Pseudomonas Aeruginosa: interactions with organisms in the environment and cells of the immune defence

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ON THE NATURE OF THINGS

Titus Lucretius Carus (c.99-55 BCE)
Rewritten into poem form by anonymous.

No single thing abides; but all things flow.
Fragment to fragment clings - the things thus grow
Until we know and name them. By degrees
They melt, and are no more the things we know.

Globed from the atoms falling slow or swift
I see the suns, I see the systems lift
Their forms; and even the systems and the suns
Shall go back slowly to the eternal drift.

You too, oh earth - your empires, lands, and seas -
Least with your stars, of all the galaxies,
Globed from the drift like these, like these you too
Shalt go. You are going, hour by hour, like these.

The seeds that once were we take flight and fly,
Winnowed to earth, or whirled along the sky,
Not lost but disunited. Life lives on.
It is the lives, the lives, the lives, that die.

Flakes of the water, on the waters cease!
Soul of the body, melt and sleep like these.
Atoms to atoms - weariness to rest -
Ashes to ashes - hopes and fears to peace!

O Science, lift aloud your voice that stills
The pulse of fear, and through the conscience thrills-
Thrills through the conscience with the news of peace-
How beautiful your feet are on the hills!

This poem is a shortened version of Titus Lucretius Carus' (c.99-55 BCE) epic "De rerum natura" which describes "everything in nature": the physical origin of the universe, the atomic structure of matter and the emergence and evolution of life forms.

While this is a rather broad topic, this thesis has a more limited focus....

Pseudomonas aeruginosa: interactions with organisms in the environment and cells of the immune defence

Ph.D. thesis
by
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BioScience and Technology
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Article 1
Rasmussen, T.B., **Skindersoe, M.E.** Bjarnsholt, T. Phipps, R.K., Christensen, K.B., Jensen, P.O. Andersen, J.B., Koch B., Larsen, T.O., Hentzer M., Eberl L., Hoiby N. and Givskov, M. *Identity and Effects of Quorum Sensing Inhibitors Produced by Penicillium Species.* Microbiology 2005 151: 1325-1340.

Article 2
Skindersoe, **M. E.,** Epstein-Ettinger, P., Rasmussen, T. B., Bjarnsholt, T., de Nys R., & Givskov M. *Quorum sensing antagonism from marine organisms*. Marine Biotech. In press.

Article 3
Skindersoe, M. E., Phipps, R., Yang, L., Jensen, P.O., Rasmussen, T. B., Bjarnsholt, T., Høiby, N. and Givskov, M. *Effects of antibiotics on quorum sensing in Pseudomonas aeruginosa* Submitted to Antimicrobial Agents and Chemotherapy.

Article 4
Skindersoe, **M. E.,** Hjerrild, L., Pedersen, S. B., Fink, L. N., Lazenby, J., Williams, P., Diggle, S., Whittall, C., Frøkjær, H., Cooley M., and Givskov M., *Pseudomonas aeruginosa Quorum Sensing Signal Molecules interfere with dendritic cell induced T cell proliferation*. (Manuscript in preparation for submission to Infection & Immunity)

Other articles by the author referred to in this thesis.

Yang, L., Barken, K.B., **Skindersø, M.E**., Christensen, A.B., Givskov, M. & Tolker-Nielsen, T. *Effects of iron on DNA-release and biofilm development by Pseudomonas aeruginosa* Microbiology 2007 May;153(Pt 5):1318-28.

Bourne, D., Hoj, L., Payne, M., **Skindersoe, M.,** Swan, J., Webster, N., Givskov, M. & Hall, M. *Towards the Development of Rock Lobster Aquaculture: Aspects of the microbiology of phyllosoma rearing of the ornate rock lobster Panulirus ornatus* Aquaculture 268 2007 274–287.

Nicholson, R. L., Marsden, D.M., **Skindersø, M.E.,** Givskov, M.C., Ladlow, M., Welch, M., & Spring, D. *Discovery of Quorum Sensing Antagonists By On–Demand Synthesis and Screening Using 3–D Small Molecule Microarrays* Submitted to Angewandte Chemie

Rasmussen, T.B., Bjarnsholt, T., **Skindersoe, M.E.**, Hentzer, M., Kristoffersen, P., Köte, M., Eberl, L., Nielsen, J., Givskov, M. *Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector*. J Bacteriol. 2005 Mar;187(5):1799-814.

Persson, T., Hansen, T., Rasmussen, T.B., **Skindersoe, M.E.,** Givskov, M., Nielsen, J. *Rational design and synthesis of new quorum-sensing inhibitors derived from acylated homoserine lactones and natural products from garlic.* Org Biomol Chem. 2005 Jan 21;3(2):253-62.

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This thesis is submitted as a partial fulfilment of the requirements to obtain the Ph.D. degree at the Technical University of Denmark (DTU). The work presented herein was carried out from July 2004 to August 2007 with Professor Michael Givskov as supervisor at the institute for BioScience and Technology (in the former division Center of Biomedical Microbiology). The Ph.D. scholarship was collectively financed by DTU, the Pseudomonas Ph.D. school (Pseudomonas, Pathogenicity and Biotechnology), and a STF grant (jour. no. 2052-03-0013).

Although doing a Ph.D. costs sweat, tears and blood(!), it has been a great time, not just because it has brought me around the world (actually several times), but also because these three years have been a journey which has brought me invaluable knowledge and personal development. But I have not travelled alone:

First of all I want to express my great gratitude to my supervisor Professor Michael Givskov for introducing me to the exciting area of interbacterial communication and for giving me excellent opportunities to do research in this fascinating field. I would also sincerely thank him for his support and encouragement during my Ph.D. Mate Mike, I owe ya a shout at Coogee beach some day in the future!

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And to all DTU ScienceShow people, I have really enjoyed our experiences, experiments and explosions. I look forward to participate in our future "edutainment" events! Kold $N_{2(l)}$!

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Kgs. Lyngby, August 2007 Mette Elena Skindersø.

ABBREVIATIONS

15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
acyl-ACP	acyl-acyl carrier protein
AHL	*N*-acyl L-homoserine lactone
APC	Antigen Presenting Cell
BC	Breast Carcinoma
BHL	C_4-HSL, *N*-butanoyl L-homoserine lactone
BM	Bone Marrow-derived
CDC	Center for Disease Control and Prevention
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
COX	Cyclooxygenase
CSBP	Cytokine Suppressive anti-inflammatory drug Binding Protein or p38
DAPI	4',6-diamido-2-phenylindole
DC	Dendritic Cell
DHL	*N*-decanoyl L-homoserine lactone
EPS	ExoPolymeric Substances or Extracellular Polysaccharides
ERK	Extracellular signal-Regulated Kinase
FDA	Food and Drug Administration
GC-MS	Gas Chromatography-Mass Spectrometry
GFP	Green Fluorescent Protein
HHL	C_6-HSL, *N*-hexanoyl L-homoserine lactone
HQNO	2-heptyl-4-hydroxyquinoline *N*-oxide
HTGS	Human submucosal Tracheal Gland Serous
HUVEC	Human Vascular Endothelial Cell/Human Umbilical Vein Endothelial Cells
IDDM	Insulin-Dependent Diabetes Mellitus, type 1 diabetes
IC_{50}	Inhibitor Concentration required for 50 % inhibition
IFN-γ	Interferon-γ
IL	Interleukin
JNK	c-jun N-terminal kinase
LB	Luria-Bertani medium
LBD	Ligand Binding Domain
LPS	LipoPolySaccharides
MAPK	Mitogen-Activated Protein Kinase
Mex	Multidrug efflux
MHC	Major Histocompatibility Complex
MIC	Minimum Inhibitory Concentration
NIDDM	Non-Insulin-Dependent Diabetes Mellitus, Type 2 diabetes
NOD	Non Obese Diabetic
OdDHL	3-oxo-C_{12}-HSL, *N*-3-oxododecanoyl L-homoserine lactone
OHHL	3-oxo-C_6-HSL, *N*-3-oxo-hexanoyl L-homoserine lactone
OHL	C_8-HSL, *N*-octanoyl L-homoserine lactone
OMP	Outer Membrane Porin/Protein
ORF	Open Reading Frames
OtriDHL	3-oxo-C_{13}-HSL, *N*-3-oxotridecanoyl L-homoserine lactone
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peroxisome Proliferator-Activated Receptor
PI-3K	Peripheral Blood Mononuclear Cell
PMN	Phosphoinositol-3-Kinase

PQS	Polymorphonuclear leucocytes
QS	*Pseudomonas* Quinolone Signal; 2-heptyl-3-hydroxy-4-quinolone
QSI	Quorum Sensing
QSIS	Quorum Sensing Inhibitor(y)
ROS	Quorum Sensing Inhibitor Selector
SAM	Reactive Oxygen Species
SAPK	S-adenosyl methionine
SDS	Stress-Activated Protein Kinases, also c-jun N-terminal kinase, JNK
STAT	Sodium Dodecyl Sulphate
TLR	Signal Transducers and Activators of Transcription
TNF-α	Toll Like Receptor
wt	Tumor Necrosis Factor alpha
	wild-type

CONTENT

SAMMENFATNING

Pseudomonas aeruginosa er en opportunistisk patogen, der forårsager kronisk lungebetændelse i patienter med cystisk fibrose og livstruende infektioner i personer med et svækket immunforsvar. Denne bakterie er i stand til at producere en lang række skadelige virulensfaktorer såsom toksiner, vævsnedbrydende enzymer samt produkter, der kan svække værtens immunsystem. *P. aeruginosa* vokser primært som vækstbelægninger, såkaldte biofilm, hvilket beskytter den mod værtens immunforsvar og mod antimikrobielle stoffer. Dertil kommer at *P. aeruginosa* har en naturlig høj tolerance overfor mange af de typer af antibiotika, der bruges mest, hvilket understreger behovet for nye strategier, der kan benyttes til at bekæmpe denne sygdomsfremkaldende bakterie.

P. aeruginosa der lever i en biofilm udviser en organiseret adfærd, der minder om flercellede organismer. *P. aeruginosa* regulerer udtryk af virulensfaktorer, elementer involveret i biofilmdannelse og produkter som påvirker immunforsvaret via en form for bakteriel kommunikation kaldet quorum sensing. Denne afhandling omhandler bl.a. en strategi, der søger at modvirke *P. aeruginosa*s virulens og sygdomsfremkaldende egenskaber ved at forhindre bakterierne i at kommunikere. En behandlingsform, der er målrettet mod bakteriel kommunikation fremfor bakteriel vækst har den fordel at den ikke medfører det voldsomme selektionspres for udvikling af resistens som eller er det primære problem ved den konventionelle brug af antibiotika.

De to første artikler i denne afhandling (artikel 1 og 2) beskriver screeningen af ekstrakter fra eukaryoter (skimmelsvampe og marine hvirvelløse organismer) for tilstedeværelsen af quorum sensing hæmmere. Som det fremgår af disse to artikler ser det ud til at molekyler med inhiberende effekt på bakteriel kommunikation er hyppigt forekommende blandt eukaryoter, hvilket indikerer at evnen til at interagere med koordineret bakteriel adfærd giver højere organismer en evolutionær fordel. Quorum sensing antagonister såsom de to mykotoksiner patulin og penicillinsyre (artikel 1) og de tre manoalid-forbindelser (artikel 2) har 2(5*H*)-furanon-strukturen tilfælles. Da denne struktur forefindes i så forskellige organismer indikerer det at evnen til at producere denne furanon-type enten er opstået parallelt i forskellige organismer eller er en reminiscens fra en gammel fælles oprindelse for såvel marine organismer som skimmelsvampe og at dette træk pga. selektion er bevaret gennem tiderne.

De fleste typer af antibiotika, der anvendes nu til dags stammer fra naturlige kilder. Det antages almindeligvis at antibiotika er evolveret i naturen som en del af et forsvar mod konkurrenter i kampen om føde og plads. Denne formodning er dog for nylig blevet udfordret og det er istedet foreslået at antibiotikas økologiske rolle er som budbringere fremfor våben.
Artikel 3 i denne afhandling undersøger anvendelsen af lave koncentrationer af naturlige samt syntetiske antibiotika som hæmmere af bakteriel kommunikation og dermed også virkende mod virulensfaktorer. Tre slags antibiotika der ofte benyttes til at behandle infektioner med *P. aeruginosa* er i stand til at nedsætte produktionen af en række quorum sensing regulerede virulensfaktorer når de benyttes i koncentrationer, der ikke påvirker bakteriernes vækst. Dette indikerer at antibiotika muligvis har multiple anvendelsesmuligheder; som stoffer der slår bakterier ihjel eller hæmmer deres vækst og som antagonister af bakteriel kommunikation og dermed inhibitorer af sygdomsfaktorer. Det er således rimeligt at formode at vi end ikke fuldt ud udnytter potentialet af det medikamenter vi allerede kender.

Som beskrevet i det sidste manuskript i denne afhandling (artikel 4) så producerer *P. aeruginosa* også stoffer med multifunktionelle egenskaber; de to quorum sensing signalmolekyler; *Pseudomonas* Quinolone Signal og *N*-3-oxododecanoyl L-homoserine lakton

kan påvirke immunforsvaret udover deres funktion som budbringere af bakteriel kommunikation. Disse to signalmolekyler er i stand til at svække aktiviteter essentielle for immunforsvarscellernes evne til at bekæmpe bakterieinfektioner, og dette fænomen bidrager muligvis til *P. aeruginosa* evne til at etablere en infektion.

Nøglen til at bekæmpe denne bakterie kan meget vel ligge i udviklingen af en ny form for behandling der søger at bekæmpe denne patogens koordinerede adfærd, dens produktion af virulensfaktorer og dens evne til at svække værtens immunforsvar. Denne nøgle findes formodentlig allerede og er ”lige til at samle op”, hvis man søger blandt eukaryoter der har udviklet evnen til at producere quorum sensing antagonister.

SUMMARY

Pseudomonas aeruginosa is an increasingly prevalent opportunistic pathogen which causes chronic pneumonia in cystic fibrosis patients and severe life-threatening infections in immunocompromised persons. This pathogen produces a range of malicious virulence factors such as toxins, tissue degrading enzymes and components capable of impairing the hosts' immunity. *P. aeruginosa* readily assumes the biofilm lifestyle which confers efficient protection against the activity of the host defence system. In addition, *P. aeruginosa* exhibit an inherent tolerance to many of the antibiotics most commonly used, which emphasises the urgent need for development of novel strategies that will help us to defeat this pathogen.

P. aeruginosa biofilm cells display a multicellular-like coordinated behaviour and control expression of virulence factors, elements involved in biofilm development and immunomodulating factors by means of signal molecule mediated communication, known as quorum sensing. This thesis explores a strategy which aims to counteract *P. aeruginosa* virulence and pathogenicity by impeding its cell-to-cell communication. A treatment regime, which focuses on targeting bacterial communication instead of growth, has the immediate advantage that it does not impose a harsh selection pressure for resistance which is the main drawback of conventional antibiotics.

The first two articles included in the present thesis (article 1 and 2) describe the screening of extracts of eukaryotic organisms (microfungi and marine invertebrates) for the presence of quorum sensing antagonists. As revealed in these two articles, production of compounds capable of interfering with bacterial communication seems to be common among eukaryotes, supporting the idea that the ability interact with coordinated bacterial behaviour provides higher organisms with an evolutionary advantage. Interestingly, quorum sensing inhibitors such as patulin and penicillic acid (article 1) and the three manoalide compounds (article 2) share the 2(5*H*)-furanone moiety. Since these furanones are found in extracts of diverse groups of organisms, it suggests that the ability to produce them either has evolved in parallel in different organisms or that the ability to produce such compounds is a reminiscence of an ancient shared ancestor (of a range of marine invertebrates and microfungi), the trait of which has been maintained through times by selective forces.

Most of the antibiotics used today originate from natural sources. The general assumption is that antibiotics have evolved in nature to counteract growth of competitors; however, this idea has recently been challenged, and it has been suggested that the ecological role of antibiotics is as communication messengers instead of defeating competitors.
Article 3 included in this thesis investigates the application of low concentrations of natural as well as synthetic antibiotics as inhibitors of bacterial communication and in turn production of virulence factors. Three renowned antibiotics which are frequently used in the clinic to treat *P. aeruginosa* infections were found to decrease the expression and production of a range of quorum sensing regulated virulence factors when administered in subminimum inhibitory concentrations. This suggest that antibiotics may display dual activities; as bactericidal or bacteriostatic agents and as antagonists of bacterial communication. Taking this into account, it is fair to assume that we have not even fully explored and exploited the potential of the drugs we know today.

As described in the final manuscript (article 4), *P. aeruginosa* also produces multifunctional compounds; two quorum sensing signal molecules; the *Pseudomonas* Quinolone Signal and *N*-3-oxododecanoyl L-homoserine lactone exhibit the ability to modulate activities of the immune defence in addition to functioning as quorum sensing mediators. The two signal molecules

impair activities of immune cells crucial for the pro-inflammatory and antibacterial responses of the host, and this phenomenon may contribute to the successful establishment of *P. aeruginosa* in its host.

The key to combat this bacterium may lay in the development of a novel chemotherapy which aims at impeding the coordinated behaviour of this pathogen, its production of virulence factors and its ability to alter the response of the immune defence. This key probably already exists and may be found among eukaryotic organisms which have evolved to produce quorum sensing antagonists.

INTRODUCTION

Last century started with the promising prospect of antibiotics as highly efficient drugs for treatment of bacterial infections, but closed with the gloomy scenario of rapid development of multi-resistant bacteria. The discovery of antibiotics undoubtedly opened one of the greatest medical breakthroughs of the last century. Antibiotics however, target the ability of bacteria to grow and proliferate, thus such compounds also induce a simple "survival of the fittest" selection for the bacteria to evolve mechanisms to withstand this effect. The American Food and Drug Administration (FDA) has proposed that if action is not taken to prevent it, the increasing emergence of antibiotic resistant bacteria could make us face previously treatable diseases that have again become untreatable, as in the days before antibiotics were developed. Moreover, according to FDA around 70 % of the bacteria causing infections in hospitals are currently resistant to one or more of the drugs most commonly used. Thus, there is an urgent need to find new strategies to defeat pathogenic bacteria based on principles that are unlikely to impose selection pressures for the bacteria to develop tolerance against them.

One strategy is the development of anti-pathogenic drugs which function to attenuate bacteria with respect to virulence rather than defeating bacterial growth. One such target is interbacterial communication known as quorum sensing, used by many Gram-negative pathogens to synchronise expression of virulence genes in response to the density of the surrounding bacterial population (287). Obstruction of cell-to-cell communication, and thus virulence factor production, may prevent bacterial establishment of a successful invasion and improve the prospects of the host's immune system to clear the bacterial infection, particularly if this strategy is used in combination with conventional antibiotics (134). Proof of this concept has been provided by several *in vivo* experiments; e.g. applying the pulmonary infection model where mice are intratracheally challenged with bacteria embedded in alginate beads resembling the initial stages of a chronic lung infection (292). Mice infected this way with *P. aeruginosa* and treated with compounds inhibiting quorum sensing clear the bacteria faster and have lower mortalities than untreated mice similarly infected. (Described in article 1 and (134, 322, 426)).

Besides being an increasing medical challenge, the opportunistic pathogen *P. aeruginosa* is an important model organism for analysing the impact of environmental conditions on the expression of virulence factors. *P. aeruginosa* is often responsible for very persistent infections especially in immunocompromised patients, partly due to its intrinsic resistance against most applied antibiotics. Another very remarkable and important feature of this organism is its ability to interact with eukaryotic cells. Notably it has been shown that *P. aeruginosa*, via quorum sensing signal molecules, affects functions of the immune system in mammalian organisms (359, 378). By interfering with the immune defence, *P. aeruginosa* may improve its prospects for surviving the hostile environment within the host. The pathogenic properties of this bacterium are however, not restricted to mammalian hosts; *P. aeruginosa* has been shown to cause disease in the nematode *Caenorhabditis elegans,* the fruit fly *Drosophila melanogaster* and the plant *Arabidopsis thaliana* (16, 62, 306, 315). In addition, *P. aeruginosa* strains have been shown to inhibit the growth of a broad spectrum of phytopathogenic fungi (21, 176).

Bacteria living in association with eukaryotes (such as soil bacteria and pathogens) often use quorum sensing to coordinate their activities, including the production of compounds potentially detrimental to other organisms. In order to defend themselves and their habitat, eukaryotes devoid of cell mediated immune defences produce bioactive compounds that can counteract menacing bacteria and/or their virulence. Thus a logical place to search for quorum sensing antagonists is in natural libraries of chemically rich organisms such as fungi, plants and

marine invertebrates. This application of one aspect of prokaryotic-eukaryotic interaction has been explored in article 1 and 2 included in this thesis. Article 1 "*Identity and Effects of Quorum Sensing Inhibitors Produced by Penicillium Species"* entail the screening of extracts of microfungi for the presence of quorum sensing inhibitors. Two secondary fungal metabolites, patulin and penicillic acid, are identified as efficient inhibitors of quorum sensing. Article 2 "*Quorum sensing antagonism from marine organisms*" describes the results of screening 284 organisms from the Great Barrier Reef where three sesterterpenoid compounds were found to be highly active. Intriguingly, one of these bioactive manoalides were already known to be a potent inhibitor of human phospholipase A_2 (IC_{50} 0.02-0.2 μM) and thus hold potential for treatments against autoinflammatory conditions such as rheumatoid arthritis along with the ability to inhibit quorum sensing (153). Other examples of drugs capable of affecting multiple targets are described in the third article "*Effects of antibiotics on quorum sensing in Pseudomonas aeruginosa*". This article demonstrates that although antibiotics are conventionally used to kill or inhibit growth of microorganisms some may also have applications as antagonists of bacterial cell-to-cell communication. The last manuscript, article 4 "*Pseudomonas aeruginosa Quorum Sensing Signal Molecules interfere with dendritic cell induced T cell proliferation*" also deal with dual functioning compounds. As is demonstrated in this study, OdDHL and PQS are not only functioning as quorum sensing signal molecules, they also have the capacity to alter the immune defence of the host.

The intention of this thesis is to explore the interaction between the prokaryote *P. aeruginosa* (mediated via its signal molecules) and cells of the immune defence. It also investigates the potential of using signal antagonists derived from natural sources (eukaryotic organisms) to prevent bacterial quorum sensing. As demonstrated in this thesis; *P. aeruginosa* is, via its cell to cell communication, intertwined with higher organisms in a network of cross-kingdom interactions, and this may - at least partly - explain why this microbe is a rather successful opportunistic pathogen which is difficult to combat with the present day technologies. At the same time these cross-kingdom interactions offer a solution to counteracting *P. aeruginosa* infections by targeting communication of this bacterium, e.g. by using naturally evolved quorum sensing antagonists.

PSEUDOMONAS AERUGINOSA

Pseudomonas aeruginosa is a single flagellated Gram-negative rod belonging to the family Pseudomonadaceae as a part of the γ-proteobacteria. (See figure 1). It is an aerobic bacterium; but during oxygen limitations it is able to exploit nitrite/nitrate as terminal electron acceptors (124, 208). The optimum growth temperature for *P. aeruginosa* is 37 °C, it can, however, grow even at temperatures as high as 43 °C. It inhabits a wide variety of ecological niches such as soil, vegetation and aquatic environments and it is capable of causing disease in many eukaryotic organisms e.g. plants, insects and humans (62, 306, 315).

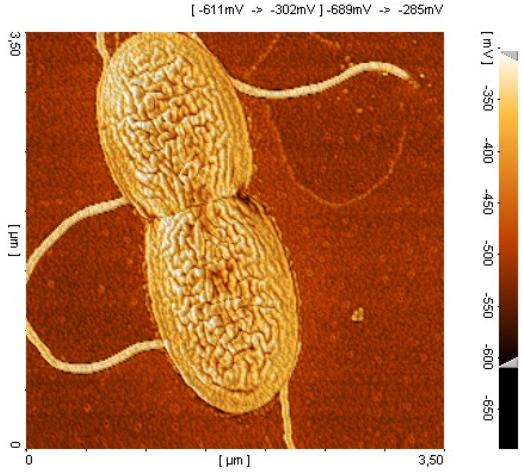


Figure 1. *P. aeruginosa* is a single flagellated aerobic rod. Each cell measures 0.5 to 0.8 µm by 1.5 to 3.2 µm. (Phase Imaging Atomic Force Microscopy of dry PAO1 cells by Anne Louise Frost, Center for System Microbiology, Biocentrum-DTU, 2007)

The sequenced *P. aeruginosa* PAO1 strain has a genome sized 6.3 million base pairs and 5570 predicted ORFs (Open Reading Frames) thus its genome is almost 50 % larger than the *Escherichia coli* genome. Moreover, the *P. aeruginosa* genome contains a high proportion of regulatory genes (8.4 % of predicted ORFs) which is presumed to contribute to its adaptability, broad host range and high intrinsic resistance to a wide range of antimicrobial substances (121, 178, 371). In comparison, only around 5 % of the genes in *E. coli* and *Bacillus subtilis* are regulatory genes (371). In addition, 0.3% of the total ORFs in PAO1 code for proteins involved in antimicrobial resistance. The pathogenic potential of this versatile bacterium is also reflected by the high number of genes encoding secreted virulence factors such as toxins and tissue degrading enzymes including proteases and elastases (371).

THE PATHOGEN *P. AERUGINOSA*

P. aeruginosa seldom infects healthy individuals, but has been found in relation to periodontitis and to be the cause of keratitis among users of contact lenses (22, 98, 383, 416). Over the last few decades, *P. aeruginosa* has emerged as a major opportunistic human pathogen accounting for a high number of hospital-acquired infections, especially at intensive care units (122, 213, 338, 387). This bacterium often infects decubitus ulcers and burns wounds thereby retarding healing or even causing enlargement of the wounds (64). *P. aeruginosa* infections are also commonly found among immune suppressed individuals e.g. AIDS patients or cancer patients undergoing chemotherapy. It is also the predominant cause of pneumonia in persons afflicted by Cystic Fibrosis where it is the main source of morbidity and mortality due to lung failure caused by prolonged *P. aeruginosa* infection (142, 171, 205)

P. AERUGINOSA AND CYSTIC FIBROSIS

Cystic fibrosis (CF) is an autosomal recessive disease and, according to Centers for Disease Control and Prevention (CDC), the incidence rate is 1: 2500 among non-Hispanic Caucasians and 1:13.000 and 1:15.000 among Hispanic Caucasians and African Americans, respectively (45). According to the Danish CF foundation there is around 435 CF patients in Denmark (www.cf-dk.org, 2007). The disease is caused by dysfunction of a cAMP-regulated chloride channel, CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) and more than 1500

different mutations leading to CF have until now been identified (1). CFTR is sited at the apical surface of epithelia cells including those located in the respiratory, gastrointestinal, hepatobiliary and reproductive systems where it is involved in electrolyte secretion and absorption across the epithelium. As the name implies the CFTR protein is a "conductance regulator" and thereby it affects many important transport systems involved in the maintenance of the basal state fluid balance. Inactivating mutations in CFTR result in an increased resorption and a decreased secretion of water, which among other serious effects leads to a lowered amount of fluid in the airways of patients suffering from CF (See figure 2). It is presumed that the reduced surface to liquid volume in the airways impairs ciliary function thereby reducing mucociliary clearance and creating the basis for recurrent infections (205). In young CF patients a variety of bacteria such as *Haemophilus influenzae, Staphylococcus aureus* and *Streptococcus pneumonia* cause pneumonia. From the age 10-14 *P. aeruginosa* becomes the predominant pathogen in the CF lung, sometimes followed by coinfection by *Burkholderia cepacia* (18, 97).

