 90	+	-
PA14_50270	Statin	Flagellar capping protein FliD	-	-
PA14_53820	Statin	Hypothetical protein	-	-
PA14_57010	Statin	Chaperone GroEL	-	-
PA14_58540	Statin	Hypothetical protein	-	+
PA14_61850	Statin	Putative TonB-dependent receptor	-	-
PA14_62710	Statin	Polynucleotide phosphorylase/polyadenylase	+	+
PA14_62970	Statin	Molecular chaperone DnaK	+	-

PA14_64840	Statin	Putative short-chain dehydrogenase	-	-
PA14_66330	Statin	Peptide methionine sulfoxide reductase	+	+
PA14_68070	Statin	Amino acid ABC transporter periplasmic binding protein	-	-

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