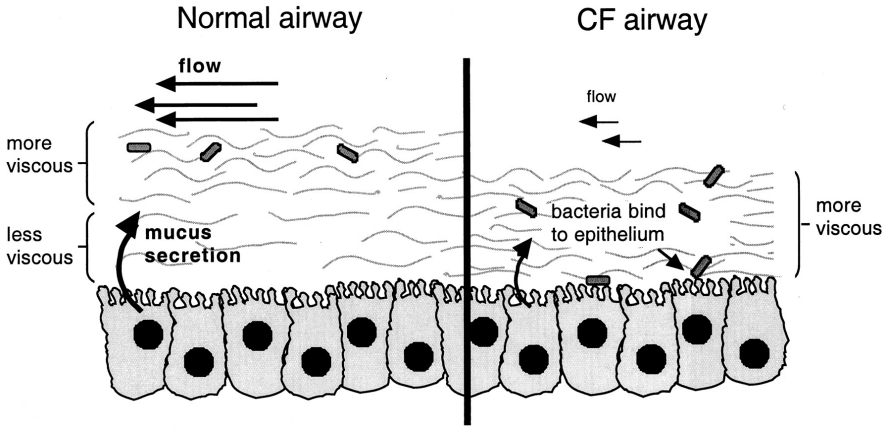


Figure 2. Mucociliary clearance in normal airway versus CF airway. In the normal airway, the epithelium is covered with a differentiated mucus layer with the upper part being more viscous. Concerted beating of the epithelial cell cilia causes the mucus to flow toward the esophagus and thereby removing the bacteria. In the CF airway the mucus layer is more uniformly viscous and the beating of the cilia are not sufficient to propel the mucus and bacteria towards the esophagus. (Figure adapted from (205)).

Other factors besides reduced bacterial clearing caused by high mucus viscosity might predispose CF patients to chronic bacterial colonisation. It has been proposed that CFTR functions as an epithelial cell receptor for *P. aeruginosa* being involved in bacterial internalization and clearance from the lung and that the lack of this host factor in CF patients may be a critical factor for the hypersusceptibility of these patients to *P. aeruginosa* chronic lung infections (302-304). The increased bacterial survival in the CF lung may in part be caused by impaired phagocytic killing by neutrophils due to the elevated chloride concentration (375). CF airway epithelia have been reported to constitutively overexpress the transcription factor nuclear factor kappa B (NF-κB) which is a central player in inflammation. Increased NF-κB activity may be involved in the exaggerated interleukin-8 (IL-8) production reported in the CF lung leading to excessive neutrophil infiltration and thus tissue destruction (29, 374). Furthermore phosphorylation by the extracellular signal-regulated kinase (ERK) has been shown to increase in CF cell lines, probably causing an enhanced expression of proinflammatory cytokines such as IL-1β, IL-6 and IL-8 (394). It thus seems that, even in the absence of a bacterial infection, factors associated with the CFTR mutation cause an intrinsic inflammation. This is supported by a recent study which showed over-expression of proinflammatory proteins in the airways of a 24-week-old CF-fetus (393).

P. aeruginosa produces several enzymes with elastolytic activity and besides causing structural damage the elastases also stimulate the production of pro-inflammatory mediators such as IL-8, which further induces neutrophilic influx. When present in excess during a prolonged period, neutrophils and their products, including released DNA from dying neutrophils, actually impair the host's ability to clear bacterial infection and enhance *P. aeruginosa* biofilm production (246, 309, 403). Elastases also impair mucociliary clearance by direct effects on ciliary function and by inducing mucus production. Moreover, elastase inhibits opsonophagocytosis by cleaving of immunoglobulin G (386). (See figure 3).

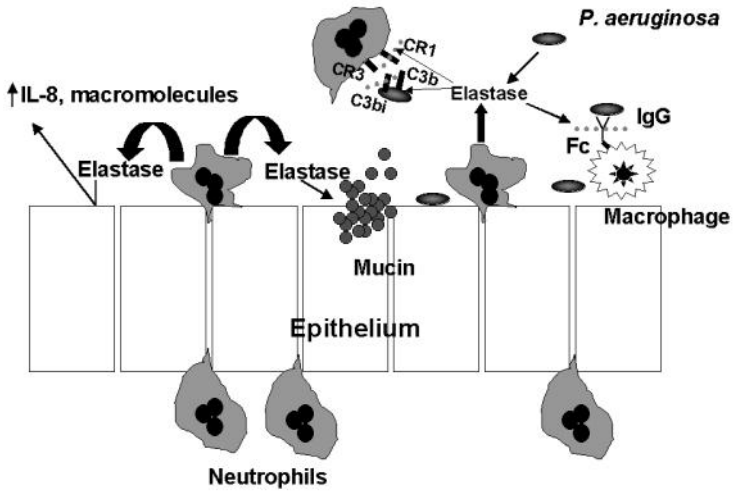


Figure 3. Effects of elastases on host defence mechanisms and inflammation. Both bacteria and neutrophils produce elastases. Besides destruction of epithelium tissue the hinge region of immunoglobulin G and complement receptors are cleaved by elastase (Figure from (54)).

CF patients receive intensive antibiotic treatments and although the bacterial load decreases shortly after a treatment period the infection is never fully eradicated. Several factors account for this phenomenon such as *P. aeruginosa*'s intrinsic high tolerance to many clinically important antibiotics as well as its ability to develop resistance against antibacterial agents. Many clinical *P. aeruginosa* strains isolated from CF patients have a mucoid phenotype and secrete high amounts of polysaccharides such as alginate, which may protect the bacterium against antibiotics and actions of the immune defence.

ANTIBIOTIC RESISTANCE IN *P. AERUGINOSA* STRAINS

Resistance can be defined as the ability of a microorganism to grow in the presence of an elevated level of antimicrobials (195). As mentioned earlier, *P. aeruginosa* has an intrinsic high resistance against most antibiotics, which significantly contributes to eradication failure. Different mechanisms accounts for this inherent multi-drug resistance against a range of structurally and functionally different xenobiotics such as chloramphenicol, kanamycin, imipenem, tetracycline and many dyes (172, 173, 229, 313).

First of all, the permeability of the *P. aeruginosa* cell membrane is 10-100 fold lower than for other Gram-negative bacteria, and this protects it from harmful substances entering the cell (264). Other important factors adding to *P. aeruginosa*'s ability to tolerate high doses of xenobiotics are the constitutively expressed multidrug efflux pumps such as MexAB-OprM and Mex pumps induced by the presence of antibiotics such as MexXY–OprM, MexCD–OprJ and MexEF–OprN (263, 308). (For an outline of the *P. aeruginosa* MexAB-OprM efflux pump see figure 4).

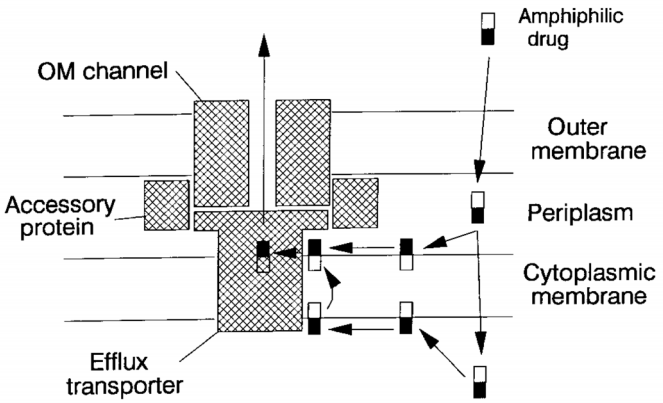


Figure 4. Schematic illustration of the MexAB-OprM efflux pump in *P. aeruginosa*. The efflux pump extrudes drugs in an energy-dependent manner by the use of proton motif force. (Figure adapted from (262).)

Efflux is usually not considered to confer high levels of resistance, but due to cross resistance it may narrow down the choice antibiotics in the clinic (202). Induced efflux of antibiotics may also favour the emergence of target mutations due to lowered intra-bacterial antibiotic concentrations (261). Predictions based on the sequenced PAO1 suggest that there may be as many as 30 efflux systems in *P. aeruginosa* and 150 outer membrane proteins (OMPs) (371). OMPs have clinical relevance as they are involved in transport of antibiotics, export of virulence factors and in anchoring mediators of adhesion and motility.

In addition to these antibiotic evading mechanisms, *P. aeruginosa* has the ability to produce enzymes which target the activity of antibiotics such as chromosomal β-lactamase present in most clinical strains and aminoglycoside acetyltransferase (67, 122). Resistance to fluoroquinolones and aminoglycosides are often mediated by gene elements which enable the bacteria to modify targets of the antibiotic such as methylation of 16S rRNA and mutations in the DNA gyrase *gyrA* gene and the topoisomerase IV *parC* gene (81, 177, 247, 437).

While *P. aeruginosa* definitely does not lack mechanisms to escape the actions of antibiotics, only few novel antibiotics with antipseudomonal activity are currently being developed (109, 229). This is certainly worrying, as infections caused by resistant strains are associated with a three-fold higher rate of mortality, a nine-fold higher rate of secondary bacteraemia and a two-fold increase in the length of hospital stay (109) thus, besides the personal consequences for the patients, resistant strains also considerably increase healthcare costs. Unfortunately it seems that the frequencies of multidrug-resistant *P. aeruginosa* (defined as resistance to at least three main classes of antipseudomonal agents such as β-lactams, carbapenems, aminoglycosides and fluoroquinolones) are increasing worldwide, reaching frequencies of up to 20 % in intensive care units in the USA and >30 % in Asia (95, 222, 229, 269, 334). Faced with such a gloomy picture, the development of new therapeutic strategies, including drugs acting on new targets is urgently needed (168, 229).

P. AERUGINOSA BIOFILMS

As outlined above, several intrinsic and acquired resistance elements add to the difficulties of eradicating infections caused by *P. aeruginosa*. Another important factor contributing to *P. aeruginosa* pathogenesis in clinical settings is the biofilm mode of growth involved in chronic as well as in acute infections (342). Centers for Disease Control and Prevention (CDC) and National Institute of Health (NIH) estimate that 65-80 % of all human bacterial infections involve biofilms. Biofilms are defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, and embedded in a matrix of self-produced extracellular polymeric substances (EPS), and exhibit an altered phenotype with respect to growth rate and gene transcription (86). This mode of growth is likely to represent the prevalent form of microorganisms in nature, and visible to the naked eye, these microbial communities were among the first to be studied. 300 years ago Antoni van Leeuwenhoek scraped dental plaque from his teeth and observed the “animalculi”

producing this coating. Despite this old finding, bacteria have most often been studied in the laboratory as planktonic cells, freely suspended in liquid growth media. Rediscovery of the phenomena that microorganisms attach to and grow on surfaces has added new knowledge to understanding the true nature of bacterial living.

Biofilm formation is a complex process involving several steps. These steps include attachment of the cells to substratum, growth and aggregation of cells forming microcolonies, development of a highly differentiated structure ("towers" or "mushrooms"), maturation and maintenance of architecture, and release of cells subsequently followed by recolonisation of new areas (267, 339). (See Figure 5 for details).

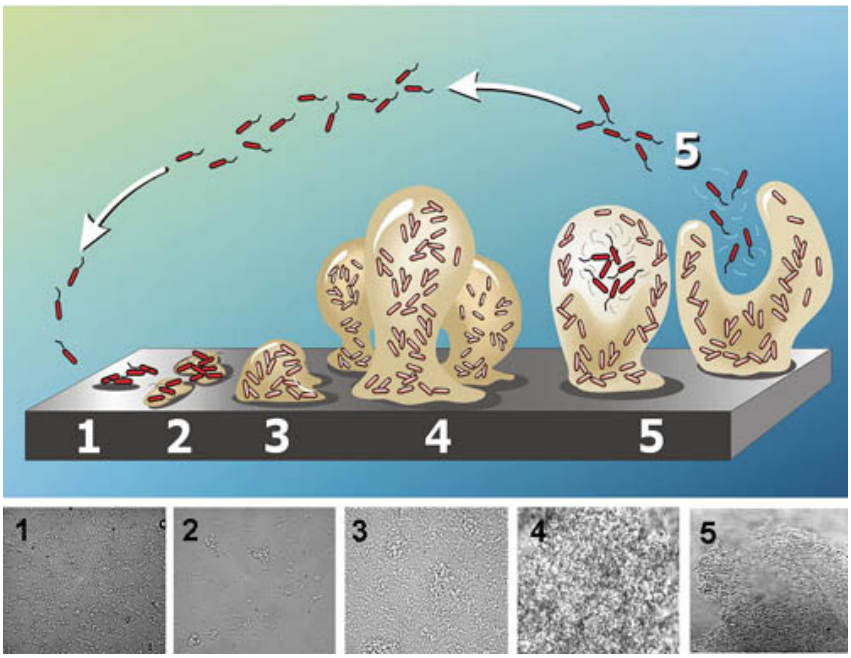


Figure 5. Biofilm development in *P. aeruginosa*. **1** Initial attachment to substratum, **2** the bacteria adhere to eachother **3** forming small microcolonies, **4** the three-dimensional structure of the mature biofim develops and **5** cells are released (sloughing off) and recolonise new areas of substratum. (Graphic and photos by Peg Dirckx and David Davies, Center for Biofilm Engineering, Montana State University, Bozeman.)

The biofilm consists of heterogeneously distributed bacterial cells (cells constitute 5-20 % of the biofilm volume) embedded in an EPS matrix. The exact composition of the matrix depends on the phenotype of the bacteria producing the EPS. Mucoid *P. aeruginosa* strains overproduce the exopolysaccharide alginate (composed of uronic acid β-D-mannuronate and its C-5 epimer α-L-guluronate), whereas in the non-mucoid matrix the predominant exopolysaccharides are polymers of glucose and rhamnose (423). Also extracellular products such as proteins, nucleic acids, metabolites and absorbed nutrients are parts of the biofim matrix (142, 411). As a consequence of its complex structure the micro milieu are very heterogeneous throughout the biofilm, with gradients present from the waterfilled channels containing dissolved oxygen and nutrient into the base of the biofilm. Hence the metabolic state of the cells differs likewise. (See figure 6 describing the heterogeneity of the physiological activity within a *P. aeruginosa* biofilm).

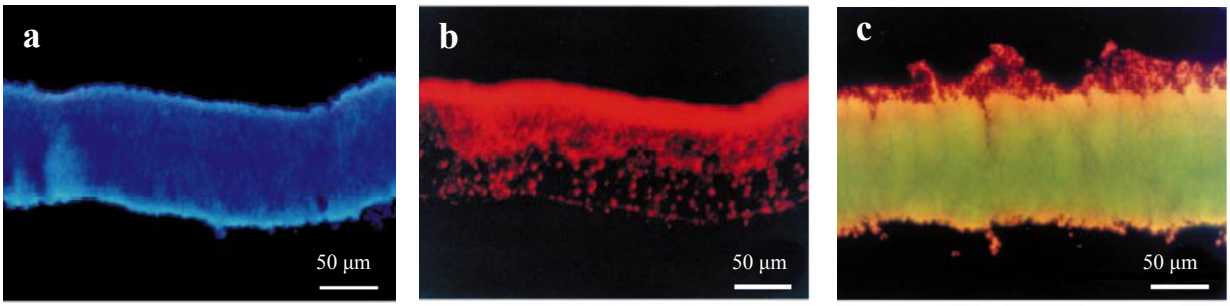


Figure 6 Heterogeneity of the physiological activity within a *P. aeruginosa* biofilm. Frozen sections of biofilms examined by epifluorescent microscopy. Media flow at the top and substratum at the bottom. **a** Biofilm section post-stained with the DNA stain DAPI. Blue indicates the distribution of all cells independent of metabolic activity. **b** Biofilm stained with 5-cyano 2,3-ditolyl tetrazolium chloride. Red indicates respiratory activity, mainly found at the top. **c.** Biofilm section post-stained with acridine orange. Orange/red indicates putative regions of rapid growth and green regions with slow or no growth. Yellow indicate an intermediate growth rate. In accordance with the respiratory activity, rapid growth is found near the media flow. (Figure adapted from (430)).

It has been shown that *P. aeruginosa* cells growing as biofilms tolerate 100-1000 times more antibiotics than their planktonic counterparts (15, 46, 145). By the conventional definition and criterion of resistance as an increased Minimum Inhibitory Concentration (MIC), biofilm cells do not necessarily show increased resistance against antibiotics. However, the *survival* of cells in biofilms treated with antibiotics might be increased compared to planktonic cultures. Thus, most of the studies reporting elevated resistance of biofilm cells describe an increased tolerance to killing rather than growth inhibition (365). The mechanisms for the increased tolerance of biofilm living cells towards antibiotics are not fully elucidated. Several factors may contribute to the protection of cells in the biofilm mode of growth, perhaps also depending on the properties of various antibiotics. The matrix is assumed to reduce the diffusion of some antibiotics in a rate depending on the nature of the applied compounds and the biofilm matrix composition. An investigation by (210) showed that a *P. aeruginosa* strain with a mutation in the *ndvB* locus, required for the synthesis of periplasmic glucans, was unable to develop high-level biofilm-specific resistance to tobramycin, gentamicin and ciprofloxacin. This suggest that the glucans sequester these antimicrobial agents in the periplasm, however this mechanism does not work for all antibiotics as the resistance of this strain against nalidixic acid was unchanged (210).

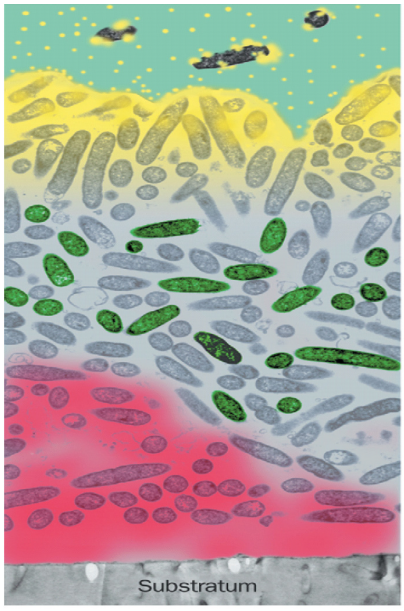


Figure 7. Mechanisms suggested contributing to antibiotic resistance in biofilms. Slow penetration. Antibiotic (yellow) may fail to penetrate beyond the surface layers of the biofilm. Resistant phenotype. Some of the bacteria may differentiate into a protected phenotypic state (green) Altered microenviroment. In zones of nutrient depletion or waste product accumulation (red) antibiotic action may be antagonized. (From (367)).

Aminoglycosides (like gentamicin and tobramycin) bind to the anionic polysaccharide in the matrix, reducing the free concentration of antibiotic being the driving force of the diffusion, and this might inhibit the distribution of these antimicrobials (152, 258). The biofilm matrix may confer a diffusion barrier and may contribute to the enhanced resistance of biofilm living cells, but it is probably not the sole explanation on the lowered sensitivity to antibiotics of biofilm living cells (366). However, if the decreased diffusion rate is combined with a rapid (enzymatic) degradation of the antimicrobial compound it could inhibit the penetration of

active antibiotics (12). Another hypothesis for the enhanced resistance is based on the earlier mentioned heterogeneous microenviroment within the biofilm, where the nutrients and waste products are unevenly distributed. The accumulation of waste products or the depletion of oxygen and/or nutrients may cause the bacteria to enter a non-growing state, and thereby protect them against killing by antibiotics targeting growth (388). (See figure 7). When the antibiotic treatment is ended, surviving cells start proliferating and restore the biofilm population. (Refer to figure 8).

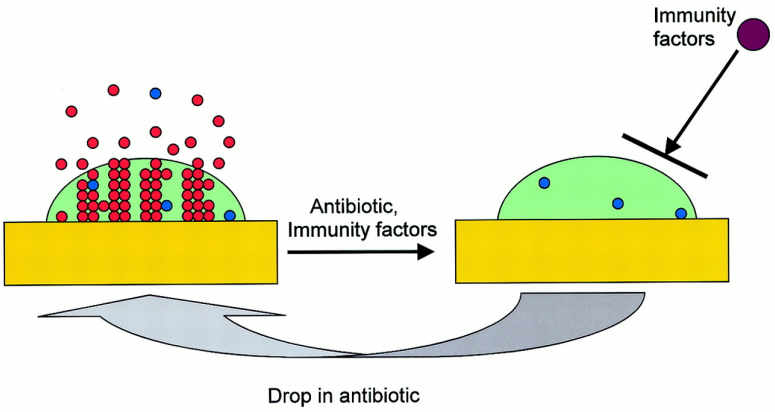


Figure 8. Model of biofilm tolerance. An initial treatment with antibiotics kills planktonic cells and the majority of biofilm cells. The immune system may eradicate planktonic survivors, but the cells persisting in the biofilm are protected from the host defences by the EPS. When antibiotic concentration drops, these cells reestablish the biofilm and the infection relapses. (Figure from (195)).

It has been observed that even newly formed - and hence thin – biofilms protect the cells against killing even though the biofilm matrices at this stage are too thin to pose a barrier to the penetration of antibiotics and metabolic substrates (59, 65). However, a recent proteomic study shows that *P. aeruginosa* biofilm cells overall mostly seem to resemble exponentially growing planktonic cells (230). Collectively, this suggests that the biofilm mode of growth causes a minor subpopulation of cells within the biofilm to differentiate into a protected phenotype analogous to spore formation.

Multiple resistance mechanisms such as those mentioned above may act together to cause the improved tolerance to bactericidal agents of biofilms however, the multicellular nature of the biofilm is likely to play a central part in the explanation model. Lowered antibiotic penetration is dependent on the ability of the microorganisms to form aggregates to affect the diffusion rate. Local variations in the concentration of nutrients and waste products only exist if the cell clusters reach a critical mass. The existence of non-growing cells is dependent on the other cells in the biofilm to propagate the genome, whereas the growing cells depend on the dormant cells to re-establish the community after the event of catastrophic killing. Thus to treat biofilm related infections, the strategy should be targeted to the multicellular level involved in the development of the heterogeneous biofilms rather than essential functions of the individual cells.

It has been demonstrated that quorum sensing is crucial for the development of a normal biofilm in *P. aeruginosa* and for the high tolerance to antibiotics of *P. aeruginosa* biofilms (66, 281, 346). Therefore, potential therapies against biofilm infections could involve treatment with inhibitors of the signalling system.

QUORUM SENSING

In order to be able to survive under changing conditions and in hostile environments, bacteria have evolved systems to monitor and respond appropriately to environmental stimuli such as nutrients, stressors and - for some bacteria – cell density. The latter depends on small diffusible signal molecules constitutively produced by the bacteria, which interact with transcriptional activators to couple gene expression with cell population density. This enables the bacteria to function as a group and to some degree exhibit multicellular behaviour. This form of cell to cell communication is termed quorum sensing. In Gram-negative bacteria this phenomenon is mainly mediated by *N*-acyl L-homoserine lactones (AHLs), consisting of a conserved homoserine lactone ring with an *N*-acyl side chain. The AHLs predominantly vary in the presence or absence of a keto- or hydroxy- group on the C3 of the acyl chain and the length and saturation of this chain. (See figure 9).

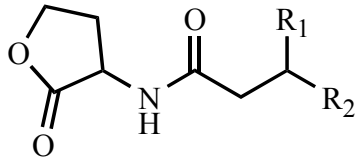


Figure 9. General structure for the AHL signal molecule. R_1 is -H, -OH or =O; R_2 is $(-CH_2)_{1\text{-}13}-CH_3$ or $-CH_2-CH_2-CH{=}CH-(CH_2)_5-CH_3$

Also a number of Gram-positive bacteria apply signal mediators to coordinate behaviour with cell density. Gram-positive bacteria use peptides as ligands for the receptor of a two-component signalling module thereby activating a complex adaptation response involving many genes (206). In the following however, the term ”quorum sensing” is exclusively used about AHL-mediated quorum sensing in Gram-negative bacteria, unless otherwise stated.

Quorum sensing relies on two key proteins; the LuxR-homologue (the LuxR-type response regulator) which is the receptor protein recognising the signal molecules and the LuxI-homologue synthesising the signal molecules (AHLs). The AHLs are produced constitutively at a basal low level, and are distributed in the cell and the surrounding environment either actively or by diffusion. Hence, the concentration of AHL increases with increasing cell density and reaching a certain level, corresponding to a "quorate" cell density, the signal molecule binds to the LuxR-homologue which thereby becomes activated. The activated complex then functions as a transcription factor, which binds to the promoter region of quorum sensing regulated genes triggering their expression (103).
Quorum sensing was first identified in the marine symbiont bacteria *V. fischeri* (formerly *Photobacterium fischeri*) where the *lux* system controls the expression of bioluminescence (251, 252, 355). The signal molecule produced via the LuxI synthetase is 3-oxo-hexanoyl L-homoserine lactone (OHHL) which binds to and activates LuxR. The OHHL-LuxR complex dimerizes and binds to the *lux* box located in the *luxI* promoter region thereby activating transcription of the *lux* operon; *luxICDABEG*, which leads to induction of luminescence. (See figure 10). Since *luxI* is a part of the *lux* operon, positive feed back regulation takes place and results in a burst of bioluminescence. Hence, the quorum sensing system is also termed an autoinducer system; even though not all of the cell-cell communication systems today known include autoinducing loops.

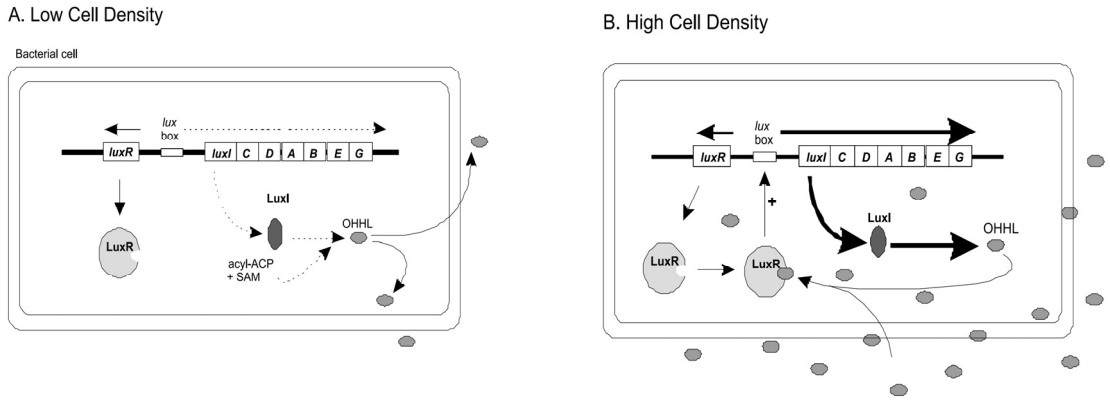


Figure 10. The regulation of bioluminescence in *V. fischeri*. **A.** At low cell density, transcription of genes encoding bioluminescence (*luxICDABEG*) is weak and insufficient for light emission due to low levels of OHHL. **B.** At high cell density OHHL reaches a high concentration indicating that the population is "quorate" and OHHL binds to LuxR leading to transcription of *luxICDABEG*. This further causes amplification of OHHL and production of luminescence. Adapted from (412).

Numerous *Vibrio* species are known to produce quorum sensing signals presumably to regulate expression of virulence factors including entero- and exotoxins and enzymes linked to pathogenicity (37, 61, 216). Full commercial exploitation of aquaculture is often hampered by bacterial infections caused by *Vibrio* species (117, 197). In collaboration with the Australian Institute of Marine Science I have recently investigated the presence of AHLs in tropical ornate rock lobster rearing tanks and in lobster larvae (phyllosomas). AHLs were first found to be produced within the biofilm attached to the rearing tank at day 7 and were then present through the remaining period of the 21 day larval-rearing run. Interestingly, quorum sensing inducing signals were first detected in the phyllosoma samples at day 9 to 12 although at low levels before being strongly expressed at day 15. This coincided with a mass mortality event within the larval-rearing tank and suggests that quorum sensing plays a significant role for the pathogenicity of aquaculture pathogens (32).

Since the discovery of the *lux* quorum sensing system in *V. fischeri,* a number of Gram-negative bacteria belonging to the α- β- and γ- subclasses of the proteobacteria have been found to produce LuxR and LuxI-type proteins (reviewed by (249)). However, not all the *luxR* type proteins are transcriptional activators, a few function as repressors of gene expression. Examples are EsaR of *Pantoe stewartii* and VirR of *Erwinia carotovora* ssp. *atroseptica* which govern the expression of specific target genes by repression and AHL-dependent derepression (39, 232, 397).

E. coli and *Salmonella enterica* serovar Typhimurium both harbour a gene encoding a signal receptor of the LuxR family, SdiA, but not a corresponding signal-generating enzyme. Instead, it seems that SdiA enables these two organisms to respond to AHLs generated by other microbial species (8, 354, 390, 436). Besides interacting with AHL, the *E. coli* SdiA transcription factor also responds to a stationary phase signal; indole which is secreted in large quantities into rich medium by *E. coli.* Indole has been shown to control several genes (e.g., *astD*, *tnaB*, *gabT*), multi-drug exporters, and an *E. coli* pathogenicity island (50, 138, 188, 404). While indole seems to function as a repressor of *E. coli* biofilm formation it stimulates biofilm formation in pseudomonads and has thus been suggested to be an inter-species signal molecule (188). Interestingly, it has been shown that indole inhibits quorum sensing in the *V. fischerii lux* system (320).

As in *V. fischeri,* where the luminescence only develops when a sufficiently high cell population is reached to allow the presence of a visible light, it is presumed that quorum sensing in other bacteria controls the production of specific products which need to be present only at high cell density. For pathogenic bacteria it may be important to reduce production of virulence factors (which alarm the immune defence) until the cell population has reached a "critical mass" that can cope with the immune defence. This strategy is likely to minimise the hosts' ability to mount a sufficient and effective defence against the invading bacteria (282, 419, 442). Moreover, it is obviously also more cost-efficient to delay expression of "expensive" proteins until they are needed, that is, at dense cell populations.

QUORUM SENSING IN *P. AERUGINOSA*

Two AHL-mediated quorum sensing circuits have been identified in *P. aeruginosa*; the LasI-LasR and the RhlI-RhlR system. They function in concert to control the expression of an array of genes, among these many genes encoding virulence factors (106, 181, 285). The final step in the synthesis of OdDHL (*N*-3-oxododecanoyl-L-homoserine lactone) and BHL (*N*-butanoyl-L-homoserine lactone) are mediated by LasI and RhlI, respectively. Each system modulates a regulon comprising an overlapping set of genes. However, it is presumed that a regulatory hierarchy exists in which LasR-OdDHL activates the transcription of *rhlR* (180). Hence, genes controlled by the RhlI-RhlR system require a functional LasI-LasR system for full activation. Under phosphate-limiting conditions various transcriptional activators, including the global regulatory gene Vfr, the alternative sigma factor σ^{54} and RhlR itself participate in the regulation of *rhlR* expression (225). It has been shown in a heterologous genetic background (*E. coli*) that OdDHL is able to compete with BHL for the binding site of RhlR, and thus inhibit the translation of RhlR activated genes, indicating that LasI - through OdDHL - controls RhlR activity at a posttranslational level (295). Advancing the explanation of posttranscriptional control of the *las* system, it has been shown that RhlR forms homodimers both in the presence and absence of BHL, whereas OdDHL induce monomerization of the RhlR homodimer (392). LasR forms multimers only in the presence of OdDHL and this form is presumed to be the one responsible for its ability to function as a transcriptional activator (30, 169).

The crystal structure of the ligand-binding domain (LBD) of LasR bound to OdDHL has recently been solved, showing that the LasR monomer structure resembles, to some degree, the LuxR homologues TraR (*Agrobacterium tumefaciens*) and SdiA (*E. coli*) but with differences in the quaternary organization and the positioning of the acyl chain (30). The LBD of the LasR protein bound to OdDHL is shown in figure 11.

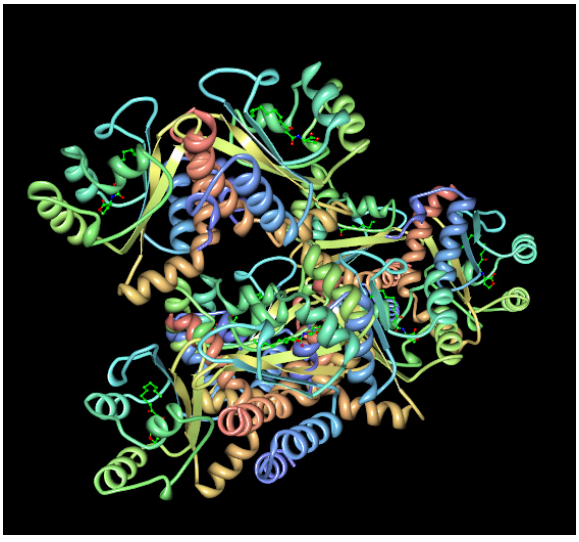


Figure 11. The tetramer structure of the ligand-binding domain of the LasR protein bound to OdDHL. (Accession number 2UV0. From www.rcsb.org/pdb Protein Databank of the Research Collaboratory for Structural Bioinformatics. PDB Simple viewer).

P. aeruginosa cells are permeable to BHL which freely diffuses into and out of the cells, whereas a membrane efflux pump is involved in active transport of OdDHL (290). A likely explanation of this difference is that the length and/or the substitution of the *N*-acyl side chain determine whether the signal molecule diffuses freely or is actively transported.

Besides the AHL signal molecules BHL and OdDHL, a third intercellular signal has been found to be part of the quorum sensing regulon in *P. aeruginosa.* This molecule; 2-heptyl-3-hydroxy-4-quinolone (designated the *Pseudomonas* quinolone signal; PQS) has been shown to play an important role in the infection of nematodes and mice (104, 182, 429). PQS has also been found in sputum, bronchoalveolar lavage fluid and mucopurulent fluid from distal airways of CF lungs removed at transplant (60). PQS is produced at the end of the exponential phase and peaks in the late phase of growth, which suggests that the production is not only controlled by the *las* and *rhl* quorum sensing system since they are activated well before onset of the main production of PQS (294). PQS acts as a coinducer for a LysR-type transcriptional activator, PqsR (formerly MvfR; Multiple virulence factor Regulator) (429). PqsR interacts with PQS and functions as a transcriptional activator until the stationary phase, when a negative feedback mechanism via the *rhl* system seems to repress expression of *pqsR* (42, 223). The transcription of the *pqsABCDE* operon is induced by PqsR-PQS thereby creating an autoinducing loop as this operon is a part of the PQS synthesis gene cluster (399, 429). PQS is also involved in the regulation of other quorum sensing related genes, among these BHL and multiple virulence factors such as pyocyanin, elastase and phospholipase (42, 224, 429). LasR has been found to be necessary for the production of PQS, which indicates that PQS play a role as an additional connecting link between the *las* and *rhl* quorum systems. Moreover, the ratio of OdDHL to BHL might have an influence on the production of PQS, suggesting a regulatory balance between the quorum sensing systems (223).
Recently it has been shown that PQS forms complexes with iron(III) at physiological pH which suggests that beside functioning as a signal molecule, PQS has a secondary function in iron entrapment to facilitate siderophore-mediated iron delivery (77). In accordance with the observation that PQS may assist iron-uptake, we have recently shown that expression of the *pqs* operon is induced in a subpopulation of biofilm cells under low-iron conditions (1 μM $FeCl_3$) (434). Along with PQS, *P. aeruginosa* produces more than 50 other quinolone compounds, many originally identified because of a third property; their antibiotic activities (126, 190, 192).

REGULATION OF THE QS CIRCUIT IN *P. AERUGINOSA*

The paradigm stating that the transcription of quorum sensing regulated genes exist as a response on a high concentration of signal molecules, is only partially adequate to explain the complex quorum sensing system in *P. aeruginosa*. Several surveys have demonstrated that the addition of AHLs to low cell density cultures of *P. aeruginosa* does not induce expression of quorum sensing regulated genes (78, 224, 226). Different genes under the control of quorum sensing are expressed at different growth phases, indicating that the AHL concentration alone does not control the expression of quorum sensing controlled genes in *P. aeruginosa* (413). Environmental factors such as medium composition and oxygen availability have also been found to influence the expression of quorum sensing regulated genes (88, 401).

Factors other than LasI-LasR and RhlI-RhlR and presumed to be involved in the quorum sensing cascade in *P. aeruginosa* are briefly described below.
The ***rpoS*** gene encodes the stationary-phase sigma factor (RpoS or σ^s) and is a central regulator controlling the expression of genes involved in cell survival in response to various stress effects, most notably stressors occurring in connection to cessation of growth (stationary phase) such as nutrient deprivation (102). RpoS also regulates the general stress response and protect the cell against oxidative stress, heat shock, increased osmolarity and low pH and other environmental challenges in a retroactive and a proactive manner (157, 201). In *P. aeruginosa*, the level of σ^s increases dramatically at the onset of the stationary phase and is regulated at the transcriptional and posttranscriptional levels. Several studies suggest a link between RpoS and

quorum sensing. Transcriptome analyses have shown that many quorum sensing regulated genes are induced at the onset of stationary phase or later during stationary phase, indicating a possible involvement of RpoS (134, 345, 401). The RhlI-RhlR system has been reported to activate the transcription of *rpoS* (180). Interestingly another study has reported that RpoS activates *rhlI* transcription (414). It thus seems that in *P. aeruginosa* RpoS and quorum-sensing are part of the same regulatory network. (See also figure 12).

RsaL is a pivotal modulator of quorum sensing and belongs together with many other important proteins to the tetrahelical superclass of helix-turn-helix (H-T-H)-containing proteins (319). *rsaL* is located downstream from *lasR* and is transcribed antisense relative to *lasR*. RsaL has been suggested to bind to the *lasI* operator region, at low cell densities -and hence low concentration of OdDHL- thereby directly repressing the expression of LasI by hindering the interaction between the *lasI* operator region and the LasR-OdDHL complex. As the concentration of LasR-OdDHL complex increases with increasing cell densities this complex outcompetes RsaL binding to the *lasI* operator and *lasI* is then expressed (69, 318).

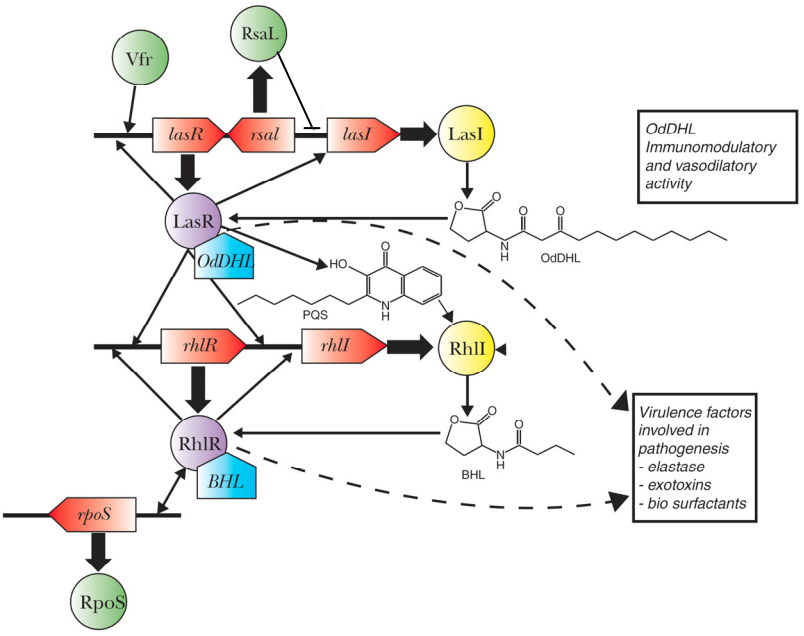


Figure 12. Central elements in the *P. aeruginosa* quorum sensing cascade. LasI-LasR and RhlI-RhlR is activated by their cognate signal molecules; OdDHL and BHL, respectively. Other factors such as PqsR/PQS, RpoS, RsaL and Vfr have also been shown to be involved in the regulation of the quorum sensing system.

RsmA (regulator of secondary metabolites) is a global posttranscriptional regulatory protein working in tandem with a small non-coding regulatory RNA molecule, RsmZ (also known as RsmB) located in the *rpoS/fdxA* intergenic region, to regulate the expression of the AHL synthase genes *lasI* and *rhlI*. RsmA functions as a negative regulator of quorum sensing by suppressing the production of OdDHL and BHL and thus decreasing levels of extracellular virulence factors such as proteases, elastases, PA-IL lectin, hydrogen cyanide and pyocyanin. RsmZ opposes the negative effect of *rsmA* on virulence factor production most likely by binding to RsmA (40, 136, 298). Another small non-coding RNA RsmY has been found to antagonize RsmA in a fashion similar to RsmZ (363). Expression of RsmZ and RsmY is dependent on RsmA and the global regulator GacA (40, 136, 163).

The **GacS/GacA** two-component system has been suggested to positively regulate the transcription of *lasR* and *rhlR* and to control the production of BHL thereby regulating

transcription of quorum sensing controlled genes and biosynthesis of virulence factors (296, 325). *P. aeruginosa gacA* mutants are less virulent compared to the wild-type in animal as well as plant models (316, 317). GacA has been found to modulate cyonogenesis positively at a post-transcriptional level. This suggest that the regulation exerted by the GacS/GacA system in *P. aeruginosa* is not functioning as a simple linear signal transduction pathway, but may involve both transcriptional and post-transcriptional mechanisms, where quorum sensing is probably only involved in the former (296, 297).

Besides LasR and RhlR a third LuxR type protein **QscR** (Quorum sensing control regulator) has been found in *P. aeruginosa* (57). QscR is proposed to form inactive heterodimers with LasR and/or RhlR in the absence of AHL; thereby inhibiting the expression of some quorum sensing controlled genes. When the concentration of signal molecules increases, the equilibrium would be changed towards the formation of the LasR-OdDHL homodimer complex and/or the RhlR homodimer bound to BHL, leading to expression of quorum sensing regulated genes (187). No cognate AHL synthase has been found for QscR, however it seems that OdDHL serves as a signal molecule for QscR (57, 193). Transcriptome analyses of a *qscR* mutant showed that QscR affects transcription of more than 400 genes, most of which are not affected by the Las and Rhl systems (193). It thus seems that the QscR regulated genes are only partly overlapping the quorum sensing regulon.

Hfq is a global regulatory protein belonging to the large family of Sm-like proteins, which forms homo-hexameric rings (265). The Hfq protein is highly conserved among bacteria, and it has been shown that Hfq from *P. aeruginosa* can functionally replace that of *E. coli* (362). Hfq controls the stability of mRNAs by interfering with ribosome binding and by affecting the length of poly-A tails (120, 398). In *E. coli*, the translation of the *rpoS* gene is stimulated by Hfq, presumably by modulation of the function of small regulatory RNAs (34, 239, 440). Hfq seems to be involved in *P. aeruginosa* virulence as an *hfq* mutant showed significantly attenuated pathogenesis compared to its wild-type parent when administered intraperitoneally in mice (361). A transcriptome analysis revealed that Hfq is involved in the regulation of around 5 % of all ORFs in *P. aeruginosa* PAO1 with around 25 % of the affected genes also being quorum sensing regulated (363). Hfq induces the expression of the BHL synthetase; this effect may be due to an Hfq mediated stabilisation of RsmY RNA (363, 364).

Another global regulatory gene, ***vfr*** (virulence factor regulator) is also presumed to be part of the complex quorum sensing mechanism of *P. aeruginosa* (410). Vfr is a homologue of the *E. coli* catabolite repressor protein CRP (cAMP receptor protein) and Vfr has been shown to bind to a CRP-binding consensus sequence (CCS) located at the promotor region of *lasR* and to positively regulate expression of exotoxin A and protease production (10, 410).

The regulator **PprB** has recently been reported to modulate quorum sensing and global gene expression in *P. aeruginosa* (84). The two-component regulatory system PprAB was first identified as a regulator of membrane permeability and sensitivity to antibiotics (405). While overexpression of *pprB* caused clinical strains to become more sensitive to antibiotics (405), knockout of the *pprB* gene caused a drastic reduction in virulence factor production and cell motility (84). Genes affected by an insertion mutation in *pprB* were mapped by transcriptomics and showed that 85.5 % of all genes activated by PprB were in addition quorum sensing regulated. Further analysis showed that PprB positively controls OdDHL influx and probably thereby regulates expression of quorum sensing controlled genes (84).

Other factors such as the AraC-type global regulator system **VqsM/VqsR**, the alginate regulator protein **AlgR2** (AlgQ), the negative virulence regulator **MvaT** and the posttranscriptional virulence factor regulator **DksA** have been found to interact with the quorum sensing cascade of *P. aeruginosa* in a complex interplay (78, 85, 158, 159, 186).

Thus, as outlined above, quorum sensing involves and is involved in a wide array of complex regulatory systems, probably being important not only for the pathogenesis of this versatile bacterium, but also for the adaptation of this organism to a wide array of environmental conditions.

QUORUM SENSING AND THE EXPRESSION OF VIRULENCE FACTORS

Many quorum sensing regulated genes of *P. aeruginosa* are involved in the infection process, such as genes encoding proteases, elastase, exotoxins and factors favouring establishment of the pathogen such as biofilm components. See table 1.

Table 1. Quorum sensing regulated virulence factors in *P. aeruginosa.* (The list is not exhaustive).

Product	Gene	Reference
Alkaline protease	*aprA*	(106, 181)
Catalase	*katA*	(125, 372)
Chitinase	*chiC*	(100, 420)
Cyanide	*hcnAB*	(181, 297, 420)
Cytotoxic lectin	*lecAB*	(421)
Elastase B	*lasB*	(105, 285)
Exotoxin A	*toxA*	(106, 369)
LasA protease	*lasA*	(385)
PvdS-regulated endoprotease, lysyl class	*prpL*	(17, 266)
Pyocyanin	*phz*	(35)
Pyoverdin	*pvd*	(368)
Rhamnolipid	*rhlABC*	(271, 289, 314)
Secretion mechanisms	*xcpPR*	(51)
Superoxide dismutase	*sodAB*	(125)

The *P. aeruginosa* elastase LasB (also called pseudolysin) degrades matrix proteins, which besides elastin (a major component of connective tissues being resistant to hydrolysis by most proteases) also includes laminin (a membrane associated glycoprotein), fibrin (a blood plasma protein) and collagen types III and IV (26, 128, 340). Consequently, LasB shows significant tissue degrading activity and are capable of destroying the structural lung proteins thereby causing extensive damage. Elastase has been suggested to be a potent inflammatory factor in mouse models (432). Besides the destruction of lung structural proteins, elastase has the capacity to inactivate crucial immune system components such as immunoglobulin, serum complement factors and the α_1-proteinase inhibitor (87, 127, 143, 234, 344). Together with

alkaline protease, elastase inactivates the human cytokines interferon γ (INF-γ) and tumor necrosis factor α (TNF-α) (280).

Another elastase; LasA has been demonstrated to enhance the elastolytic activity of other elastases, including human leukocyte elastase and LasB, in a dose-dependent manner (299, 300). It is also a staphylolytic protease, which lyses *S. aureus* cells by cleaving the peptidoglycan pentaglycine interpeptides, and LasA is therefore also known as staphylolysin (166). LasA enhances the shedding of syndecan-1 from the surface of epithelial cells (277). Shedding of cell surface molecules as soluble extracellular domains is one of the host responses activated during tissue injury, and it seems that *P. aeruginosa* exploits this mechanism to promote its establishment within the host (276). Together with elastase B, LasA was one of the first factors of *P. aeruginosa* found to be controlled by quorum sensing, hence the name of the LasR protein (105, 181, 385).

The endoprotease PrpL (protease IV, Piv) has also been found to have elastolytic activity (415). Besides cleaving elastin, PrpL able is to degrade casein and proteins of the transferrin family, including lactoferrin and decorin. PrpL has been shown to contribute to *P. aeruginosa´*s ability to persist in a rat chronic pulmonary infection model (415). PrpL production is controlled by quorum sensing like most other secreted virulence factors of *P. aeruginosa* (17, 266).

Alkaline protease (AprA) is a metalloprotease and a member of the serralysin family. AprA has maximal enzymatic activity at alkaline pH, hence the name (272). The *apr* locus encodes beside AprA also an AprA specific small protease inhibitor (AprI) (89). The inhibitory protein consists of 131 residues, including a signal peptide of 25 residues which is cleaved off during secretion into the periplasm. The inhibitor is entirely located in the periplasm of *P. aeruginosa*, where its presumed physiological function is to protect the periplasmic proteins against the secreted protease (89, 129). AprA has together with LasB been found to inhibit activities of Natural Killer cells and reduce lymphocyte proliferation, probably via cleavage of IL-2 (291, 380). The two proinflammatory cytokines, IL-6 and IL-8 have also been shown to be degraded by the two proteases AprA and LasB (219).

Chitin is a homopolymer of β-(1-4)-*N*-acetyl-D-glucosamine which is highly abundant throughout the natural world, and is found for instance in the exoskeletons of insects, in the shells of crustaceans, in nematodes and in the cell walls of many fungi and algae. Degradation of chitin converts the polymer into acetate, NH_3 and fructose-6-phosphate. *P. aeruginosa* produces an extracellular (endo)chitinolytic enzyme encoded by *chiC*. The production of chitinase is regulated – at least in part – by quorum sensing (100, 266, 420). Interestingly water-soluble chitin, as well as chitosan (deacetylated chitin), has been found to accelerate wound healing (55, 231).

An important part of the defence against bacteria is the phagocytes which release hydrogen peroxide and other Reactive Oxygen Species (ROS) in order to defeat invading microorganisms. However, many bacterial pathogens, including *P. aeruginosa*, exploit superoxide dismutase and catalytic enzyme systems to survive the oxidative stress within the host. *P. aeruginosa* produces at least three catalases; KatA, KatB, KatE and KatN and two superoxide dismutases SodB and SodM (the latter also known as SodA) (371). KatA and, to a lesser extent, KatB, but not KatE, have been shown to be required for resistance to peroxide and osmotic stresses in *P. aeruginosa* PA14 (189). Both superoxide dismutase and catalase have been found to be required for full virulence of *P. aeruginosa* in different *in vivo* models (150, 189). Production of superoxide dismutase (SodM and SodB) and catalase (KatA) – and

thus the ability to withstand ROS produced by cells of the immune defence – has been found to be controlled by quorum sensing (27, 125).

A characteristic of *P. aeruginosa* compared to other fluorescent pseudomonads is the production of substantial quantities of the phenazine-derivative pyocyanin. (See proposed synthesis of pyocyanin and phenazines in figure 13). The production of pyocyanin is controlled by quorum sensing, iron and phosphate availability (155). Pyocyanin displays redox activity and induces oxidative stress in cellular systems and thus has the potential to impair tissue repair (35, 242). Pyocyanin has been isolated from sputum of infected patients at levels above 100 μM and is reported to modify several host cell responses including altering the platelet eicosanoid metabolism and inhibit prostacyclin release (160, 174, 243-245, 418). When pyocyanin is reduced by the reaction with NAD(P)H, the reduced pigment immediately reacts with molecular oxygen and thereby produces superoxide and by dismutation, hydrogen peroxide (68). The ability of *P. aeruginosa* to generate phenazines has been found to be critical for pathogenicity in both a worm model (*C. elegans*) and a murine model of septicaemia (211).

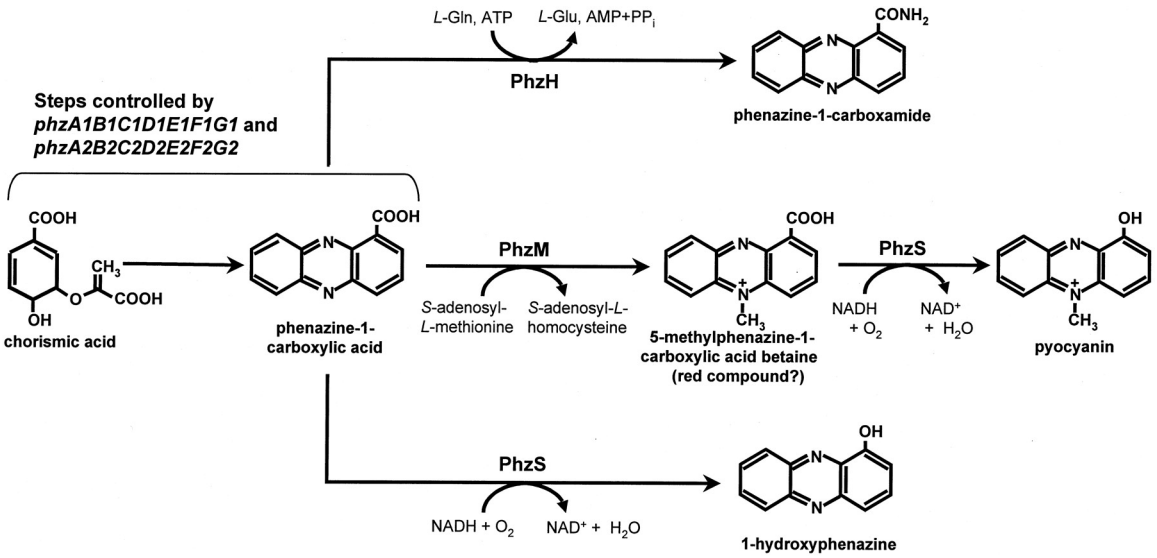


Figure 13. Proposed mechanism for the synthesis of pyocyanin, 1-hydroxyphenazine, and phenazine-1-carboxamide in *P. aeruginosa* PAO1. Two steps are suggested to be involved in the synthesis of pyocyanin from phenazine-1-carboxylic acid. In the first step, catalyzed by the SAM-dependent methyltransferase PhzM, phenazine-1-carboxylic acid is converted to 5-methylphenazine-1-carboxylic acid betaine. The second step, catalyzed by the NAD(P)H-dependent flavoprotein monooxygenase PhzS, involves hydroxylative decarboxylation of 5-methylphenazine-1-carboxylic acid betaine to pyocyanin. (From (220)).

Exotoxin A is a cytotoxic enzyme, which is able to ADP-ribosylate the eukaryotic elongation factor II, thereby inhibiting protein synthesis within the target host cell leading to cell death (149). Expression of exotoxin A (encoded by *toxA*) is complex and involves a cascade of regulators that results in the maximal production of exotoxin A in the stationary growth phase. Besides being dependent on low iron concentration, the *toxA* expression is presumed to be regulated by factors such as PvdS (pyoverdine sigma factor) and connected to cell density and growth phase by the *las* encoded quorum sensing system and *vfr* (106, 203, 270, 410).

In 1961 Liu *et al*. noticed that the "slime fraction" from burn wounds were more toxic than the cellular fraction (200). The toxicity of the "slime" was probably due to a high amount of hydrogen cyanide produced by *P. aeruginosa* infecting the wound. Goldfarb & Margraf, 1967 found that *P. aeruginosa* isolates from burn wounds produced high levels of hydrogen cyanide (1.5-3.2 μg/ml or 56 to 118 μM). They also found cyanide directly in burn wounds infected by

P. aeruginosa and suggested that this could be implicated in the "almost universal lethality of *P. aeruginosa* septicaemia in burn patients" (116). This has later been supported by the finding that cyanide is the mediating factor in the paralytic killing model of *C. elegans* by *P. aeruginosa* (104). The toxicity of the cyanide ion is caused by its ability to bind to cytochrome c oxidase, thereby denaturing the enzyme and disrupting the electron transport chain. In *P. aeruginosa* hydrogen cyanide biosynthesis is catalyzed by the membrane-bound enzyme HCN synthase, which forms hydrogen cyanide and CO_2 from glycine (43). Besides being controlled by quorum sensing the synthesis of hydrogen cyanide seems to be partly regulated by oxygen availability via the anaerobic regulator ANR (297).

P. aeruginosa produces different lectins which contribute to virulence. The galactophilic lectin PA-IL was the first to be characterized. It is a broad-spectrum hemagglutinin and agglutinates all types of erythrocytes from humans and various animals, as well as thrombocytes and leukocytes. Lectins are presumed to participate in the initial infection process where they facilitate the binding to the respiratory, gastrointestinal or other epithelial surfaces (110, 310). It has been demonstrated that RhlR-BHL - probably orchestrated by RpoS - controls expression of lectins (421).

As outlined above, *P. aeruginosa* produces many virulence factors, the majority being exoproteins requiring transport mechanisms to be able to cross both membranes of the cell envelope. Several pathways facilitating the transport of exoproteins and cellular appendages proteins (e.g. type IV pili and flagella) have been identified, including the type III (or contact) secretion system and the autotransporter pathway (13, 96, 131). Many exoproteins are exported by the type II secretion machinery; the Xcp system, which encompasses 12 different proteins encoded by *xcpA* and *xcpP-Z* (96). Considering that the transport of the majority of the factors facilitated by the Xcp system are quorum sensing regulated it is not surprising that the Xcp system itself has been found to be quorum sensing regulated (51).
The type III secretion system (TTSS) is involved in the secretion and translocation of several cytotoxic products into the cytosol of eukaryotic cells (reviewed by (431)). Expression of TTSS increases pathogenicity of *P. aeruginosa* and is associated with more severe infections and a higher mortality rate (5, 335, 341). Quorum sensing has been shown to negatively modulate expression of the TTSS in *P. aeruginosa* (28), that is; the TTSS system is down-regulated under high cell density conditions and thus this system probably primarily plays a role in the early stages of an infection.

P. aeruginosa synthesises extracellular glycolipids composed of L-rhamnose and 3-hydroxy-alkanoic acid. The rhamnosyl transferases taking part in the rhamnolipid pathway are encoded by *rhlAB* and *rhlC* (214). Besides being a heat stable hemolysin, rhamnolipid rapidly lyse neutrophils, macrophages and different animal cells (154, 221, 350). Rhamnolipids are found in high concentrations in sputa of *P. aeruginosa* infected CF-patients where they impair ciliary beating and thus mucociliary transport (175, 323). The concentration of di-rhamnolipid in *P. aeruginosa* over night cultures is in the range of 50-100 µg/ml being sufficient to lyse erythrocytes (see article 3). Swarming motility has been demonstrated to be dependent on the *rhlA* gene which is required for the production of the 3-hydroxy-alkanoic acid moiety of rhamnolipid and *rhlA* has also been found to be important for the development of normal biofilms (74, 275). RhlR-BHL promotes the transcription of *rhlAB*, and since the *lasI-lasR* quorum sensing system influences the *rhlI-rhlR* system the production of rhamnolipid is at least in part dependent on the *lasI-lasR* system and perhaps also PQS (224, 227).

As outlined above, many virulence determinants playing crucial parts in the infection process are regulated by quorum sensing. Also many *in vivo* studies emphasise that quorum sensing is indeed involved in pathogenesis, as quorum sensing deficient *P. aeruginosa* strains are much less virulent in animal model systems (56, 151, 194, 287, 424, 425). Interestingly, mutations in *lasR* are often found in isolates from chronically infected CF patients. While the *lasR* mutant and its corresponding wild-type show similar growth rates initially, the *lasR* mutant seems to have a higher fitness than the wild-type in late (alkaline) cultures (135). This is supported by the observation that loss of *lasR* functionality may confer the bacterium with a growth advantage as a mutation in *lasR* increases expression of the catabolic pathway regulator CbrB (63). Moreover, mutations are found more frequently in *lasR* than e.g. in *gacA* and *rhlR* (204) again suggesting a selective advantage for *lasR* mutations during an adaptation to an environment (either a culture flask or the CF airways), indicating that quorum sensing may only be important in the initial stages of an infection.

QUORUM SENSING AND BIOFILMS

Since quorum sensing regulated genes are mainly activated at high cell densities it would be reasonable that quorum sensing is *not* involved in the early phase of biofilm development, where the cells attach to the substratum forming the first microcolonies, and the cell density has not yet reached a "quorate" concentration. However, this is an area of controversies. Glessner *et al.,* 1999 applied the mutants PDO100 (Δ*rhlI*), PAO-JP1 (Δ*lasI*) and PAO-JP2 (Δ*rhlI*-Δ*lasI*) to demonstrate that the *las* and *rhl* quorum sensing systems were required for type IV pilus-dependent twitching motility (115). Moreover, the ability of *P. aeruginosa* cells to adhere to human bronchial epithelial cells was also found to be dependent on the *rhl* quorum-sensing system (115). A later survey of *las* and *rhl* knock-out mutants showed that the mutants ability to move by twitching were unaltered compared to the wild-type, however during culturing, secondary mutations occurred in the *lasI* and *rhlI* mutants which caused accumulation of twitching-defective variants (25). Thus, the lack of twitching motility in quorum sensing mutants found by Glessner *et al.* 1999, might be explained by secondary mutations occurred as a consequence of a compensatory mechanism, instead of being caused by the strains' quorum sensing deficiency.
The impact of flagellar-mediated motility and type IV pili on biofilm development are disputed. Heydorn *et al*., 2002 studied continuously-fed biofilms and concluded that the development of microcolonies is independent of twitching motility (137). Contrary to this, O'Toole and Kolter, 1998 found that flagellar-mediated motility and type IV pili are necessary for biofilms formation (268), however the latter was based on investigations using a set-up without flow and a much shorter time frame for the experiment than Heydorn *et al*., 2002.
Reimmann *et al.,* 2002 constructed a *P. aeruginosa* PAO1 strain harbouring a gene encoding an AiiA homologue (an AHL lactonase). The expression of the AHL lactonase reduced the amount of OdDHL and completely prevented the accumulation of BHL. The strongly reduced AHL content correlated with decreased expression and production of several virulence factors such as elastase, rhamnolipids, hydrogen cyanide and pyocyanin. Swarming was also reduced, but there was no observed effect on flagellar swimming or twitching motility, and the strains ability to adhere to a polyvinylchloride surface was not affected (326).

The initial step of biofilm formation is a very complex, fairly controversial and not fully understood process, and whether or not quorum sensing takes part of this stage is a question which still needs further investigations to be answered. However in later stages of the biofilm development, high cell densities occur, and several studies have demonstrated the importance of quorum sensing coordinated activities for the maturation of a normal biofilm. Davies *et al.,* 1998 demonstrated that a specific *lasI* -mutant (the PAO-JP1 also used by Glessner *et al*.,

2002) formed flat, thin and undifferentiated biofilms that, unlike wild-type biofilms, were sensitive to the biocide sodium dodecyl sulphate (SDS). The mutant biofilms appeared as the wild-type when grown in the presence of exogenous OdDHL (66). Based on the mutants utilized by Glessner *et al.,* 1999 and Davies *et al.,* 1998 (PDO100, PAO-JP1 and PAO-JP2), Shih & Huang, 2002 confirmed the importance of quorum sensing - and especially the *las*-system - for the development of normal biofilms. Moreover, quorum sensing was also shown to be important for the high tolerance of PAO1 biofilms when treated with kanamycin, since the quorum sensing mutants were much more susceptible to this antimicrobial agent (346). However, whether secondary mutations in the applied strains have any impact on the conclusions drawn in these papers, are unknown.

Heydorn *et al.,* 2002 found that the biofilm made by the *lasI*-mutant PAO-JP1 was indistinguishable from the dense wild-type biofilm at all time points, when both strains were grown under the same conditions and with citrate as carbon source (137). This suggests that quorum sensing may not be necessary for *P. aeruginosa* biofilm architecture under all conditions. This is supported by recent findings showing that quorum sensing mutants depending on the carbon sources either forms biofilms identical to the wild-type strain or biofilms with defects in the early steps of biofilm formation (349). This study further demonstrated that swarming plays a role in biofilm formation in agreement with findings by Pamp and Tolker-Nielsen, 2007 who observed that early microcolony formation and late migration-dependent structural development are dependent on biosurfactants and the ability of the bacteria to migrate (275).

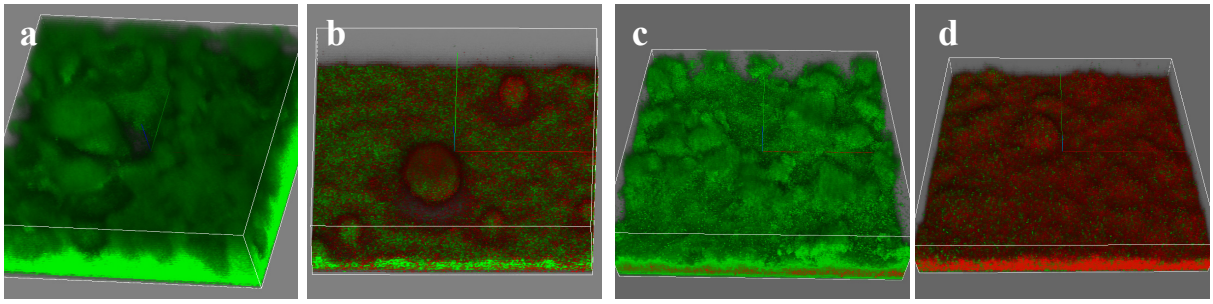


Figure 14. The quorum sensing mutant develops more flat and more tobramycin-sensitive biofilms than the wild-type. Three days old Gfp-tagged *P. aeruginosa* biofilms were treated with 10 μg/m tobramycin and stained with propidium iodide to differentiate live/dead/ cells. **a** PAO1 wild-type biofilm before and **b** after treatment with 10 μg/ml tobramycin. **c** Δ*lasR-rhlR* mutant before and **d** after treatment with 10 μg/ml. (From (27)).

It has been shown that a biofilm formed by a *lasR-rhlR*-mutant of *P. aeruginosa* is much more susceptible to killing by tobramycin and hydrogen peroxide than its wild-type parent (27). (See figure 14). Moreover, while neutrophils readily phagocytosed the quorum sensing mutant biofilm, the wild-type biofilm escaped the grazing by neutrophils. (See figure 15, next page). Recently it has further been demonstrated that rhamnolipid released by a wild-type *P. aeruginosa* biofilm caused the neutrophils to rapidly undergo necrosis, and thereby hindered their normal antimicrobial actions (154). This suggests that rhamnolipid is a key virulence factor of *P. aeruginosa.*

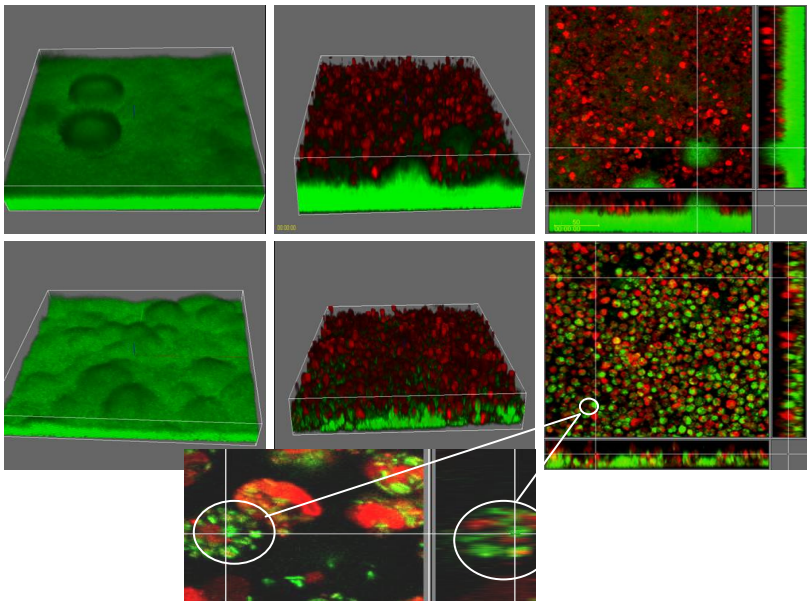


Figure 15. Quorum sensing dependent tolerance of *P. aeruginosa* biofilms towards neutrophils. Three days old Gfp-tagged *P. aeruginosa* biofilms (wild-type (upper panel) and a *lasR-rhlR* mutant (lower panel)) were exposed to neutrophils for 2.5 hours (second and third picture in both panels). Neutrophils (red) were stained with SYTO 62. While the wild-type biofilm escaped neutrophil grazing, the *lasR-rhlR* mutant biofilm were fully penetrated by neutrophils. As seen from the enlargement, neutrophils phagocytised the quorum sensing deficient bacteria. (27).

The *pel* operon involved in the production of exopolysaccharides is important for formation of the biofilm matrix. The transcription of this operon has been found to be controlled by quorum sensing in *P. aeruginosa* PA14 (336). Intriguing, it was also found that *pelA* transcription and the formation of biofilm decreased dramatically when the temperature was changed from 25 °C to 37 °C (336). Thus, the *pelA-G* genes are probably not involved in biofilm formation in pneumonia and other host associated infections. (See colony morphologies of *P. aeruginosa* PA14 wild-type and the Δ*pelA*, Δ*rhlI* and Δ*lasI* mutants in figure 16).

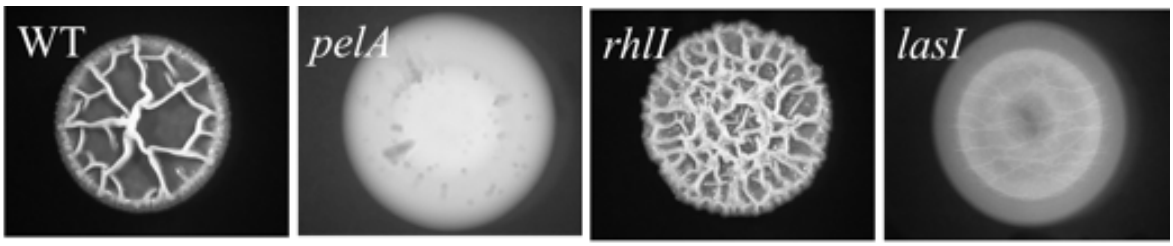


Figure 16. Colony morphologies of *P. aeruginosa* PA14. WT (wild-type) and the Δ*pelA*, Δ*rhlI* and Δ*lasI* mutants. The Δ*pelA*, and Δ*lasI* mutants do not exhibit the wrinkled morphology of the wild-type. (336).

Whitchurch *et al*., 2002 showed that treatment with DNase I inhibited development of *P. aeruginosa* biofilms and dissolved already established biofilms, thereby suggesting that extracellular DNA is an important factor of *P. aeruginosa* biofilms, perhaps as a structural component (411). A more recent study showed that the extracellular DNA is generated via a mechanism dependent on quorum sensing, flagella and type IV pili (11). Moreover, biofilms formed by quorum sensing mutants, a *fliMpilA* mutant and a DNase I treated wild-type biofilm were more susceptible to SDS than the untreated wild-type biofilm (11). This suggests that the quorum sensing controlled release of extracellular DNA may be involved in the enhanced tolerance of wild-type *P. aeruginosa* biofilms against antimicrobial treatments.

As several studies suggest that quorum sensing may be involved in the formation of structured and antimicrobial-tolerant biofilms, the possibility of inhibiting quorum sensing of biofilm forming bacteria and thereby promoting their clearance has been explored. Hentzer *et al*., 2003 showed that the quorum sensing inhibitory compound 30 (C30) increased biofilm susceptibility to tobramycin and SDS (134). C30 was also used to treat mice in a pulmonary infection model and greatly promoted clearance of the infecting bacteria (134, 426). In article 1 it is described how the treatment of mice with the quorum sensing inhibitor patulin caused a 20-fold lower bacterial count in the lungs of the patulin treated mice compared to the placebo treated group, showing that this quorum sensing inhibitor significantly promotes bacterial clearing (322). Also other studies have demonstrated the concept and prospects of bacterial quorum sensing as a new drug target (56, 320).

QUORUM SENSING INHIBITORS

As outlined in the previous parts, an approach to decrease bacterial virulence and enhance biofilm susceptibility could be to target quorum sensing and thus attenuate the microorganism's ability to establish a successful infection. Different strategies are conceivable in order to inhibit quorum sensing:

QSI STRATEGY I – TARGETING THE AHL-SYNTHASE

One strategy aiming to inhibit quorum sensing could be to impede the signal molecule generation mediated by the LuxI-homologue. Key elements in the synthesis of the different AHLs are acyl-ACPs (acyl-acyl carrier proteins) as the primary source of acyl chain and SAM (S-adenosyl methionine) as the amino donor for the homoserine lactone ring moiety. (See the model representing OdDHL formation mediated by LasI in figure 17).

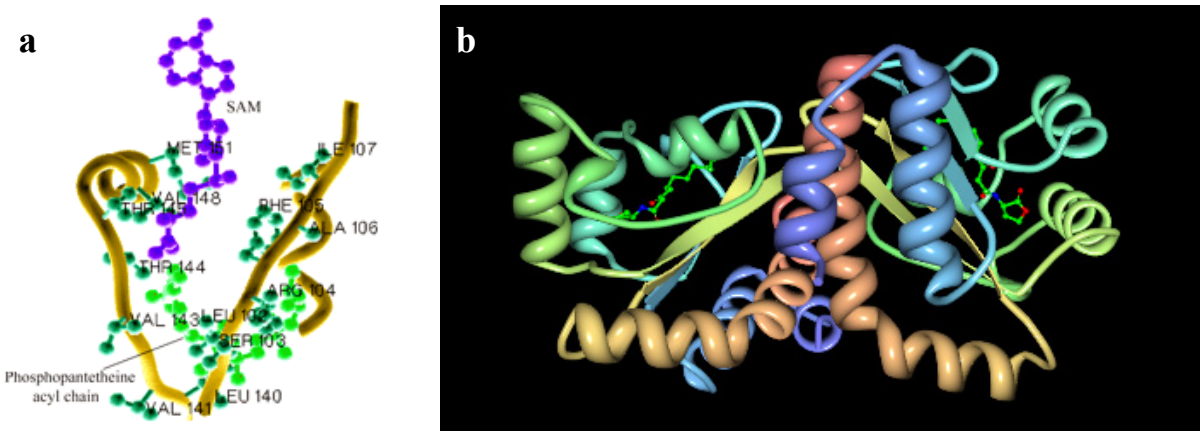


Figure 17. Representation of the model structure of the OdDHL synthetase LasI. **a.** Close-up of LasI. Interacting residues are shown in green, SAM in purple and acyl-phosphopantetheine in orange (48). **b.** Structure of LasI based on the crystal structure solved by (119). (From www.rcsb.org/pdb Protein Databank of the Research Collaboratory for Structural Bioinformatics Accession number 1RO5. PDB Simple viewer).

Parsek *et al*., 1999 demonstrated that it was possible to block the RhlI mediated synthesis of BHL by the use of SAM analogues such as S-adenocylcystein and buteryl-SAM (283). In a biological system however, the concept of using SAM analogues may have an unwanted pleiotropic effect, as SAM is an important cofactor involved in various reactions, not limited to AHL synthesis.

QSI STRATEGY II – THE SIGNAL MOLECULE

In a very interesting study Chun *et al.,* 2004 demonstrated that human airway epithelia have the ability to inactivate OdDHL. The inactivation is selective for long chain AHLs, and does not influence BHL concentration. The process responsible for the long chain AHL inactivation was not elucidated, but enzymatic degradation was suggested to be involved (58). Yang *et al.,* 2005 found quorum quenching activity in mouse, rabbit, horse, goat, human and bovine sera, but not in sera from chicken and fish. By the use of HPLC/MS analyses lactonase-like enzymes with properties reminiscent of paraoxonases were revealed in the six active mammalian sera samples (433). Paraoxonases 1-3 (PON1-3) are esterases and have the capacity to retard the accumulation of lipid peroxides in low-density lipoprotein (LDL), thus retarding the oxidation of these lipoproteins and attenuating their pro-inflammatory effects (257, 324, 406). Interestingly, it has been demonstrated that PON1-3 also have the ability to degrade OdDHL and thereby inhibit quorum sensing (274).

Several soil living bacterial species which produce enzymes capable of degrading AHLs have been isolated, among these *Arthrobacter, Bacillus, Comamonas, Ralstonia, Rhodococcus* and *Variovorax* species (82, 185, 198, 278, 279, 389). AHLases hydrolysing the ester bond of the homoserine lactone ring as well as amino-acylases hydrolysing the amide bond has been identified. Apparently, some strains are able to degrade and utilize AHLs as the sole energy, carbon and nitrogen source (185, 279).

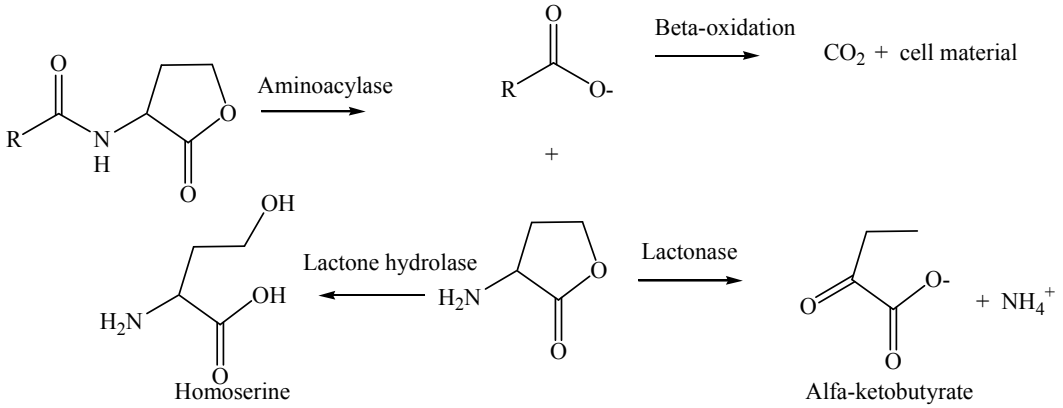


Figure 18. Proposed enzymatic degradation pathways of AHLs. The complete break down and utilization of the signal molecules requires several different classes of enzymes. It is possible that only some of the enzymes (e.g. acylases) are present in the strains identified as having AHL degrading activity (After (185)).

Interestingly, *P. aeruginosa* has been shown to produce at least two acylases PvdQ (PA2385) and QuiP (PA1032) with OdDHL-degrading activity (147, 148, 353). This suggests that PvdQ and QuiP may be involved in quorum sensing regulation.

The soft-rot phytopathogen *Erwinia carotovora* relies on OHHL to control and coordinate activation of genes encoding extracellular plant cell wall degrading enzymes in a cell density-dependent manner (39). Using an experimental system with transgenic tobacco plants producing OHHL[1] it was shown that the OHHL releasing plants had enhanced resistance to infection by *E. carotovora.* Exogenous addition of OHHL to wild-type plants similarly

[1] In the article by Mae *et al*., 2001 is the *E. carotovora* oxo-AHL abbreviated as OHL. To avoid confusion is the already introduced abbreviation OHHL for *N*-3-oxo-hexanoyl-L-homoserine lactone applied here, and OHL is reserved for *N*-octanoyl-L-homoserine lactone.

increased the resistance against the pathogen (209). It is possible that the enhanced resistance of the tobacco plants supplied with OHHL (either transgenic or exogenous supplementation) is due to a premature production of virulence factors by *E. carotovora* before the bacteria population are quorate and thus earlier induction of the plant defence systems.

In another model, plants were infected with *E. carotovora* transformed to express an AHL-lactonase from *Bacillus* sp. The transformed *E. carotovora* strain released less AHLs, had decreased production of pectolytic enzymes and attenuated pathogenicity compared to the wild-type parent (83). Whether AHLases or acylases is applicable for the treatment of human infections is still an open question. In particular it is important to ensure that acylases are not interfering with other substrates in the body than the AHL produced by infecting bacteria.

A new method to inhibit quorum sensing via binding of the signal molecule to AHL-specific monoclonal antibodies has recently been suggested. An antibody named RS2-1G9 is proposed to be capable of inhibiting quorum sensing of *P. aeruginosa* by encapsulating the polar lactone moiety of AHLs. This suggests future antibody-based therapy that target quorum sensing (73).

QSI STRATEGY III – TARGETING THE REGULATOR PROTEIN

Another way of attenuating quorum sensing signal transduction is to impede the interaction between the LuxR-homologue and the signal molecule.

Much effort has been done to develop competitive inhibitors by synthesising AHL analogues designed to fit to the LuxR-homologue receptor site without activating the protein. Studies of the structure-activity relationship of AHL analogues have revealed that the ability to bind and activate LuxR-homologues depends on the length and flexibility of the acyl chain and on the presence of the 3-oxo moiety (286). Reverchon *et al.,* 2002 modified the extremity of the acyl chain in OHHL by introducing ramified alkyl, cycloalkyl or aryl substituents at the C-4 position. Most of the analogues with either acyclic or cyclic alkyl substituents had agonist activity in the applied *lux* quorum sensing system of *V. fischeri.* However, the phenyl substituted analogues displayed significant antagonist activity suggesting a mechanism where the aryl group interacts with an aromatic amino acid in the receptor site of LuxR, thereby preventing it from forming the active dimer (327). Smith *et al.*, 2003 synthesised a focused library of AHL analogues, based on changes in the homoserine lactone moiety. By replacing the lactone with other ring structures such as aniline derivatives or cyclohexanol, the agonist effect of the original signal molecule was changed into an antagonistic effect of the analogue (356, 357). By investigating a range of lactones with a sulphur containing acyl chain we have recently identified *N* (heptylsulfanylacetyl) L-homoserine lactone as a potent inhibitor of quorum sensing (293).

IDENTIFYING INHIBITORS OF THE REGULATOR PROTEIN

One approach to identify novel quorum sensing inhibitors is the use of *in silico* screening methods. Taha *et al.,* 2006 used modelling based on OdDHL to predict a collection of chemical functionalities in a 3D space likely to be involved in OdDHL-LasR interaction. Training on known quorum sensing inhibitors led to the prediction of novel quorum sensing inhibitors. Six out of 19 tested compounds selected among the best hits of the model were found to inhibit quorum sensing. Four of the active compounds contained either mercury or tetravalent lead, thereby representing a novel class of quorum sensing inhibitors (376).

The crystal structure of Ligand Binding Domain (LBD) of LasR has recently been solved (30) and this makes it possible to perform huge *in silico* screenings of libraries with the aim of identifying new potential quorum sensing inhibitors.

Muh *et al.,* 2006 used an Ultra-High-Throughput screen based on an *rsaL*-reporter fusion to screen a library of approximately 200,000 compounds for inhibitors of LasR dependent gene expression. Although the chemical compounds in the library covered a large structural variety, the two most potent quorum sensing inhibitors both resembles OdDHL (241). In connection to this library screening a triphenyl compound (TP-1) was identified as an agonist of quorum sensing. Though structurally very different from OdDHL it was able to activate multiple LasR dependent promotors in a *lasI-rhlI* mutant of *P. aeruginosa* (240). Contrary to OdDHL, TP-1 is insensitive to high pH and the action of paraoxonases and thus suitable as a scaffold for the development of inhibitors of quorum sensing (240).

I have recently contributed to a study applying alternative high-throughput screens for identification of potential quorum sensing antagonists. 1200 AHL analogues were synthesised and cross linked to 3D hydrogel microarray slides in a spatially-separated and -addressable way. Soluble LuxR-type receptors from the plant pathogen *E. carotovora* subsp. *carotovora* (Ecc), $CarR_{1-167}$ and $EccR_{1-182}$, were purified, labelled with fluorescent tags (Cy3 or Cy5) and used in microarray–based protein–binding screens. Fluorescent spots indicate protein-ligand interaction and the molecules identified by this screen are potential antagonists of quorum sensing (260). While this concept is very elegant, it has two major drawbacks: 1.) It selects equally well for inducers as well as inhibitors of quorum sensing. 2.) Since the molecules are identified by the use of a regulator protein, information about the ability of the molecules to cross the bacterial cell membrane is not achieved. Overall a system like this based solely on protein-ligand interaction may be useful as a first high-throughput screen but needs to be accompanied by at least one screen implying quorum sensing at a cell level, e.g. as outlined in the following section. My contribution to this study was to investigate potential inhibitors by the use of quorum sensing monitor strains. Two different quorum sensing antagonists were identified via this combined approach. (Manuscript submitted to Angewandte Chemie).

Besides the generalised strategies for targeting quorum sensing described above, other concepts are possible. As outlined earlier, the quorum sensing system of *P. aeruginosa* consists of a network of regulators, and each of these regulators may hold the key for novel concepts of inhibiting quorum sensing in this organism. One way to inhibit quorum sensing in *P. aeruginosa* is discussed in article 3, where it is described how antibiotics seems to decrease expression of the two-component regulator PprAB, which regulate membrane permeability, sensitivity to antibiotics and OdDHL efflux. Interaction with PprAB may be a novel way to counteract *P. aeruginosa* virulence via the quorum sensing system.

SCREENING FOR QUORUM SENSING INHIBITORS

Three of the articles included in this thesis (article 1-3) entail screening for quorum sensing inhibitors. In the following sections, different concepts for screening for quorum sensing inhibitors applied in these articles will be briefly discussed.

The simplest way to screen for quorum sensing inhibitors is to use an easily measurable/detectable quorum sensing regulated trait, e.g. violacin production by *Chromobacterium violaceum*, prodigiosin biosynthesis by *Serratia marcescens* and pigment (pyocyanin) production by *P. aeruginosa* and then investigate changes in the phenotype as a response on potential quorum sensing inhibitors. Another approach, based on the same concept, is to fuse a reporter gene expressing an easily detectable product (e.g. *gfp* or *lacZ*) to the promoter -including the *lux* box - of a gene regulated by quorum sensing.
The stability of the original Gfp makes it unsuitable for monitoring fluctuations in gene expression. Changes introduced to the C-terminal end of Gfp, such as in Gfp(ASV), makes the

protein more unstable as it is rendered susceptible to the bacterium's intracellular tail specific housekeeping proteases (14). The half-life of Gfp(ASV) is about 110 min. allowing online monitoring of changes in gene expression to be seen within a time span of a few hours (14). The construct consisting of a quorum sensing regulated gene fused to a reporter gene can be maintained in an AHL producing strain such as the P_{lasB}::*gfp(ASV)* chromosomal insert in PAO1 used in article 1 and 2. The addition of a quorum sensing inhibitor to this type of construction will result in a lowered expression of reporter gene to an extent proportional to the efficiency of the inhibitor. Besides monitoring expression of the phenotypic trait or expression of the reporter, it is important to observe growth in order to avoid false-positives/false-negatives due to toxic/nutritional effects.

An alternative screening approach is the Quorum Sensing Inhibitor Selector (QSIS) (320). This system is basically constructed from the same key elements as described above. One important difference is that the reporter gene has been replaced with a gene encoding a lethal product. The quorum sensing regulator protein (the LuxR homologue) will, in the presence of AHL signals, induce expression of the toxic gene product, thereby impeding cell proliferation. Hence, growth will only commence if quorum sensing is blocked by the presence of exogenous, non-toxic inhibitors.

The QSIS1 system is employed in article 1-3 to screen for quorum sensing inhibitors in natural libraries (article 1-2) and among antibiotics (article 3). The QSIS1 selector system is based on the *lux* quorum sensing system from *V. fischeri*. The construct is harboured by a plasmid containing *luxR* controlled by its own promoter and *phlA* under the control of the *luxI* promoter (See figure 19 A). *phlA* encodes phospholipase A which originates from *Serratia liquefaciens* MG1 (114). Since the quorum sensing circuit in *V. fischeri* is an autoinducer system, the expression of the *luxI* gene is under the control of the regulator protein LuxR. When the signal molecule interacts with LuxR it induces the transcription of the gene under the control of the *luxI* promoter and in QSIS1 this leads to the expression of phospholipase A thereby causing lysis of the cell. If a quorum sensing inhibitor is present along with the signal molecule the production of phospholipase will be abolished, allowing the bacteria to grow (320). This rescuing effect of a functional inhibitor is easily visualized in a diffusion assay by maintaining QSIS1 in an *E. coli* constitutively expressing β-galactosidase (see figure 19 C). Another selector system based on the *P. aeruginosa* quorum sensing system, named QSIS2, has been used in addition to the QSIS1 system in article 1 and 2 to screen for quorum sensing inhibitors. QSIS2 harbours a plasmid carrying the *lasB* promoter fused to *sacB* which encodes the secretory levansucrase of *Bacillus subtilis* (See figure 19 B). SacB catalyses a transfructosylation reaction by which sucrose is hydrolysed, and along with the release of glucose the reaction contributes to the production of the polymer levan (107). Levan is lethal to Gram-negative bacteria, but the exact killing mechanism is not clarified (183). As in QSIS1, presence of quorum sensing inhibitors will rescue the cells from expression of the suicidal gene product. QSIS2 also carries a plasmid containing a part of the *lux* cassette (*luxCDABE*), thus growth of the QSIS2 causes bioluminescence and indicates the presence of a quorum sensing inhibitor.

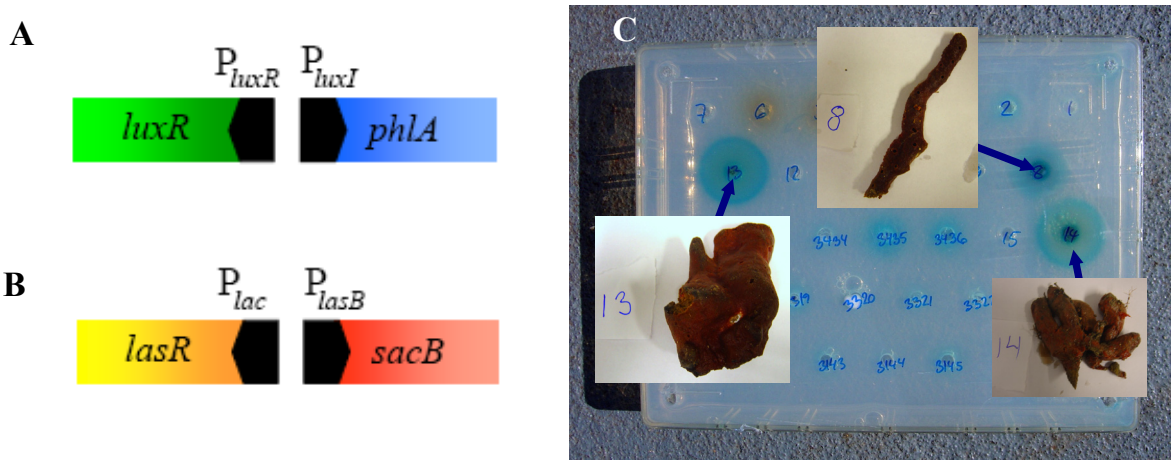


Figure 19. The Quorum Sensing Inhibitor Selector systems; QSIS1 and QSIS2. **A**. Schematic outline of the essential parts of the plasmid in QSIS1. The gene encoding *luxR* is controlled by its own promoter, while expression of the lytic factor phospholipase A is regulated by quorum sensing via the *luxI* promoter. **B.** Outline of the QSIS2 system. The transcriptional regulatory protein LasR is constitutively expressed as it is controlled by the *lac* promoter. Expression of the cell toxic product SacB is controlled by the quorum sensing regulated *lasB* promoter. **C.** Extracts of marine organisms tested using the QSIS1 system. Extracts are loaded in wells in the agar and the plates are incubated over night. Three marine sponges showed strong quorum sensing inhibitory activities as seen from the blue rescuing zones around the wells marked with arrows.

In the screening assay, the QSIS strains are cast into agar along with signal molecules. Test samples consisting of either extracts or pure compounds are added to wells made in the agar, creating a concentration gradient of the test sample when diffusing into the agar. Samples to be extracted could be microfungi as in article 1 or marine organisms (article 2) and pure compounds could be different antibiotics as in article 3 or compounds purified from extracts e.g. patulin, penicillic acid or manoalides. Depending on the nature (molecular weight, solubility etc.) of the components in the extract, the compounds diffuse with different rates and a crude separation may take place. Moreover, due to the concentration gradient, a range of concentrations are tested at the same time. After incubation, samples containing quorum sensing inhibitory compounds can be identified by a zone of growth (rescuing zones) around the application wells. Toxic compounds present in the test sample give a clearing zone near the well where growth is not possible. Compounds and extracts which possess quorum sensing inhibitory as well as toxic properties give a clearing zone near the well, where the highest concentration is present, followed by a zone of growth indicating quorum sensing inhibitory activity. When applying the screening system QSIS1, a blue rescuing zone emerges along with growth due to the presence of hydrolyzed X-Gal in the plate (See figure 19 C). Growth of QSIS2 can either be recorded as bioluminescence or by using tetrazolium as viability reporter. Most bacteria, among these *P. aeruginosa*, are capable of reducing the colour-less tetrazolium to the red-coloured triphenyl formazan (TPF) by using tetrazolium as an electron acceptor. Thus by adding a solution of tetrazolium to the QSIS2 test plate, wells containing quorum sensing inhibitory compounds can be identified due to a red zone around the well, where the active extract/compound was loaded.

One additional example of this concept is a selector system based on the quorum sensing system of *Burkholderia cepacia* outlined in figure 20. I constructed this selector to be able to identify inhibitors of quorum sensing functioning against the *B. cepacia cepI-cepR* quorum sensing system. However, results obtained using this system were almost identical to results achieved by the use of QSIS1 and the *cepI-cepR* based selector was not further used, but shown here as an example of the Quorum Sensing Inhibitor Selector System.

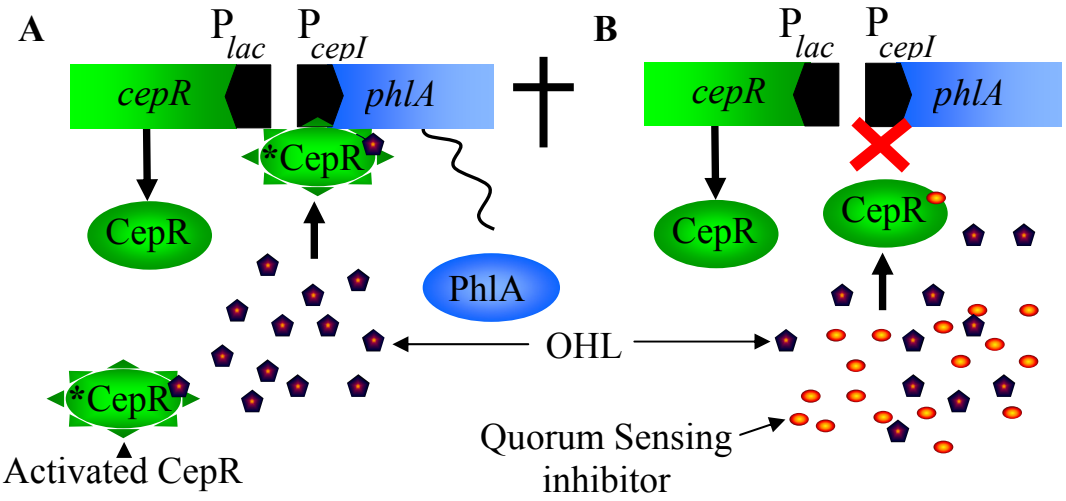


Figure 20. Quorum Sensing Inhibitor Selector based on the *B. cepacia* cepI-cepR quorum sensing system. **A.** OHL binds to and activates CepR thereby inducing transcription of *phlA*. The expression of phospholipase leads to lysis of the cell, which dies. **B**. If a quorum sensing inhibitor is present along with OHL it abolishes the production of phospholipase, and the cell is able to proliferate.

QUORUM SENSING INHIBITORS FROM NATURAL SOURCES

As an alternative to synthesising and screening large compound collections, novel quorum sensing inhibitors may be identified by screening natural libraries. Nature is indeed ingenious and compounds with complex structures, difficult to synthesise in the laboratory, can be produced by living organisms via enzymatic processes. Moreover, as many plants, fungi and invertebrate organisms use chemical activities in the competition with other organisms, many of the secondary compounds they produce have strong bioactive properties and thus potentially medical applications. This view is supported by Newmann and Cragg by this quotation: "natural products play a dominant role in the discovery of leads for the development of drugs for the treatment of human diseases" and by the fact that more than 60 % of all new pharmaceuticals introduced the last 25 years are either natural products or derivatives/mimics of natural products (254, 256). Also many of the clinically important antibiotics used today such as aminoglycosides, cephalosporins, chloramphenicol and colistin are of natural origin (4, 90, 370, 402, 408). It has been widely assumed that antibiotics in nature have been evolved by various microorganisms to fight competitors as an element in a Darwinistic "struggle for life". However, a recent study questions this and put forth the argument that the concentrations of free antibiotics present in soil compared with laboratory conditions are very low (199). Antibiotics applied in concentrations not affecting growth (subminimum inhibitory concentrations, subMIC) have been shown to increase cytotoxicity, enhance bacterial motility and biofilm formation (139, 199, 443). This suggests that the ecological role of antibiotics in naturally occurring low concentrations may be as signalling mediators, organising surface colonization and fighting predators by enhanced cytotoxicity, rather than targeting growth of rivals (199).

As described in article 3, three different antibiotics (azithromycin, ceftazidime and ciprofloxacin) decrease expression of many quorum sensing regulated genes of *P. aeruginosa* when applied in concentrations not affecting the growth rate of this bacterium. Moreover, the three antibiotics also impede the production of quorum sensing regulated virulence factors such as chitinase and protease. This supports the hypothesis that –at least some- antibiotics have dual activities; as bactericidal drugs when applied in (artificial) high concentrations and interacting with cell signalling when applied in low, non-toxic concentrations.

Antibiotics derived from various soil organisms are only representing one example of the success of naturally derived medicines; approximately one third of today's best selling drugs are either natural products or have been developed based on lead structures provided by nature (255). Although around 70 % of the world is covered by oceans, almost all medicinally used natural products or derivatives thereof have been obtained from terrestrial organisms, and only few marine natural products are currently used as pharmaceuticals or are in clinical trials. However, due to the uniquely diverse and novel chemistry found among marine natural products, the oceans have been proposed to be the treasury of future drug development (93, 307).
In article 2 extracts of 284 marine organisms are investigated for quorum sensing inhibitory properties. Interestingly, many of the extracts being active in the QSIS screening systems also caused a clearing in the vicinity of the well where the extract was added, indicating bactericidal activity. Extracts of the marine sponge *Luffariella variabilis* and its metabolites manoalide, secomanoalide and manoalide monoacetate showed strong quorum sensing inhibitory properties, but weak to no toxicity against the two Gram-negative selector bacteria used in QSIS1 and 2 systems. However, the sesterpenoid manoalide and the two manoalide derivatives produced by this sponge have been reported to possess antibiotic properties against the Gram positive bacteria *S. aureus* and *Streptomyces pyogenes* (71, 248). Thus it seems that manoalide, secomanoalide and manoalide monoacetate also belong to the group of bioactive compounds with multiple functionalities.

The archetypical example of quorum sensing inhibitors derived from marine sources are the very efficient inhibitors of quorum sensing derived from the benthic marine macro-alga *Delisea pulchra* which produces a suite of biologically active brominated furanones (113, 321). Synthetic derivates of these furanones (C56 and C30) have been shown to attenuate *P. aeruginosa* infections *in vivo* and inhibit quorum sensing *in vitro* (133, 134).
Considering the functionalities of the halogenated furanones and the fact that the bromo groups represent excellent leaving groups, these inhibitors may function via irreversible binding to the LuxR (homologue) receptor site. Even though the halogenated furanones are capable of displacing OHHL from the LuxR protein it has not been possible to isolate a stable complex of the regulator protein bound to the inhibitor (215, 217). Western immunoblot analysis has shown that halogenated furanones dramatically enhance turnover of the LuxR protein, hence, it is likely that the lowered level of LuxR protein is the mode of action of the algae compounds (217). Also the halo-furanones of *D. pulchra* possess numerous activities; in addition to their ability to inhibit quorum sensing they hold anti-fouling and antibacterial properties (70) as additional examples of multiple functioning compounds.

Other examples of eukaryotic organisms possessing quorum sensing inhibitory activities are extracts of grapes and strawberries and exudates from pea seedlings (*Pisum sativum*) (101, 379). Also garlic, a well known universal panacea in herbal medicine, possesses strong quorum sensing inhibitory activity (320). As described in article 1 fungi also produce quorum sensing inhibitors. From an ecological point of view it is not surprising that microfungi have developed the ability to produce compounds interfering with bacterial quorum sensing, as most of the antifungal products of *P. aeruginosa* (and of other pseudomonads as well) such as chitinase, phenazines and pyocyanin are under quorum sensing control (165, 305). By screening extracts of microfungi (*Penicillium* and *Aspergillus* sp.) two mycotoxins; patulin and penicillic acid were identified as strong quorum sensing inhibitors.

Even though we did not fully elucidate the mechanism by which patulin and penicillic acid exert their action on quorum sensing, results obtained by Western protein blotting suggest that

the two mycotoxins decrease the half life of the LuxR-protein in a way similar to the degradation mediated by the halogenated furanones (See article 1).

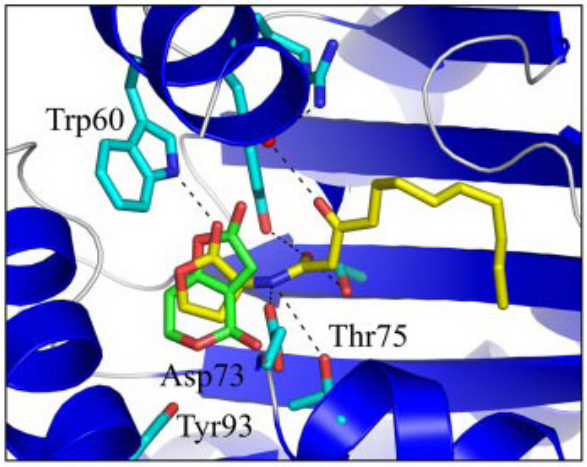


Figure 21. LBD of LasR and interactions with OdDHL (in sticks, yellow; carbon) and with superimposed patulin (in sticks: green; carbon) (30).

Penicillic acid and patulin are capable of forming covalent cross links with amino acids, e.g. cysteine, lysine, histidine and α-amino acids and thereby inactivate protein functionality (99). If such a cross linking reaction occurs within the receptor site of LasR or another LuxR homologue it may change the protein conformation making the protein a substrate for house keeping proteases. However, another possibility is that the stability of the LasR protein upon binding a ligand requires an acyl chain to form a hydrophobic core, and as the halogenated furanones, patulin and penicillic acid lack this moiety, they destabilise the protein (30). (See figure 21 showing OdDHL and superimposed patulin in the LBD of the LasR protein). The latter hypothesis fits with the observation that only brominated furanones lacking the acyl chain are capable of inhibiting quorum sensing in *P. aeruginosa.* (Personal communication, M. Hentzer and T.B. Rasmussen).

P. AERUGINOSA QUORUM SENSING IN CROSS-KINGDOM INTERACTIONS

Communication between cells by means of signalling is universal in nature. In mammals structurally diverse molecules such as peptides (e.g. insulin) and small molecules (e.g. epinephrine) bring messages from one cell to another, insects use pheromones such as (R,*E*)-9-hydroxy-2-enoic acid to control swarms and 2,6-dichlorophenol as sex attractants. In fungi important cell signalling factors include farnesol and rhodotorucine A, while some plants use phytohormones such as the cytokinin zeatin to regulate plant development. As described in the previous section, bacteria also use signalling systems to coordinate socio-microbial behaviour. Considering that diverse organisms such as fungi and bacteria often inhabit the same ecological environment, it is not surprising that signals may be perceived across-kingdoms.

As outlined in the previous sections, several eukaryotic organisms produce compounds capable of interfering with *P. aeruginosa* quorum sensing. Studies also indicate that *P. aeruginosa* is able to sense and respond to signalling compounds produced by its host. Wu *et al.,* 2005 showed that the inflammation cytokine interferon γ (INF-γ) binds to an outer membrane protein of *P. aeruginosa*; OprF, resulting in an enhanced expression of quorum sensing regulated virulence factors (427). In a study not yet finished, I have investigated the effect of human recombinant INF-γ on rhamnolipid production in *P. aeruginosa* PAO1 wild-type and its Δ*lasI*-Δ*rhI* derivative using the erythrocyte lysis assay described in article 3. Treatment with INF-γ (300 ng/mL) induced an earlier and higher pigment and rhamnolipid production (the latter measured as hemolysis capacity) by the *P. aeruginosa* wild-type than its untreated counterpart. Moreover, adding INF-γ to the *lasI-rhI* mutant, normally devoid of rhamnolipid production, induced the QS mutant to produce rhamnolipid, although very late (7 hours after the entrance to stationary phase and around 5 hours later than the INF-γ treated wild-type). See Figure 22 for details.

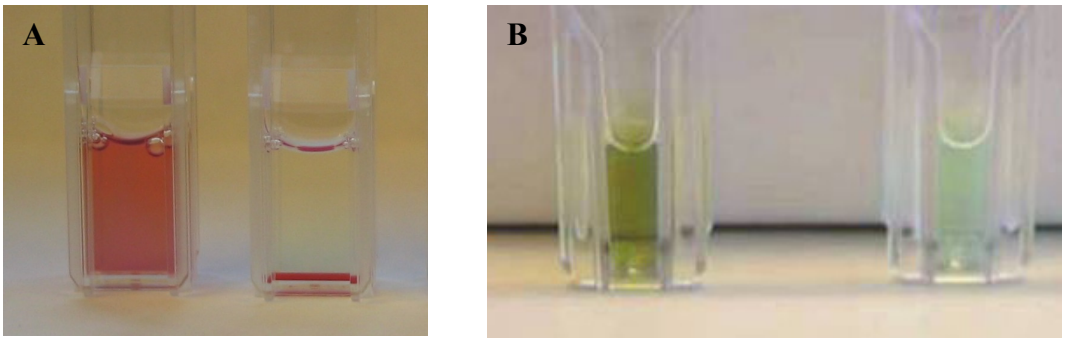


Figure 22. Effect of INF-γ on *P. aeruginosa* virulence factor production. **A** Hemolysis capacity of supernatant from a *P. aeruginosa* PAO1 *lasI-rhI* mutant grown in the presence of INF-γ (left) or absence (right). Precipitation of the red blood cells (right) means no lysis whereas uniformly distribution of the red colour (left) indicates lysis. **B** Pigment production by *P. aeruginosa* PAO1 wild-type grown in the presence of INF-γ (left) or absence (right). *P. aeruginosa* pigment production is – at least partly - controlled by quorum sensing (see assay in (381)).

I also performed a global DNA microarray analysis of the *lasI-rhI* mutant treated with INF-γ vs. an untreated *lasI-rhI* mutant, both harvested at the entry into stationary phase. The INF-γ treatment induced expression of several PQS controlled genes 20-70 fold, and changed the "absolute call" of the PQS genes from absent (undetectable) in the untreated Δ*lasI*-Δ*rhI* mutant to present (detectable) in the INF-γ treated mutant (according to the A/P/M evaluation by the GCOS DNA microarray analysis software vs.1.4). Likewise, did INF-γ treatment induce *rhlA* (PA3479) expression 10 fold. (The study is presently unpublished, but the results have been

presented at the conference "A genomic perspective to host-pathogen interaction" 7th September 2006 in Hinxton, England).
The exact mechanism by which INF-γ exerts its effect on *P. aeruginosa* quorum sensing has not yet been fully revealed. Changes in OprF expression have been associated with quinolone resistance in *P. aeruginosa*, and another membrane porin of *P. aeruginosa*; OpmD (part of the MexGHI-OpmD pump) has been shown to be involved in PQS efflux (7, 112, 301). It is possible that binding of INF-γ to OprF somehow decreases PQS efflux, thereby increasing the intracellular PQS concentration which, in turn, via activation of PqsR further induces transcription of the PQS operon and *rhlA*. Regardless of the mechanisms by which *P. aeruginosa* perceive and react to the host inflammation cytokine INF-γ, *P. aeruginosa* is apparently able to detect and respond to changes in the host status. Another example is the ability of *P. aeruginosa* to sense the host produced κ-agonist dynorphin and react to the release of this stress opioid by enhanced expression of virulence factors by a mechanism involving quinolone signalling (438). Dynorphin is an opiate-like stress factor released by neutrophils and other cells in connection to injury or inflammation. Thus, in order to be able to target the host when it is most susceptible, *P. aeruginosa* seems to have evolved the ability to monitor the presence of host factors indicative of inflammation and stress and time expression of virulence factors accordingly.

Communication also proceeds in the opposite direction. *P. aeruginosa* signal molecules (in particular OdDHL) have been found to be implicated in interactions with eukaryotic cells: *Candida albicans* is a prevalent fungal pathogen causing infections in immunocompromised patients and may be found coexisting with *P. aeruginosa*. Studies in patient with mixed *C. albicans* and *P. aeruginosa* infections have shown that attenuation of *P. aeruginosa,* e.g. after treatment with antibiotics*,* is followed by an increase in the *C. albicans* population and the growth of *C. albicans* has been shown to be limited by the presence of *P. aeruginosa* (38, 164). *C. albicans* exists in a commensal yeast form and in a filament form associated with virulence and pathogenicity. Interestingly, *P. aeruginosa* only bind to and kills the filamentous form of *C. albicans* (140). Additionally, OdDHL inhibits *C. albicans* filamentation in concentrations not affecting fungal growth rates in a way resembling *C. albicans* own signal molecule farnesol (141). It thus seems that *P. aeruginosa* only tolerates habitat sharing with *C. albicans* in its non-virulent – and thus non-competing – yeast form.

THE INFLAMMATORY PROCESS

The inflammation process is another example of host-pathogen interaction. Microbial components such as lipopolysaccharide (LPS), CpG DNA and other PAMPs (Pathogen-Associated Molecular Patterns) are recognised by Toll-like receptors (TLRs) whereupon proinflammatory cytokines and other soluble factors are released activating the complement pathway and attracting phagocytic cells such as neutrophils to the site of infection (6, 284). Through binding to TLR4, LPS also triggers cascades of intracellular signalling events, including those that lead to activation of Nuclear Factor κ B (NF-κB) which plays a key role in regulation of the immune response to infection (130, 439). The NF-κB proteins are normally sequestered in the cytoplasm as inactive complexes with specific inhibitors, such as IκBα (20). Upon activation by cellular stimuli, IκB kinase (IKK) phosphorylates IκBs, targeting these inhibitors for degradation. The released NF-κB can then migrate to the nucleus and induce expression of specific genes that have DNA-binding motifs for NF-κB, typically genes involved in inflammation and cell survival responses (162). LPS also triggers the three classes of mitogen-activated protein kinases (MAPK): the extracellular signal-regulated kinase (ERK) (also known as p42/44 MAPK), the c-jun N-terminal kinase (JNK) (also known as stress-activated protein kinases (SAPKs)), and p38 MAPK (also known as cytokine suppressive anti-

inflammatory drug binding protein (CSBP)). These MAPKs are in turn involved in the activation and induction of a large array of proinflammatory genes, such as those encoding the cytokines tumor necrosis factor α (TNF-α) and IL-8 and the prostaglandin-generating enzyme cyclooxygenase 2 (COX-2) (9, 179, 373). Binding of the inflammatory cytokines to their receptors on cells of the immune defence further amplify the innate immune response targeting the invading pathogen. Antigen presenting cells (APCs) such as dendritic cells (DCs) are induced to migrate to the site of infection by microbial products, inflammatory chemokines and TNF-α. The APCs internalize pathogens and display the antigens together with MHC (major histocompatibility complex) to B and T lymphocytes thereby also initiating and shaping the immune response.

While the presence of any pathogen in general would lead to activation of the immune defence including the events outlined above, *P. aeruginosa* has been found to interfere with many of these cellular events associated with host immunity. Most remarkable are the effects of *P. aeruginosa*'s quorum sensing signal molecules.

THE EFFECT OF ODDHL ON MAMMAL SIGNALLING PATHWAYS

As described earlier, *C. albicans* reacts on the *P. aeruginosa* signal molecule OdDHL. Several studies also document the effect of this signal molecule on mammalian cells. One example is the pharmacological effect of OdDHL on the cardio vascular system. Experiments with porcine pulmonary and coronary blood vessels have demonstrated that OdDHL possesses vaso-relaxing activity, which might increase the nutrient supply to an area infected with *P. aeruginosa* (184). OdDHL is also a very efficient inhibitor of ATP and UTP-induced secretion by CF human submucosal tracheal gland serous (HTGS) cells (IC_{50} in the picomolar range). The lack of responsiveness of HTGS cells in the presence of OdDHL (and to a lower degree other AHLs) were found to be related to the defect in CFTR, since adenovirus-mediated CFTR transfer conferred resistance to the effect of the signal molecules (337). This finding is highly interesting as *P. aeruginosa* is frequently found in the lungs of CF patients and OdDHL has been detected in CF lungs and sputum (92, 352).

Human macrophages have been shown to increase their phagocytic activity when exposed to OdDHL; however OdDHL did not affect respiratory (oxidative) burst exhibited by stimulated macrophages (395). This stimulatory effect of OdDHL was found to be mediated via the p38 MAP kinase pathway without involving the ERK pathway (p42/44 MAP kinase) (395). The p38 MAP kinase is involved in the regulation of many cellular processes including inflammation, cell differentiation, cell growth and death, while ERK (p42/44 MAP kinase) is a key effector in signalling proliferative responses in various cell types (49).

It has also been demonstrated that human epithelial Caco-2 cells exposed to OdDHL show decreased transepithelial electrical resistance and reorganisation of F-actin indicating that OdDHL disrupts barrier integrity (396). F-actin is a filamentous polymer consisting of G-actin subunits. Actin is an important structural part of the cytoskeleton, involved in the formation and maintenance of tight junctions. OdDHL was also found to disrupt cell-cell adherence, probably due to the effect on F-actin (396). (See figure 23 for the effect of OdDHL on the apposition of Caco-2 cells). The authors concluded that this effect of OdDHL is, at least, partly mediated via the p38 and p42/44 MAP kinase pathways. Based on these observations, the authors suggest that OdDHL plays an important role in the destruction and dysfunction of epithelia cells during *P. aeruginosa* infections (396).

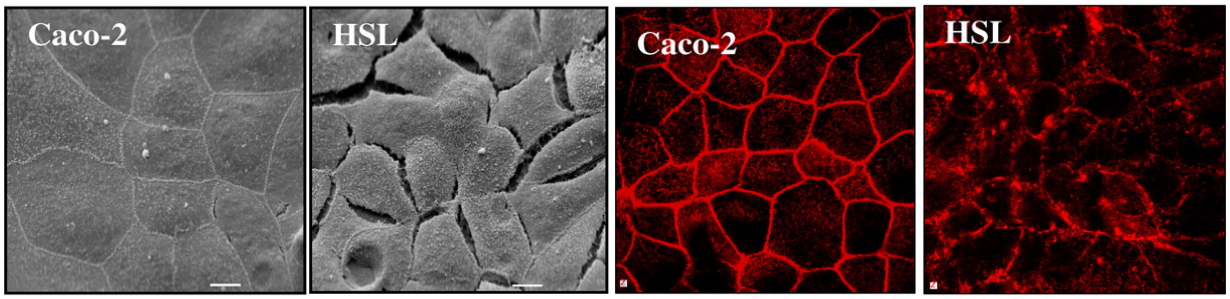


Figure 23. Disruption of the close apposition of Caco-2 cells by OdDHL. Examination by scanning electron microscopy (grey). Cells were either untreated (Caco-2) or stimulated for 2 h with 200 μM OdDHL (HSL). Effect of OdDHL on the organization of the actin cytoskeleton (red) in Caco-2 cells (396).

Also calcium signalling has been found to be involved in OdDHL mediated interaction between *P. aeruginosa* and host cells. In a study investigating the effect of OdDHL on murine fibroblasts and human vascular endothelial cells (HUVECs), OdDHL was shown to modulate expression of immune mediators (IL-6 and COX-2) and to induce apoptosis accompanied by an increase in cytosolic calcium (348). Calcium ions act as second messengers in signal transduction, and changes in calcium concentration can be an intermediate event in several signal transduction cascades e.g. the PI3K (phosphoinositol-3-kinase) and MAPK signalling pathways. Ca^{2+} dependent signalling was found to mediate OdDHL induced apoptosis, while the OdDHL-imposed immunomodulatory effects was not linked to calcium signalling (348). Based on these data the authors suggested that OdDHL elicits at least two distinct cellular responses, one involving calcium signalling mediating apoptosis, and another implicated in an immunomodulatory response mediated by a pathway independent of calcium signalling.

The effect of OdDHL on breast carcinoma (BC) cells has recently been investigated, and it seems that OdDHL blocks cell proliferation and induces apoptosis in BC cells (196). Investigating the effect of OdDHL on signalling pathways in the BC system, it was revealed that while OdDHL had only minor or no effect on MAPK pathways, OdDHL seemed to partially inhibit the protein kinase B pathway and eliminate STAT3 (Signal Transducer and Activator of Transcription 3) activity. Protein kinase B/Akt (PKB/Akt) is a serine/threonine kinase involved in various cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration. Activation of PKB/Akt occurs downstream of PI3K and is regulated via cytokine receptors and as earlier mentioned, calcium signalling (259). STAT3 is a transcription factor and mediator of the switch from survival to death signalling activated by certain cytokines and growth factors including G-CSF and IL-6 (2). Since OdDHL completely inhibits STAT 3 activity in BC cells and partially inhibit PI3K signalling this may explain OdDHL's ability to induce apoptosis in BC cells (196).

Although the various studies report on different and at times contradicting observations on OdDHL's effects on mammal signalling systems, it is worth remembering that MAP kinases, calcium signalling, PKB/Akt and PI3K pathways, and STAT3 are all entangled systems which interact with eachother: Calcium signalling is involved in MAP kinase and PI3K pathways, with PI3K being an activator of PKB/Akt, while PI3K/Akt and STAT3 are two parallel pathways both induced by IL-6 (41). Moreover, results obtained by the use of one type of cells may not correspond to observations achieved by using a totally different cell system.

ODDHL AND CELLS OF THE IMMUNE DEFENCE

DiMango *et al.,* 1995 first reported on immunomodulatory properties of OdDHL and showed that OdDHL stimulate respiratory epithelial cells to produce IL-8 (79). Also Smith *et al.,* 2001 demonstrated that OdDHL promotes IL-8 production by human lung fibroblasts and epithelial cells (358). IL-8 is a neutrophil attracting chemokine released by macrophages and other cell types such as epithelial cells. In the early stages of an inflammatory response neutrophils are the predominant cells at the site of infection. Neutrophils are attracted to the site of an infection by chemokines (e.g. IL-8) and by complement split products, prostaglandins and leukotrienes. Also certain PAMPs such as formyl peptides are chemotactic for neutrophils (reviewed by (422)). Neutrophils are important phagocytes and when activated, they produce biocidal oxygen radical species including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) (produced via the NADPH-oxidase complex) and hypochlorous acid (HOCl) and chloramines, via myeloperoxidase. Though neutrophils are mostly known for their central role in phagocytosis and production of oxidative burst, these cells also make vital contributions to the recruitment, activation and programming of APCs e.g. by proteolytical activation of prochemerin to generate chemerin, a dendritic cell attracting chemokine. Neutrophils also influence whether macrophages differentiate to a predominantly pro- or anti-inflammatory state (250). As neutrophils represent the first line of defence against bacterial infections, the interaction between neutrophils and *P. aeruginosa* has attracted some attention. In a recent study by Wagner *et al.,* 2007 it was demonstrated that OdDHL works as an attractant on neutrophils; moreover the expression of the immunoglobulin receptors CD16 and CD64 and the adhesion protein CD11b were increased by the presence of OdDHL (400). In another article, the authors further investigated OdDHL as a neutrophil chemoattractant and found that neutrophils bind radioactivity labelled OdDHL in a specific and saturable fashion, suggesting that neutrophils express a receptor which OdDHL is able to bind to (444). Based on inhibition of signalling pathways it was further demonstrated that OdDHL induces neutrophil chemotaxis via a pathway involving tyrosine kinase, phospholipase C, protein kinase C and ERK as specific inhibition of each of these factors impeded OdDHL-induced chemotaxis (444). Inhibition of several factors crucial for IL-8-promoted chemotaxis however, did not interfere with OdDHL-induced chemotaxis indicating that OdDHL attracts neutrophils via a pathway at least in part different than that of IL-8. It is indeed quite intriguing that OdDHL both induces production of the neutrophil chemokine IL-8 while functioning as a neutrophil attractant *per se*.

In an attempt to further identify factors affected by *P. aeruginosa* signalling, I have (in a preliminary study) investigated the effects of pure AHL signal molecules and sterile filtered supernatants from *P. aeruginosa* PAO1 on the transcriptome of human neutrophils by the application of the GeneChip Human Genome U133 Plus 2.0 Array from Affymetrix. In brief, neutrophils were purified from peripheral blood, resuspended in RPMI buffer and incubated for 1 hour either with 5 µM BHL and 5µM OdDHL or mixed with 50 % supernatant from *P. aeruginosa* exponentially growing cultures (either from the wild-type or from the *lasI-rhlI* mutant). Following this the neutrophils were subjected to DNA microarray analysis. Both treatments had a profound effect on the expression of the PI3K gene. In neutrophils, PI3K is involved in various activities such as actin assembly and events leading to phagocytosis, chemotaxis and cytokine synthesis (94, 228). Neutrophils which had been challenged with pure AHL molecules showed a 12.6 fold decreased PI3K expression, while PI3K was 3.6 fold down-regulated by addition of wild-type supernatant compared to treatment with supernatant from the *lasI-rhlI* mutant. The wild-type supernatant differs from the *lasI-rhlI* mutant supernatant in several ways. One difference is that the wild-type supernatant contains significantly higher levels of various quorum sensing regulated products such as phenazines and proteases than the supernatant from the *lasI-rhlI* mutant. Another important difference is

that only the wild-type supernatant contains quorum sensing signal molecules. Thus the decrease in PI3K imposed by the wild-type supernatant may be caused by signal molecules in the supernatant.
Calcineurin, a central element in calcium signalling and the target for the immunosuppressant cyclosporin, was down-regulated 4.42 times by pure AHLs and down-regulated 3.5 times by the wild-type supernatant. As PI3K is in part controlled by calcium signalling, the effect on PI3K is likely to be a down stream effect of the decreased expression of calcineurin. Expression of genes involved in actin assembly, COX-2 regulation, phagocytosis and chemotaxis were also altered by exposure to pure AHLs and *P. aeruginosa* wild-type supernatant.
In eukaryotes, many environmentally induced changes take place at a post-transcriptional level, such as gene silencing by microRNAs. It has been estimated that mRNA concentrations only determine the concentration of the corresponding protein by 20%–40% (36, 384). Thus conclusions should not be based on transcriptome analyses only. The above mentioned transcriptome analyses were mainly performed to guide further investigations on the effect of *P. aeruginosa* quorum sensing regulated products - including signal molecules - on neutrophil activities. I am currently investigating the effects of AHLs on neutrophil protein expression by Fluorescence 2-D Difference Gel Electrophoresis (DiGE) proteomics in collaboration with Cambridge Centre for Proteomics. Phenotypic tests, e.g. the Boyden migration chamber test and phagocytosis tests could also be used to further explore the effect of AHL signal molecules and quorum sensing regulated products on neutrophil activities involved in the elimination of bacteria.

ODDHL AND THE T_H1/T_H2 CONTROVERSY

Most $CD4^+$ T cells (T helper cells) belong to one of the two functional subsets T_H1 and T_H2, and can be classified on the basis of their cytokine profiles. T_H1 cells are characterized by the production of IFN-γ, TNF-β and IL-2, and are pivotal in the cell-mediated immunity, while T_H2 cells are characterized by the production of IL-4, IL-5 and IL-13 and play an important role in antibody-mediated humoral immunity (reviewed in (118, 332)). T_H1 cytokines antagonise development of the T_H2 subset and *vice versa*. Cytokines produced by other cell types than T_H cells have also been assigned to belong to either the T_H1 or the T_H2 response. For instance IL-1, IL-8 and TNF-α are associated with the T_H1 subset, and IL-6 with the T_H2 subset (75, 332). While Shiner *et al*., 2006 found that OdDHL induces expression of IL-6, indicating that OdDHL supports a T_H2 type response, the observation that OdDHL induces production of IL-8 (79, 358) suggests that OdDHL imposes a T_H1 subset. This exemplifies that *in vitro* investigations and in particular the conclusions drawn are often contradicting with respect to the effect of OdDHL on the immune system. The studies and conclusions can be divided in two groups; A) studies suggesting that OdDHL will shift the host protective T_H1 response into a T_H2 response potentially leading to less bacterial clearance and B) studies indicating that OdDHL might support a proinflammatory T_H1 response likely to lead to uncontrolled and prolonged inflammation and subsequent destruction of host tissue and pathogen dissemination. Among the reports supporting that OdDHL promotes the T_H2 subset is the study by Telford *et al*., 1998 showing that OdDHL *in vitro* decreases the production of the T_H1 cytokines IL-12 and TNF-α by LPS stimulated macrophages (378). Lymphocyte proliferation was also found to be hindered by the presence of OdDHL, while immunoglobulin E production was promoted by OdDHL, collectively suggesting that OdDHL influences the balance between T_H1-T_H2 to the advantage of the T_H2 subset.
Contrary to these findings, OdDHL has been shown to induce proinflammatory responses in human lung fibroblasts and epithelial cells e.g. by mediating an induced IL-8 transcription as earlier mentioned (358). Moreover, OdDHL has been demonstrated to induce production of

INF-γ by antigen and APC activated T cells, promoting a T_H1 response (359). The latter study also showed that OdDHL, but not BHL, injected into the skin of C57BL/6 mice caused extensive inflammation (infiltrating cells and edema) and tissue destruction (359).
Studies have shown that the immune response to chronic *P. aeruginosa* lung infection in CF patients is predominantly of the T_H2 type response; it seems however, that the T_H1 type response is associated with an enhanced bacterial clearance, milder lung inflammation, and improved survival (33, 123, 235-238). Considering that these studies conclude that a T_H2 response is associated with more persistent inflammation, a T_H1 response mediated by OdDHL should lead to a better prognosis for CF patients.

PQS has recently been shown to exert antiproliferative effects on hPBMCs (human Peripheral Blood Mononuclear Cells) in a ConA driven T cell proliferation assay (144). Moreover, PQS and OdDHL also decreased the release of the T_H1 cytokine IL-2 by mitogen stimulated PBMCs (144). However, only OdDHL had an effect on the release of IL-2 by T cells activated via the T cell receptor (stimulation via anti-CD3/anti-CD28 antibodies). While OdDHL in doses above 50 μM suppressed secretion of TNF-α of *E. coli* LPS treated hPBMCs, high doses of PQS (above 25 μM) induced TNF-α expression (144). Thus, also PQS seems to have the capacity to modulate the immune response in both the T_H1 and the T_H2 direction. This is also reflected in manuscript "*Pseudomonas aeruginosa Quorum Sensing Signal Molecules interfere with dendritic cell induced T cell proliferation*" which reports on the effect of BHL, OdDHL and PQS on activities and functions of murine bone marrow derived dendritic cells. OdDHL and PQS both decreased the IL-12 production by dendritic cells, however expression of T cell stimulatory and maturation related surface markers by LPS-stimulated dendritic cells were impeded by OdDHL but seemed to be promoted by PQS. OdDHL and PQS both decreased the capacity of dendritic cells to induce T cell proliferation, whereas BHL was found not to interfere with DC functions (Article 4).
Though the T_H1 response is crucial for cell-mediated immunity, misregulation of the T_H1 response is the cause of several severe diseases. Multiple sclerosis, type 1 diabetes, rheumatoid arthritis and scleroderma are all T_H1-driven autoimmune diseases. In type 1 diabetes (insulin-dependent diabetes mellitus, IDDM) autoaggressive T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) infiltrate the pancreas and induce islet β-cell destruction and insulinitis. Studies showing that OdDHL down-regulate immune responses in an apparent T_H1 selective manner suggest that OdDHL may function as a therapeutic in T_H1 driven diseases. In an interesting study, female non-obese diabetic (NOD) mice were treated with OdDHL, OHHL and OtriDHL (N-3-oxo-tridecanoyl homoserine lactone), PBS and DMSO and evaluated for the onset of diabetes by test of glycosuria. OdDHL prophylaxis was shown to significantly retard the cumulative incidence of diabetes, while OHHL and OtriDHL had no significant effect (312). (Refer to figure 24 showing the effect of OdDHL on pancreatic islet morphology compared to placebo treatment).
Whether OdDHL may have applications as a pharmaceutical agent in IDDM and other autoimmune diseases remains to be established.

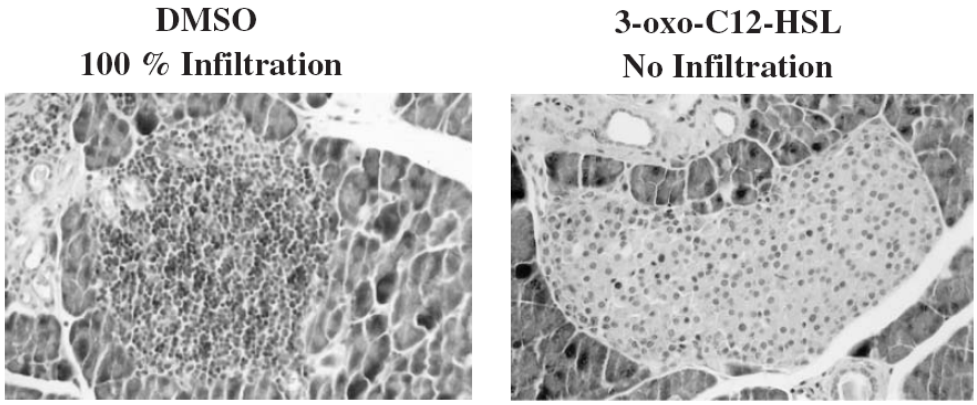


Figure 24. The morphology of pancreatic islets treated with vehicle (dimethyl sulphoxide; DMSO) (left) and with OdDHL (3-oxo-C12-HSL) (right). The DMSO treated control shows the mononuclear insulitis characteristics of NOD mice (311, 312).

Ritchie *et al.,* 2003 investigated the effect of OdDHL on C57BL/6 mice (T_H1-biased) and BALB/c mice (T_H2-biased) and found that the cytokine profile was altered depending on the underlying immune bias of the mouse strain used. Mice of the C57BL/6 strain showed a relative increase of the T_H1 cytokine IFN-γ as response to OdDHL, while BALB/c mice exhibited a relative increase of the T_H2 cytokine IL-4 (331). Moreover, a similar pattern was seen with T lymphocytes from the two strains. The authors thus suggested that the effect of OdDHL on T-cell cytokine production is mostly non-specific and accentuates an underlying immune response bias rather than specifically altering either T_H1 or T_H2 responses (331). In another study the authors demonstrated that OdDHL *in vitro* inhibits differentiation of both T_H1 and T_H2 cells and that the balance of cytokine production by T_H cells stimulated in the presence of OdDHL varies with both the antigen concentration and its affinity for the T cell receptor (329). Thus the use of different systems may be the source of conflicting observations on the effect of OdDHL on the T_H1/T_H2 equilibrium. Another related explanation on the contradicting reports is proposed by Pritchard, 2006 (311), who suggests that the effect of OdDHL on the T_H1/ T_H2 balance is determined by the OdDHL concentration. High concentration of OdDHL (100 μM) is mainly used in studies pointing at a T_H1 directing effect of OdDHL such as Smith *et al*., 2001-2 while low concentrations of OdDHL (>10 μM) have been applied in studies demonstrating promotion of the T_H2 response such as Telford *et al*., 1998 (358, 359, 378). Among the observations which contradict this hypothesis is that OdDHL decreases IL-12 production by LPS stimulated macrophages in concentrations ranging from 0.1 to 100 μM, moreover production of the T_H1 supporting cytokine TNF α by LPS stimulated macrophages has been shown to be down-regulated by OdDHL in concentrations above 40 μM (378). Thus, while there is growing evidence that OdDHL possesses immunomodulatory properties, the effects of OdDHL on elements of the immune response *in vivo* as well as *in vitro* still call for further investigations.

THE CONCENTRATION OF QUORUM SENSING SIGNAL MOLECULES IN CULTURES AND *IN VIVO*

Although virulence factor production by *P. aeruginosa*, as well as the clinical relevance of immunomodulatory effects of quorum sensing signal molecules may depend on their concentration, only a very limited number of studies have sought to quantify the concentration of signal molecules in *P. aeruginosa* liquid cultures and biofilms. This may be partly due to the problems of measuring heat labile 3-oxo-AHLs such as OdDHL by the use of GC-MS (44). The concentration of OdDHL in PAO1 cultures have been reported to be in the order of 5 μM (288). Concentrations of BHL and OdDHL in exponentially growing *P. aeruginosa* PAO1 cultures have been quantified by HPLC-(UV) and the concentration of BHL was determined to

be in the range of 0.12 μM to 2.01 μM, while OdDHL under the same conditions was found to be 0.45 μM to 1.16 μM (325). In biofilms the concentration of OdDHL has been found to be surprisingly higher; 632 μM (± 381 μM) (52).
The concentrations of PQS and the related 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) in overnight cultures of *P. aeruginosa* PAO1 and PA14 have been measured to be 17.0-17.4 μM and 32.8-35.8 μM for PQS and HQNO, respectively (191). PQS, HQNO and the two main AHLs of *P. aeruginosa* BHL and OdDHL have all been found in lung tissue and sputum of CF patients (60, 207, 352). The concentration of PQS in CF sputum has been estimated to be around 2 μM, and the concentration of OdDHL in sputum and lung tissue of CF patients have been found to be in the pico- to nanomolar range (60, 92, 352). Thus the PQS level in sputum is roughly 10 % of the PQS concentration in liquid *P. aeruginosa* cultures, whilst the concentration of OdDHL is 1000 fold lower in CF sputum compared to liquid cultures and drastically lower compared to biofilm samples. The reason for the immense difference between the OdDHL levels in liquid cultures and in sputum could be that the *P. aeruginosa* strains infecting the CF lung produce less OdDHL than the laboratory strains used for quantifying AHLs in liquid cultures. The low concentration of OdDHL found in the CF sputum could also be caused by continuous degradation of OdDHL by host paraoxanases, perhaps even after sampling (58, 433).
It thus seems that further measurements of the OdDHL concentration in medical settings such as in burn wounds and in acute and chronic lung infections are still needed in order to define a biologically relevant OdDHL concentration.

WHAT IS THE MECHANISM(S) BEHIND ODDHL'S IMMUNOMODULATORY EFFECTS?

In order to determine which structural key features of OdDHL is essential for its immunosuppressive activity Chhabra *et al.,* 2003 investigated a range of AHLs and analogues and showed that the optimal structures in regard to immunosupression were AHLs with 11-13 carbon acyl chains containing either a 3-oxo or a 3 hydroxy group and a high lipophilicity of the molecule (53). This is supported by several studies where short and middle chain AHLs were investigated along with OdDHL, but not found to possess immunomodulatory activity (144, 377, 378). Since the hydrophobicity seems to be involved in the immunomodulatory activity of OdDHL, it has been suggested that insertion of the signal molecule within the lipid bilayer is the cause of OdDHL's effects on cells of the immune defence including its ability to induce apoptosis. While it is still unknown whether PQS is also capable of crossing the eukaryotic cell membrane, several studies have demonstrated that AHLs (e.g. BHL, OdDHL and *N*-(3-oxooctanoyl)-L-homoserine lactone, OOHL) are able to cross the cell membrane of various cell lines and to function in mammalian cells; thus the target of OdDHL may be an intracellular factor (253, 330, 347, 417).

IL-8 secretion is transcriptionally upregulated by binding of the transcription factor NF-κB to its promoter (351) and OdDHL increases NF-κB leading to the induced IL-8 transcription found by (79, 358). Production of the MAP kinase p42/44 MAPK (ERK1/2) by human epithelial cells has also been reported to be induced by the presence of OdDHL (395). Addition of a specific MAP kinase inhibitor abolished the OdDHL mediated induction of IL-8 transcription, thus it seems that MAP kinases are also involved in the OdDHL induced activation of NF-κB leading to increased IL-8 secretion (358). OdDHL also induces expression of genes encoding Activator Protein 2 (AP-2), cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE_2). COX-2 can be induced by a wide variety of inflammatory stimuli (360) and a number of pathways have been described to be involved in transcriptional regulation of COX-2 in response to inflammatory mediators, among these NF-κB and factors being a part of MAP kinase cascades (72, 108). COX-2 has long been regarded as a mediator of proinflammatory

responses as this enzyme converts arachidonic acid to prostaglandin H_2, the precursor of thromboxane and the series-2 prostanoids e.g. PGE_2 which promote inflammation, fever, and pain (391). However, recent studies suggest that COX-2 also plays an important role in the process of resolving inflammation and in healing by mediating production of cyclopentenone prostaglandins including 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) (111, 161, 233). This naturally occurring eicosanoid displays a broad spectrum of biological activities, including regulation of apoptosis and cell proliferation, suppression of IL-6-induced STAT3 phosphorylation, stimulation of ERK (p42/44 MAP kinases) via H-Ras, and induction of anti-inflammatory effects (91, 167, 273, 343, 428). (See figure 25).

It has been proposed that 15d-PGJ_2 exerts its anti-inflammatory activity through the activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) and also via the inactivation of NF-κB by directly inhibiting its effector kinase IκB kinase (IKK) or the p50 subunit (47, 156, 328, 333). PPAR-γ is a phosphoprotein and its activity is regulated by ligand binding and phosphorylation. The phosphorylation is presumed to be accomplished by MAPKs, particularly ERK (146, 441). Ligand-activated PPAR-γ mediates its anti-inflammatory effects on various immune cells. PPAR-γ has been shown to inhibit IL-2 production by T cells, down-regulate dendritic cells' IL-12 production and maturation, and attenuate endothelial cells' production of T cell-active CXC chemokines which play an important role in T cell recruitment to the site of inflammation (170, 218, 435). Thiazolidinediones, a group of PPAR-γ agonists, are routinely applied to treat patients with non-insulin-dependent diabetes mellitus (NIDDM, Type 2 diabetes mellitus), moreover PPAR-γ agonists have been shown to reduce the incidence of diabetes in NOD mice (19, 23, 24).

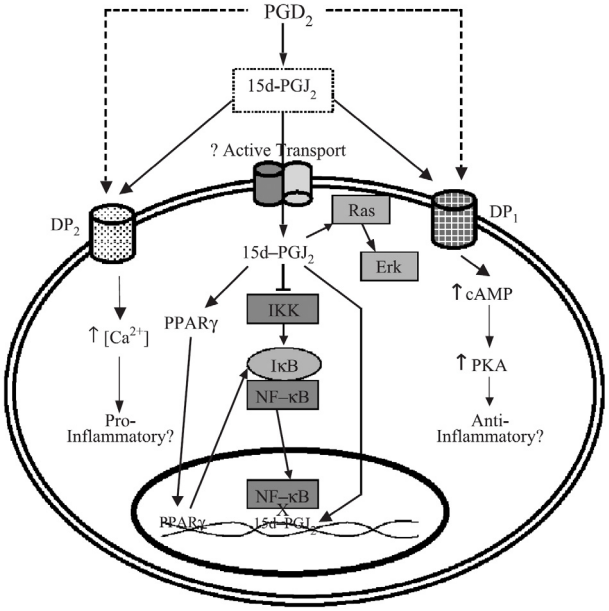


Figure 25. Pro-inflammatory and anti-inflammatory actions of 15d-PGJ_2. The prostacyclin 15d-PGJ_2, derived from PGD_2, may interact with the PGD_2 receptors DP_1, DP_2 (the latter also known as Chemoattractant Receptor-homologous molecule expressed on T_H2 cells (CRTH2)) to activate protein kinase A (PKA) (anti-inflammatory) and stimulate Ca^{2+} influx (inflammatory). In the cytosol, PGJ_2 may directly activate the Ras/Erk pathway, and bind to and inhibit IκB kinase (IKK), the activator of NF-κB. Additionally, 15d-PGJ_2 may inhibit NF-κB activity either by directly blocking NF-κB DNA binding sites in the nucleus, or activating PPAR-γ to stimulate synthesis of the NF-κB inhibitory subunit IκB. (Adapted from (343)) .

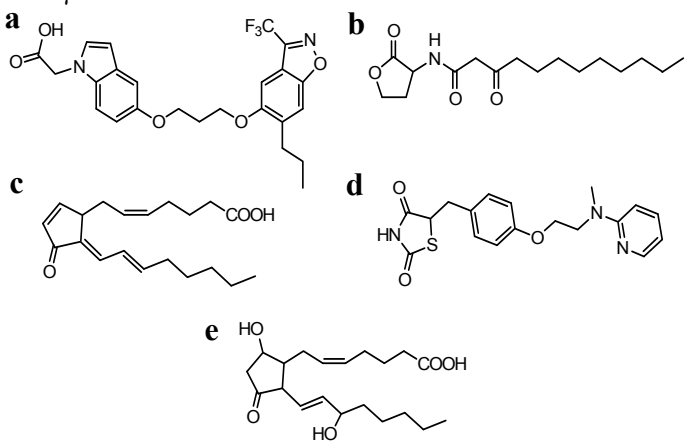
As mentioned earlier, OdDHL also has the capacity to prevent autoimmune diabetes in NOD mice, by inhibiting IL-2 production in T cells and, as shown in article 4, decreasing IL-12 production in dendritic cells and maturation of dendritic cells. Moreover, OdDHL has also been shown to induce MAPKs such as ERK (p42/44 MAP kinase), suppress STAT3 and induce calcium influx leading to inflammatory events, activities also carried out by 15d-PGJ_2. Thus one hypothesis for the immunomodulatory mechanisms exerted by OdDHL could be that OdDHL induces COX-2, which either generates proinflammatory prostaglandins such as PGE_2 promoting a proinflammatory response, or mediates generation of prostacyclins such as 15d-PGJ_2, which in turn mediates an anti-inflammatory effect via PPAR-γ and NF-κB.

OdDHL may also interact directly with PPAR-γ. Structural overlap between OdDHL and an array of agonists and antagonists (including 15d-PGJ_2 and the thiazolidinedione Rosiglitazone)

suggests that OdDHL may have the capacity to bind to the PPAR-γ receptor. Moreover, *in silico* docking of OdDHL against the x-ray crystallographic structure of PPAR-γ bound to its agonist BPR1H036 ligand 3EA (212) shows that OdDHL has high affinity for PPAR γ. See table 2 and figure 26.

Table 2. Docking of OdDHL and known agonists to PPAR γ and mean of random molecules.

Molecule	MolDock	Rerank
a BPR1H036	-144.5	-62.2
b OdDHL	-104.0	-89.7
c $15dPGJ_2$	-84.5	3.3
d Rosiglitazone	-81.3	-54.6
e PGD_2	-65.9	57.9
*Diversity mean	116.8	51.4



The x-ray crystallographic structure of the ligand binding domain of PPAR-γ bound to its agonist BPR1H036 (2-[5-[3-[7-propyl-3-(trifluoromethyl)-benzo[d]isoxazol-6-yl]oxypropoxy]indol-1-yl]ethanoic acid) (RCSB PDB code 2ATH, ligand 3EA) was used for docking of OdDHL and PPAR-γ agonists using the Template Docking Model with default settings (Molegro Virtual Docker (MVD2007) (382)). The PPAR-γ agonist BPR1H036 was set as the template ligand. Ligands were ranked by using the MVD MolDock score. The re-rank scoring is also shown. Positive scores indicate low affinity.
*Diversity mean is the average score of 25 random molecules from NCI Diversity Set of small molecules.

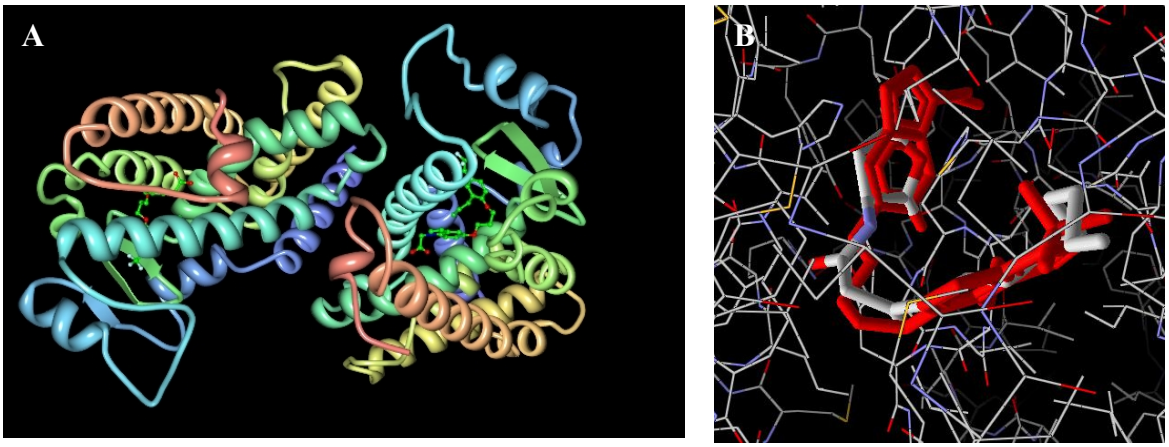


Figure 26. A. The PPAR γ dimer bound to its agonist BPR1H036. From www.rcsb.org/pdb Protein Databank of the Research Collaboratory for Structural Bioinformatics RCSB PDB code 2ath, ligand 3EA. (PDB Simple viewer). **B.** OdDHL (carbon atoms grey, oxygen atoms red and nitrogen atom blue) superimposed in the LBD of PPAR γ with BPR1H036 (red) as template. (Molegro Virtual Docker, MVD2007).

It is intriguing that the effect of OdDHL on various mammalian pathways resembles those mediated by $15dPGJ_2$. This could be due to direct interaction between OdDHL and transcription factors (activation of PPAR-γ and/or inhibition of IKK and thus NF-κB) or because OdDHL interacts with a factor upstream of $15dPGJ_2$, such as COX-2, thereby inducing production of $15dPGJ_2$ or another prostanoid with similar effects. The possibility however, that OdDHL either directly or indirectly interacts with PPAR-γ remains to be investigated by experimental studies.

FINAL COMMENTS

Here in the beginning of the 21st century, *P. aeruginosa* has definitely emerged as a serious opportunistic pathogen. This prevalent bacterium has multiple ways to escape the antagonistic actions of the immune defence; *P. aeruginosa* readily forms biofilms and the biofilm matrix confers physiological protection against aggressive host factors. Moreover, this microbe produces an array of cell destructive and toxic virulence products which weaken the host barriers and fortification against this intruder. Just to mention a few examples on the harsh armament of this bacterium: *P. aeruginosa* produces rhamnolipids which can impose immediate lysis of neutrophils and other mammalian cells, and also generates several tissue degrading enzymes such as lipases, elastases and proteases. Additionally this pathogen is capable of producing hydrogen cyanide which is a true chemical warfare agent, as it inhibits mitochondrial cytochrome oxidase and *P. aeruginosa* hydrogen cyanide has been shown to rapidly paralyse and kill nematodes. *P. aeruginosa* is being found at an increasing frequency in connection to serious infections and with its inherent high tolerance to antimicrobial agents, together with its ability to constantly evolve new resistance mechanisms, emphasize the clinical problems associated with this organism. Collectively, there is an urgent need for the development of new drugs targeting the various protective and aggressive survival mechanism of this bacterium.

One attractive strategy is to target its "line of command" which the bacterium uses to coordinate and regulate complex activities involved in biofilm formation, immuno-modulation, and virulence factor production. The concept of treating noxious activities by interfering with signal pathways is commonly used in many endogenous diseases to treat the condition or alleviate symptoms, for instance Selective Serotonin Reuptake Inhibitors (SSRIs) are used to treat depression by increasing the level of serotonin in the synapses, and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are used to ease the pain in rheumatoid arthritis by counteracting COX-2's production of inflammatory prostaglandins. To reassign this concept and use it to treat bacterial infections is however, novel. Microbial diseases are traditionally defeated by the use of bactericidal or bacteriostatic agents although, as outlined previously, this strategy has major disadvantages due to the selective pressure for development of resistance and tolerance to the drugs used. Pharmaceuticals designed to interfere with bacterial communication can be developed based on structured design using known inhibitors or using the recently solved LBD of the LasR crystal. Another option is to screen natural libraries for antagonists of bacterial communication as described in articles 1 and 2.

Natural libraries are valuable in the development of novel drugs because they provide a vast array of bioactive compounds. And in many cases natural selection is superior to combinatorial chemistry for discovery of novel substances with drug potential. Natural sources are very rich on diverse secondary metabolites. Plants have been reported to produce more than 100,000 small-molecule compounds and this high diversity is -at least in part- caused by an evolutionary process driven by selection for improved defence against microbial attack or insect/animal predation (80). Another important feature of naturally derived drugs is that besides being biologically active, they have also evolved to function in the target organism. Antibiotics derived from natural sources (for example, aminoglycosides, tetracycline and chloramphenicol) are able to bypass the dual barrier-extrusion mechanism of Gram-negative bacteria, while synthetic compounds optimised to inhibit e.g. DNA gyrase may be excluded by the membrane or omitted by the Mex pumps and thereby fail.

Natural products are, on average, sterically more complex, with more rings and chiral centres, incorporate fewer nitrogen, halogen, or sulphur atoms but more oxygen atoms and have higher molecular weights than combinatorial compounds (132). Thus screening of natural libraries is a very strong alternative or supplement to combinatorial chemistry.

INTRODUCTION TO THE EXPERIMENTAL WORK

Article 1 entails a screening for quorum sensing inhibitors in a library consisting of microfungi. Crude extracts of different isolates of *Penicillium* sp. were tested for the presence of quorum sensing inhibitory compounds by the use of the bacterial screening systems Quorum Sensing Inhibitor Selector (QSIS) 1 and 2. Two mycotoxins, patulin and penicillic acid were identified as strong inhibitors of quorum sensing, and their effect on *P. aeruginosa* gene expression was further investigated by whole genome transcriptomics. This analysis revealed that quorum sensing inhibitory genes were highly overrepresented among the genes down-regulated by the two mycotoxins. Patulin was shown to enhance biofilm susceptibility to tobramycin treatment, moreover both patulin and penicillic acid allowed neutrophils to develop oxidative burst when exposed to *P. aeruginosa* biofilms, while the activation of neutrophils was hindered by the untreated wild-type. Patulin was also shown to function *in vivo*; in a mouse pulmonary infection model *P. aeruginosa* was cleared faster from the mice treated with patulin compared with the placebo group.

Article 2 describes the screening of 284 marine organisms collected at the Great Barrier Reef, Australia. Using the *luxI-luxR*-based QSIS1 system, extracts of marine organisms belonging to various taxonomic groups (algae, ascidians, hard coral, soft corals and sponges) were screened for the presence of quorum sensing inhibitors and 23 % of the extracts were found to be active. About half of the active extracts were also positive in the *lasB-lasR*-based QSIS2 system. Extracts of the sponge *Luffariella variabilis* proved active in both systems. The secondary metabolites manoalide, manoalide monoacetate and secomanoalide isolated from this sponge were tested using a quorum sensing regulated biomonitor system, and the three manoalides were found to have strong quorum sensing inhibitory properties. Interestingly, the manoalides, patulin, penicillic acid (in the predominant lactone form) and the halogenated furanones all share the 2,5-dihydro-2-furanone (or 2-buten-1,4-olide) structure suggesting that this substructure is important for their quorum sensing inhibitory properties e.g. via interaction with the LuxR(-homologue). Also protoanemonin, a toxic by-product formed during the biodegradation of some chloroaromatic compounds, e.g. polychlorinated biphenyls, contain the 2(5H)-furanone and exhibit tremendous quorum sensing inhibitory activity. (Article in preparation).

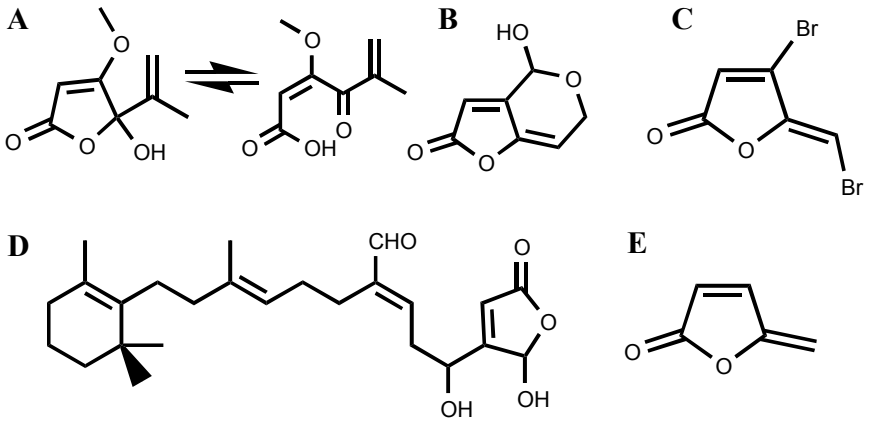


Figure 27. Quorum sensing inhibitors sharing the 2,5-dihydro furanone structure. **A**) Penicillic acid (lactone and acid in tautomeric equilibrium) **B**) Patulin **C**) C30 **D**) Secomanoalide **E**) Protoanemonin

The submitted manuscript (article 3) describes the screening of 12 antibiotics using the QSIS1 selector system. Three of the antibiotics screened; azithromycin (AZM), ceftazidime (CFT) and ciprofloxacin (CPR) exhibited strong quorum sensing inhibitory activities and were further studied by transcriptomics and phenotypic tests. These investigations revealed that the three

antibiotics efficiently down-regulated several quorum sensing regulated virulence factors of *P. aeruginosa*. However, in contrast to most other quorum sensing inhibitors identified until now, AZM, CFT and CPR most likely do not interact directly with LasR as *in silico* docking shows that the antibiotics have low affinity for the LBD of the LasR protein due to spatial effects. The transcriptome analysis suggests that the quorum sensing inhibitory properties of the investigated antibiotics at least in part is mediated by a change in membrane permeability influencing the OdDHL flux.

Most antibiotics developed until today are of natural origin or are derivatives of natural products. In the last 25 years (1981-2006) 64 out of the 109 antibacterials introduced to the market, have been based on natural compounds or derivatives thereof (254). This is also reflected in the three antibiotics investigated; AZM is a azalide derivative of erythromycin, a metabolic product of a *Streptomyces erythreus* strain (designation changed to "*Saccharopolyspora erythraea*") (407). Ceftazidime belongs to the group of cephalosporins originally purified from *Cephalosporium acremonium* (synonymous with *Acremonium chrysogenum*) (3). The fluoroquinolone ciprofloxacin is a derivative of nalidixic acid, a synthetic inhibitor of DNA gyrase, synthesised as a precursor of the antimalarial agent quinine. The knowledge that naturally occurring quinolones possess antibacterial properties, dates a long way back: In 1889 *P. aeruginosa* (at that time named *Bacillus pyocyaneus*) cultures were shown to prevent anthrax in rabbits, and later on in 1952 the active compounds responsible for the prophylactic effect were identified as quinolones (31, 76, 126, 409).

It has been widely accepted that the function of antibiotics in nature is as weapons targeted against competitors however, a recent article questions this assumption (199). Using a focused subgenomic array (555 genes) and phenotypic tests, the authors demonstrated that tobramycin, tetracycline, and norfloxacin (structurally very similar to ciprofloxacin) applied in subMIC concentrations activated expression of virulence determinants in *P. aeruginosa,* for instance expression of the type III secretion system (TTSS) was induced by the presence of tetracycline. These findings prompted the authors to suggest that the ecological role of antibiotics are not only to slay competitors but also - in low concentrations – to function as signalling molecules regulating the homeostasis of microbial communities (199), and the reverse may also be the case: As mentioned, *P. aeruginosa* quinolones have been reported to have antibacterial activity as well as being involved in quorum sensing. It is thus likely that antibiotics may, beside their traditional bactericidal applications, have the potential to be used to regulate cell to cell communication in bacteria.

While a range of compounds produced by eukaryotic organisms such as the mycotoxins patulin and penicillic acid, as well as the three manoalide compounds produced by the sponge *L. variabilis* seem to interact with quorum sensing in *P. aeruginosa* there is also evidence that the communication goes the other way round. The final manuscript (article 4) describes the effect of *P. aeruginosa* quorum sensing signal molecules on the development and maturation of stimulated dendritic cells. Interestingly it seems that PQS and especially OdDHL are able to change the pro-inflammatory T_H1-directing response elicited by the dendritic cells to an anti-inflammatory T_H2-directing response. Other studies have also shown that *P. aeruginosa* quorum sensing signal molecules possess the ability to shape cellular processes – including activities of the immune defence – of eukaryotic cells.

Thus to sum up; *P. aeruginosa* applies quorum sensing signal molecules to control virulence factors important for its infectious capacity, including rhamnolipids capable of lysing phagocytes and enzymes with host tissue and complement degrading activities. These signal molecules also seem to be capable of directly changing the immune response of the host, while host factors such as INF γ affect *P. aeruginosa* quorum sensing. The key to successful control may lie in the obstruction of the capability of this pathogen to sense, modulate and cripple the immune defense. As outlined in articles 1 and 2, eukaryotes such as fungi and marine organisms produce compounds which interfere with bacterial cell-to-cell signalling. These quorum sensing inhibitory compounds have the potential to be used to target *P. aeruginosa* virulence and thereby decrease tissue and host cell destruction as well as the quorum sensing signal molecule mediated change of the host immune response.

Thus the ability of *P. aeruginosa* to respond to host factors and manipulate actions of the immune response may contribute to the success of this pathogen. At the same time eukaryotic organisms may offer a solution for overcoming *P. aeruginosa* infections by providing us with inhibitors of bacterial communication.

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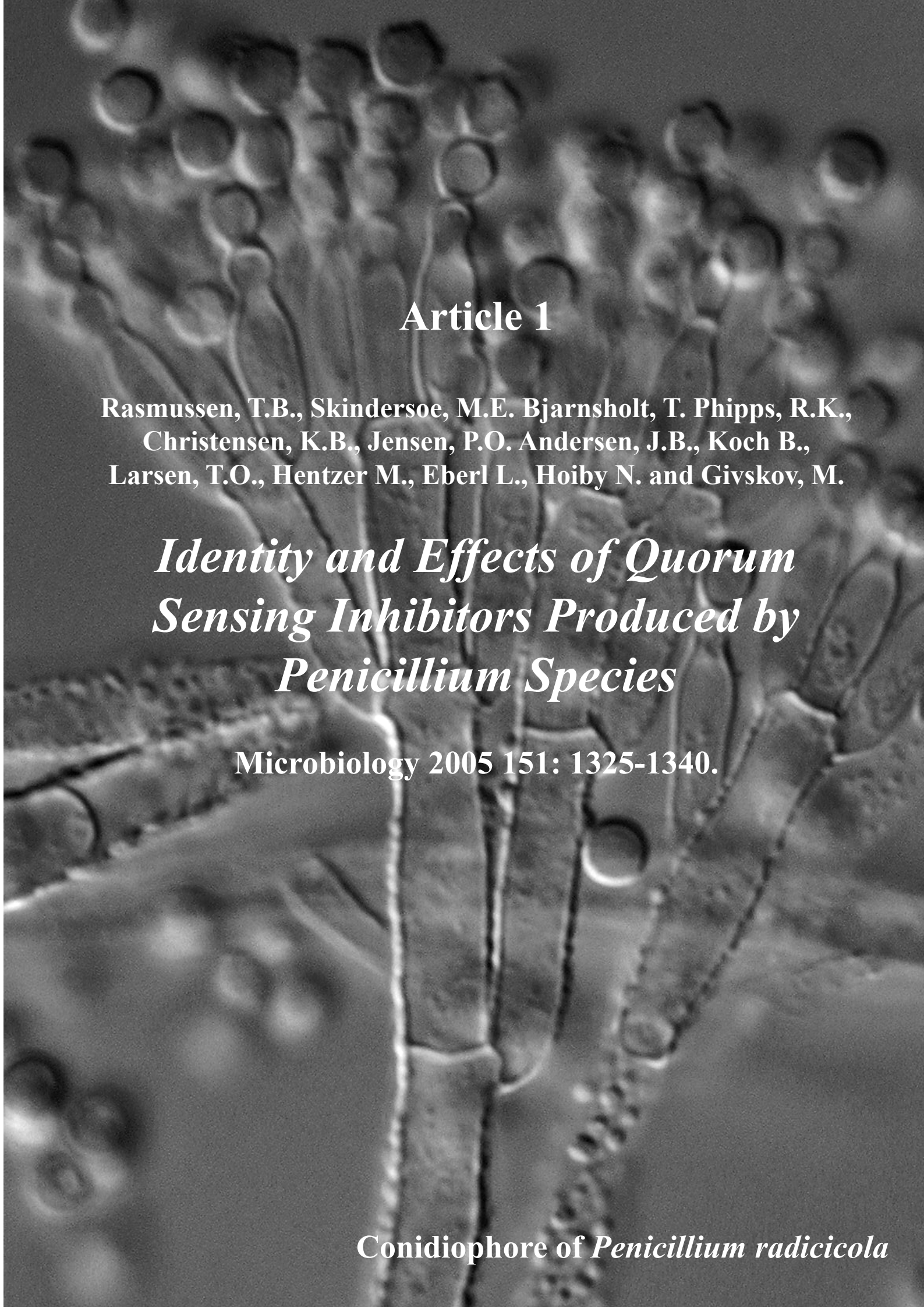

Article 1

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Identity and Effects of Quorum Sensing Inhibitors Produced by Penicillium Species

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Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species

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Quorum sensing (QS) communication systems are thought to afford bacteria with a mechanism to strategically cause disease. One example is *Pseudomonas aeruginosa*, which infects immunocompromised individuals such as cystic fibrosis patients. The authors have previously documented that blockage of the QS systems not only attenuates *Ps. aeruginosa* but also renders biofilms highly susceptible to treatment with conventional antibiotics. Filamentous fungi produce a battery of secondary metabolites, some of which are already in clinical use as antimicrobial drugs. Fungi coexist with bacteria but lack active immune systems, so instead rely on chemical defence mechanisms. It was speculated that some of these secondary metabolites could interfere with bacterial QS communication. During a screening of 100 extracts from 50 *Penicillium* species, 33 were found to produce QS inhibitory (QSI) compounds. In two cases, patulin and penicillic acid were identified as being biologically active QSI compounds. Their effect on QS-controlled gene expression in *Ps. aeruginosa* was verified by DNA microarray transcriptomics. Similar to previously investigated QSI compounds, patulin was found to enhance biofilm susceptibility to tobramycin treatment. *Ps. aeruginosa* has developed QS-dependent mechanisms that block development of the oxidative burst in PMN neutrophils. Accordingly, when the bacteria were treated with either patulin or penicillic acid, the neutrophils became activated. In a mouse pulmonary infection model, *Ps. aeruginosa* was more rapidly cleared from the mice that were treated with patulin compared with the placebo group.

INTRODUCTION

The 20th century initially offered the promising prospect of penicillin and other antibiotics to fight bacterial infections, but ended with the gloomy scenario of emerging multi-resistant bacteria. The efficiency of conventional antibiotics in preventing bacterial proliferation is the source of their success, but at the same time is also the cause of their failure. In many cases, selective pressure imposed by the use of conventional antibiotics leads to increased expression of degrading enzymes and development of drug-efflux systems which operate with increased efficiency and therefore actively reduce the internal concentration of the antibiotics. Furthermore, research over the last two decades has revealed that bacteria in the biofilm mode exhibit a higher tolerance to antimicrobial treatments (Anwar *et al.*, 1990). The biofilm mode of growth also protects opportunistic pathogens, such as *Pseudomonas aeruginosa*, against the action of the host immune system, which in turn facilitates establishment of chronic infections (Costerton *et al.*, 1999).

Abbreviations: AHL, *N*-acylated homoserine lactone; 123-DHR, dihydrorhodamine 123; GFP, green fluorescent protein; PMN, polymorphonuclear leukocyte; QS, quorum sensing; QSI, QS inhibitor(y).

The prospect of the 21st century as a post-antibiotic era highlights the importance of novel strategies to control bacterial diseases (Camara *et al.*, 2002). Many Gram-negative bacteria utilize *N*-acylated homoserine lactones (AHLs) to coordinate expression of virulence in response to the density of the surrounding bacterial population in a process termed quorum sensing (QS). The AHL molecules are produced by LuxI homologues and constitute, in complex with LuxR homologues, transcriptional regulators. AHLs consist of a conserved homoserine lactone ring with a variable *N*-acyl chain. The predominant AHL variations include presence or absence of a keto or hydroxy group on the C-3 carbon atom, as well as the length and saturation of this chain (Fuqua *et al.*, 1994; Salmond *et al.*, 1995). A number of recent publications have pointed to QS as a new drug target. Fighting bacteria by interfering with their command language and thereby disrupting virulence expression instead of inhibiting growth could serve as an alternative to the conventional ways of combating bacterial infections (Finch *et al.*, 1998; Habeck, 2003; Smith & Iglewski, 2003; Hentzer & Givskov, 2003). The strategy would be based on small molecules with variations in their chemical composition that would allow them to block the AHL receptor site of the LuxR homologues or alternatively block the formation of active dimers that are required for binding to and expression of target genes. A number of studies have identified several molecules that function as QS inhibitors (QSI) (Smith *et al.*, 2003a, b; Reverchon *et al.*, 2002; Olsen *et al.*, 2002; Hentzer & Givskov, 2003). Much effort has been spent on synthesis of AHL analogues which antagonize the cognate signal molecules. Varying the length of the acyl side chain was found to be important; for example AHLs with extended side chains generally caused inhibition of the LuxR homologues (Chhabra *et al.*, 1993; Passador *et al.*, 1996; Schaefer *et al.*, 1996; Zhu *et al.*, 1998). Other modifications to the AHLs included alteration of the acyl chain by introducing ramified alkyl, cycloalkyl or aryl/phenyl substituents at the C-4 position, resulting in both inducers (analogues with non-aromatic substitutions) and antagonists (analogues with phenyl substitutions) (Reverchon *et al.*, 2002). Modification of the lactone ring of AHLs by adding substituents to C-3 or C-4 did not give rise to strong QSI activity (Olsen *et al.*, 2002). However, exchanging the homoserine ring with a five- or six-membered alchohol or ketone ring, Smith *et al.* (2003a, b) generated a number of activators and inhibitors, some of which blocked of *Ps. aeruginosa* QS *in vitro*. Their target specificity for the QS regulon was not verified by transcriptomics.

In nature, eukaryotes live closely associated with virulent prokaryotes. This has forced mammals to evolve different defence systems. Plants and fungi, however, do not possess active immune systems; instead they have to rely on physical and chemical defences. A well-studied example of this is the production of halogenated furanone compounds by the Australian alga *Delisea pulchra* (Givskov *et al.*, 1996). This species produces the compounds in the central vesicle of gland cells, from which they are released to the surface of the plant (Dworjanyn & Steinberg, 1999), where they prevent extensive surface growth by bacteria and higher fouling organisms (Steinberg *et al.*, 1997; Maximilien *et al.*, 1998). The halogenated furanones have been shown to inhibit several QS-controlled phenotypes, including swarming motility of *Serratia liquefaciens*, toxin production of *Vibrio harveyi* and bioluminescence of *Vibrio fischeri* (Givskov *et al.*, 1996; Kjelleberg *et al.*, 1997; Manefield *et al.*, 2000; Rasmussen *et al.*, 2000). In a more clinical context, a synthetic derivative of the furanones (C-30) was found to downregulate expression of more than 80 % of the QS-regulated genes found in *Ps. aeruginosa*, many of which encode known virulence factors. This effect is not limited to planktonic bacteria: it also applies to biofilm-dwelling *Ps. aeruginosa*. Biofilms developed in the presence of furanone compounds become more susceptible to treatments with antibiotics and disinfectants (Hentzer *et al.*, 2002, 2003). This is highly interesting given that *Ps. aeruginosa* is an opportunistic pathogen often found in people with compromised immune systems, such as cystic fibrosis patients, where it is responsible for persistent, chronic infections probably caused by biofilm formation within the host (Hoiby & Koch, 2000; Hoiby, 2000; Van Delden & Iglewski, 1998). Attenuating this bacterium with respect to virulence and persistence is undoubtedly desirable; the first proof of this concept was delivered by Hentzer *et al.* (2003), who were able to attenuate and eradicate bacteria colonizing mouse lungs.

In the present study, selected members of the filamentous fungal genus *Penicillium* were investigated for production of QSI activity. Microfungi produce a huge variety of secondary metabolites, including complex molecules such as alkaloids and polyketides. Some of these are mycotoxins, compounds which can cause disease and sickness in vertebrates at fairly low concentrations. The β-lactam antibiotics, which include penicillin G and cephalosporin, were originally isolated from *Penicillium* species. The immunosuppressants cyclosporin and tacrolimus used in connection with organ transplants also originate as fungal metabolites. The ability to produce a wide variety of bioactive compounds makes the microfungi obvious candidates to screen for QSI products. Using a recently published screening system consisting of bacteria that will grow only when the growth medium is supplemented with a QSI compound (Rasmussen *et al.*, 2005), extracts of 50 members of the genus *Penicillium* have been screened. Several fungi were found to produce QSI activities and two of the compounds were identified and examined for their specificity for the *Ps. aeruginosa* QS regulon, and their effects on both biofilm tolerance to antimicrobial treatments and pulmonary infections.

METHODS

Bacterial strains. The two QSI selector strains, QSIS1 (Ap^r, *luxI–phlA*) and QSIS2 (Ap^r Gm^r, *lasB–sacB*) were described by Rasmussen

et al. (2005). We also used a quorum-sensing reporter strain such as *Ps. aeruginosa* PAO1 harbouring a *lasB–gfp* fusion (Hentzer *et al.*, 2002). The *Ps. aeruginosa* PAO1 used for the *in vitro* experiments was obtained from the *Pseudomonas* Genetic Stock Center (www.pseudomonas.med.ecu.edu, strain PAO0001). This PAO1 isolate has served as DNA source for the *Pseudomonas* Genome Project (www.pseudomonas.com) and, subsequently, as template for design of the *Ps. aeruginosa* GeneChip (Affymetrix, Inc.). The Δ*lasI rhlI* mutant and the Δ*lasR rhlR* mutant were constructed using previously described knock-out systems (Beatson *et al.*, 2002a, b). The knock-out mutants were verified by Southern blot analysis and by screening for absence of AHL production. When green fluorescent protein (GFP) was employed as a bacterial tag, we used a constitutively expressed stable GFP version encoded by a gene present on the plasmid pMRP9 (Davies *et al.*, 1998).

The *Ps. aeruginosa* used for *in vivo* experiments was obtained from B. Iglewski, University of Rochester, Medical Center, Rochester, NY, USA. The Δ*lasR rhlR* mutant of the Iglewski PAO1 was constructed using the same knock-out systems as for PAO0001 (Beatson *et al.*, 2002a, b). The knock-out mutants were verified by Southern blot analysis and by screening for absence of AHL production.

Growth medium and conditions. ABT minimal medium (AB medium of Clark & Maaløe, 1967, plus 2·5 mg thiamine l^{-1}) supplemented with 0·5 % glucose and 0·5 % Casamino acids was used. Media were supplemented with antibiotics where appropriate, and unless stated otherwise, all strains were incubated at 30 °C.

Fungal strains. Fungal isolates (Table 1) were obtained from the IBT Culture Collection at BioCentrum-DTU, Technical University of Denmark. The cultures were inoculated in triplicate on Czapek yeast (autolysate) agar (CYA) (Pritt, 1979) and yeast extract sucrose agar (YES) (Frisvad & Filtenborg, 1983) and incubated at 25 °C for 7 days in the dark.

Preparation of fungal extracts. Procedures for micro-extraction of fungal secondary metabolites were similar to those given by Smedsgaard (1997) with the following modifications. Plug extracts were prepared from nine plugs and extracted with 2 ml ethyl acetate/methylene chloride/methanol (3 : 2 : 1, by vol.) containing 50 p.p.m. trifluoroacetic acid.

Screening for QSIs. The screening assays using QSIS1 and QSIS2 were performed as described by Rasmussen *et al.* (2005). QSIS1 is an *E. coli lac*$^+$ strain harbouring pTBR2iB, which encodes an AHL-induced killing system. QSIS2 is a *Ps. aeruginosa lasI rhlI* double mutant harbouring pLasB-SacB1, encoding an AHL-induced killing system; this strain also harbours pSU2007, encoding constitutively expressed *luxCDABE*, giving rise to bioluminescence (Rasmussen *et al.*, 2005).

Microfractionation of extracts. An Agilent 1100 series HPLC with fraction collector coupled to a G2250A Micro plate sampler was used for microfractionation. Samples were fractionated on a Phenomenex Luna II C_{18} column (100 mm × 4·6 mm, 5 μm) at 40 °C with a flow rate of 1 ml min^{-1} and the following H_2O/CH_3CN solvent gradient: 2 min at 10 % CH_3CN/H_2O; a linear gradient to 70 % CH_3CN/H_2O for 12 min; isocratic at 70 % for another 10 min; a linear gradient for 2 min to 100 % CH_3CN, followed by isocratic at 100 % CH_3CN for 4 min then returned to 10 % CH_3CN/H_2O in 2 min and re-equilibrated for 8 min. Solvents were HPLC-grade acetonitrile and MilliQ water, both with 50 p.p.m. trifluoroacetic acid added. HPLC eluate was collected into Nunculon polystyrene microtitre plates (Nunc) for HPLC samples containing less than 70 % acetonitrile. Solvent-resistant U96 PP 0·5 ml polypropylene plates (Nunc) were used for collection of HPLC eluate containing more than 70 % acetonitrile.

Identification of active compounds by LC-DAD-MS. Fungal metabolites were analysed using an Agilent HP 1100 liquid chromatograph with a DAD system (Waldbronn) coupled to an LCT oaTOF mass spectrometer (Micromass) using a Z-spray ESI source and a Lock Spray probe. A Phenomenex Luna II C_{18} column (100 mm × 2 mm, 3 μm) at 40 °C and a flow rate of 0·3 ml min^{-1} was used for separation. Solvents were HPLC-grade acetonitrile and MilliQ water. The water was buffered with 10 mM ammonium formate (analytical grade) and 20 mM formic acid, and the acetonitrile with 20 mM formic acid.

Dose response. To establish a dose–response relationship of patulin and penicillic acid, dilution rows were made with growth medium (ABT with 0·5 % Casamino acids) in a microtitre dish. Each well contained 100 μl diluted QSI and 200 μl of a 1 : 100 diluted overnight culture of PAO1 *lasB-gfp*(ASV) (Hentzer *et al.*, 2002), grown in ABT with 0·5 % Casamino acids, was added. Growth was monitored as OD_{450} over a time-course of 14 h, and GFP expression was measured at 515 nm.

DNA array analysis. ABT minimal medium supplemented with 0·5 % Casamino acids (200 ml) was inoculated with exponentially growing *Ps. aeruginosa* PAO1 cells ($OD_{600} < 0·5$) at an OD_{600} of 0·05. At an OD_{600} of 0·7, the culture was split into two 100 ml cultures, which were grown on in 500 ml conical flasks on an orbital air shaker operating at 200 r.p.m. at 37 °C. To one culture QSI compound was added (at a non-growth-inhibitory concentration); the second culture served as an untreated control. Patulin was added to 8 μM and penicillic acid was added to 147 μM, with both treated and untreated cultures showing similar growth rates. Samples were retrieved at OD_{600} 2·0, mixed with 2 vols RNA Later (Ambion) and stored at −80 °C until RNA extraction. RNA extraction was performed with the Qiagen RNeasy Purification kit, using the bacterial protocol. To remove all DNA, the purified RNA was treated for 1 h with 11 U DNase I; RNA was then retrieved by using the Qiagen RNeasy Purification kit. cDNA was synthesized by mixing 10 μg RNA with 250 ng random primers (Invitrogen Life Technologies) in a total volume of 30 μl. The rest of the assay was performed according to the protocol supplied by Affymetrix.

Preparation of polymorphonuclear leukocytes (PMNs). Human blood samples were obtained from normal healthy volunteers by venous puncture, and collected in BD Vacutainers coated with heparin and lithium (Becton-Dickinson, 388330). The blood was mixed with dextran (T-500), 1 : 5, and the erythrocytes were sedimented for 40 min. The supernatant was applied to Lymphoprep (Axis-Shield Poc.) and centrifuged at 800 ***g*** for 15 min at 5 °C. The supernatant was discarded and the PMN neutrophils were treated with 2 ml 0·2 % NaCl in order to lyse remaining erythrocytes. Lysis was terminated by adding 2 ml 1·6 % NaCl and 6 ml Eagle-MEM (Bie & Berntsen). The cells were centrifuged at 350 ***g*** for 10 min at 5 °C, the supernatant was discarded and the PMNs were resuspended in Eagle-MEM.

Biofilm assays. Biofilms were grown in continuous culture, once-through flow chambers, perfused with sterile ABtrace minimal medium containing 0·3 mM glucose. Patulin (1·2 μg ml^{-1}) or penicillic acid (1·2 μg ml^{-1}) was added to the medium when appropriate. Biofilm development was examined by confocal scanning laser microscopy (CSLM) using a Zeiss LSM 510 system (Carl Zeiss) equipped with an argon laser and a helium-neon laser for excitation of fluorophores. Bacterial viability in biofilm cultures was assessed by using the BacLight live/dead staining kit (Molecular Probes) as described elsewhere (Huber *et al.*, 2001).

PMN treatment of biofilms. In order to inoculate PMNs into the biofilm chambers, the flow was stopped and the flow cells were

clamped off. PMNs in the order of $1{\cdot}5 \times 10^6$ were inoculated in each flow channel. The flow cells were incubated top down in a 37 °C water bath with shaking, until microscopic inspection.

Monitoring the oxidative burst of PMNs. Isolated PMNs were incubated for approximately 30 min in Eagle-MEM (3×10^7 cells ml^{-1}) with 10 % normal human AB serum, 5 μM SYTO 62 (Molecular Probes) to stain the nuclei (dsDNA). For detecting the oxidative burst of the PMNs 0·1 mg dihydrorhodamine 123 (123-DHR) ml^{-1} (D-1054, Sigma) was added to the PMNs in order to stain the H_2O_2 in the phagosomes (Bassoe *et al.*, 2003). One hundred microlitres of the PMN mixture was added to each biofilm.

Experimental animals. Female BALB/c mice were purchased from M&B Laboratory Animals at 10–11 weeks of age. The mice were of equal size and were maintained on standard mouse chow and water *ad libitum* for 1 week before challenge. All animal experiments were authorized by the National Animal Ethics Committee.

Immobilization of *Ps. aeruginosa* in seaweed alginate beads. *Ps. aeruginosa* was immobilized in seaweed alginate beads as described by Pedersen *et al.* (1990). The suspension was adjusted to $2{\cdot}5 \times 10^8$ c.f.u. ml^{-1} and confirmed by colony counts; 0·04 ml of the suspension was inoculated into the left lung of each mouse.

Challenge procedure. The mice were anaesthetized by subcutaneous injection of 0·2 ml Hyp/Mid [2·5 mg Hypnorm ml^{-1} (Janssen) and 1·25 mg midazolam ml^{-1} (Roche) in sterile water]. Sedated mice were immobilized and the trachea was exposed and penetrated with an 18G needle. The inoculum was introduced into the left lung approximately 11 mm from the tracheal penetration site with a bead-tipped needle (Moser *et al.*, 1997). The incision was sutured with silk and healed without any complications. Pentobarbital (DAK), 2·0 ml per kg body weight, was used to kill the animals, 5 days after infection.

Bacteriology. Lungs from mice were prepared for bacteriological examination as described by Moser *et al.* (1997). Isolated lungs were

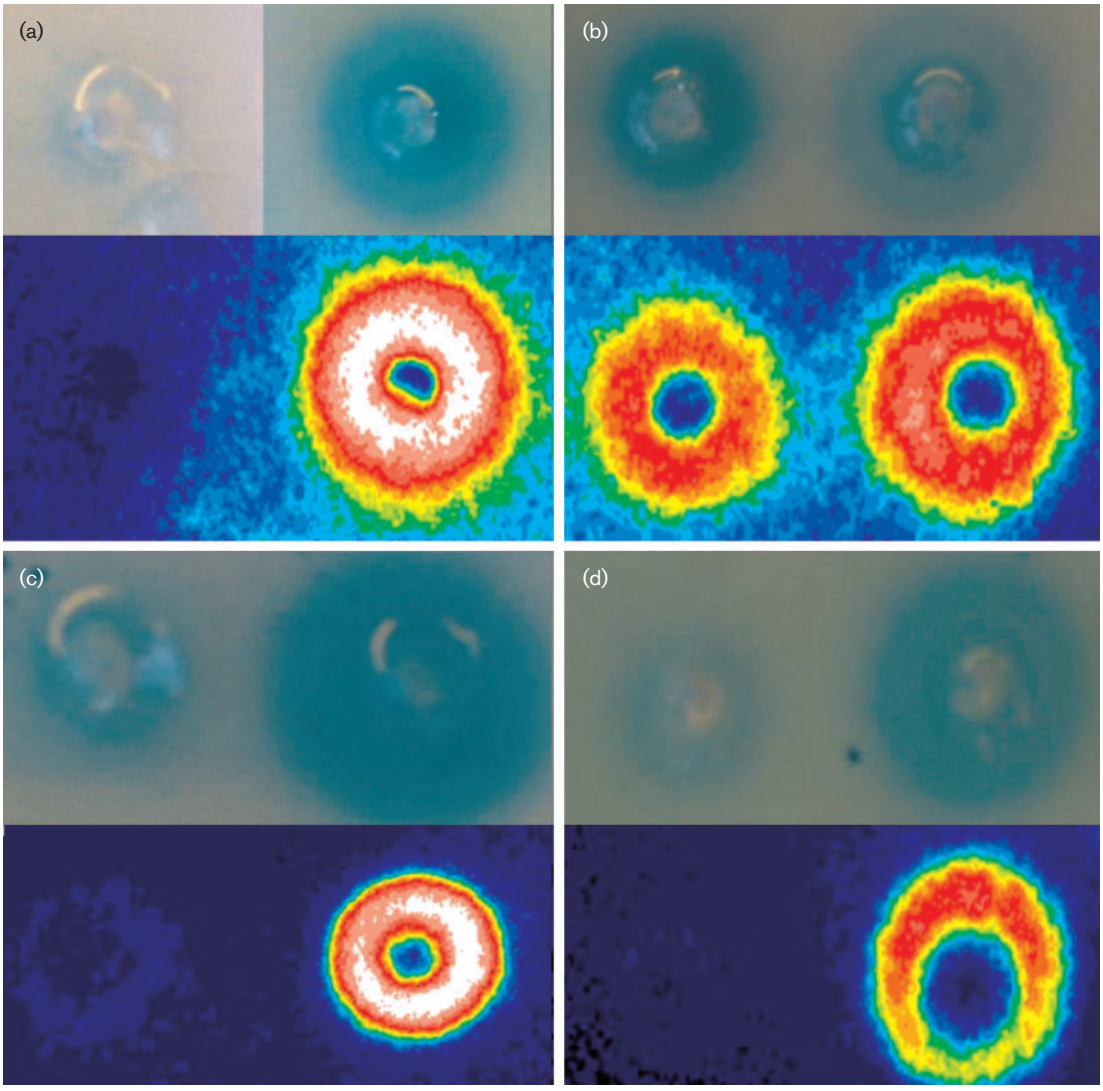

Fig. 1. QSIS1 (top rows) and QSIS2 (bottom rows) assays on extracts from four *Penicillium* spp.: (a) *P. olsonii*, (b) *P. roqueforti*, (c) *P. hordei* and (d) *P. carneum*. The fungi were grown on two media, CYA (left columns) and YES (right columns). For QSIS1, a blue ring indicates presence of QSI compound(s). QSIS2 indicates presence of QSI by emitting light visualized by a photosensitive CCD camera.

homogenized on ice. A serial dilution of the lung homogenate was performed and plated on blue agar plates (Statens Serum Institut, Copenhagen, Denmark), selective for Gram-negative bacilli, for colony counting (Hoiby, 1974).

RESULTS AND DISCUSSION

Screening of *Penicillium* extracts

Extracts of the fungi (Table 1) were prepared as described in Methods. As an initial, promiscuous screen, we employed the QSIS1 selector, which identifies inhibitors of the *lux* QS system. The selector bacterium harbours a QS-controlled gene that causes cell death when expressed. In the assay, the QSIS bacteria are cast into agar along with signal molecules that activate the killing gene. A well is made in the agar in which the test sample is placed. The sample diffuses into the agar from the well, creating a concentration gradient allowing for screening of several concentrations of the sample. Presence of a QSI is signalled by growth of the selector bacteria; therefore only non-toxic compounds are identified. The QSIS2 selector was employed as a second screen for inhibitors of the *Ps. aeruginosa* QS systems. It is based on a QS-controlled *sacB* gene which leads to cell death when expressed in the presence of sucrose (Rasmussen *et al.*, 2005). By means of these two screens, a number of QSI-producing *Penicillium* species were identified (Fig. 1, Table 1).

Fractionation of extracts and identification of active compounds

In order to identify the individual fungal metabolites inhibiting the QS systems of *Ps. aeruginosa*, *Penicillium radicicola* (IBT 10696) and *Penicillium coprobium* (IBT 6895) were selected for further studies using microfractionation and LC-DAD-MS. QSIS1 screening of IBT 6895 showed a single active well in the microtitre plate assay of the collected fractions (Fig. 2), whereas IBT 10696 extract resulted in activity in five wells (data not shown).

The corresponding UV data from the microfractionation samples suggested that the QSI compounds from *P. radicicola* and *P. coprobium* were penicillic acid and patulin respectively (Fig. 3). This was confirmed by LC-DAD-MS analysis of the remaining contents of the active fractions and by comparison with authentic samples. This is believed to be the first report of these two well-known fungal metabolites (Frisvad *et al.*, 2004) acting as QSI compounds.

The identification and presence of these two compounds

Table 1. Fungal extracts tested for QSI content

+, QSI activity, −, no activity. Extracts were prepared from fungi cultured on CYA and YES media (see Methods).

Penicillium species	IBT no.	CYA	YES	*Penicillium* species	IBT no.	CYA	YES
P. aethiopicum	21721	−	−	*P. gladioli*	14699	−	−
P. albocoremium	22521	−	−	*P. griseofulvum*	21530	−	−
P. allii	21503	−	−	*P. herqui*	24483	−	+
P. atramentosum	11801	−	−	*P. hirsutum*	10623	+	+
P. aurantiogriseum	22509	−	−	*P. hordei*	15999	−	+
P. brasilianum	12951	−	−	*P. italicum*	21533	−	+
P. brevicompactum	4342	−	−	*P. janczewskii*	16238	−	−
P. carlsbadiense	18341	−	−	*P. johanii*	24550	−	−
P. carneum	19478	−	+	*P. manginii*	19538	−	−
P. chrysogenum	14462	−	+	*P. miczynskii*	14839	+	+
P. clavigerum	18977	−	+	*P. nalgiovense*	15040	+	−
P. commune	17345	−	−	*P. neoclavigerum*	19355	−	+
P. concentricum	5623	−	−	*P. neocoprobium*	24375	−	−
P. coprobium	6895	+	+	*P. neogriseofulvum*	16848	−	−
P. coprophilum	5551	−	+	*P. neovulpinum*	18360	−	+
P. decumbens	21919	−	−	*P. nordicum*	22024	−	+
P. digitatum	13068	−	−	*P. novae-zelandiae*	21935	−	−
P. dipodomyicola	16571	−	+	*P. olsonii*	21538	−	+
P. dipodomyis	17759	+	+	*P. paneum*	11839	+	+
P. discolor	21523	−	−	*P. paxilli*	16202	−	+
P. echinulatum	7000	−	−	*P. radicicola*	10696	−	+
P. marinum	16712	−	−	*P. roqueforti*	21543	+	+
P. expansum	21525	−	+	*P. smithii*	6647	−	+
P. fennelliae	19338	+	+	*P. tulipae*	3458	−	−
P. glabrum	17691	+	−	*P. venetum*	21549	−	+

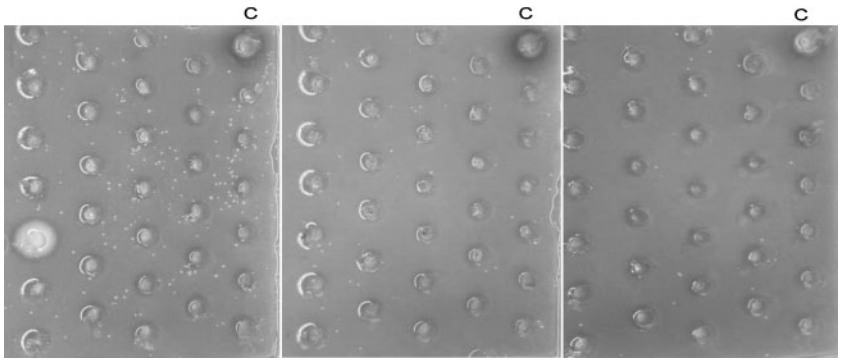

Fig. 2. Screening of 96 fractions of *P. coprobium* (IBT6895) extract. The wells marked C contain a positive control (furanone C-30).

probably explains why several of the other species also showed QSI activity. Recently the following terverticillate *Penicillium* species were reported as penicillic acid producers: *P. aurantiogriseum*, *P. carneum*, *P. cyclopium*, *P. freii*, *P. melanoconidium*, *P. neoechinulatum*, *P. polonicum*, *P. radicicola*, *P. tulipae*, *P. viridicatum*. The following species were reported as patulin producers: *P. carneum*, *P. clavigerum*, *P. concentricum*, *P. coprobium*, *P. dipodomyicola*, *P. expansum*, *P. glandicola*, *P. gladioli*, *P. griseofulvum*, *P. marinum*, *P. paneum*, *P. sclerotigenum*, *P. vulpinum* (Frisvad *et al.*, 2004). However, extracts prepared from some of these fungi did not appear active in the QSI screens used in the present study. One likely explanation is that some of the fungal strains produce only trace amounts of either patulin or penicillic acid, which is therefore below the detection limit of the QSI screen. The search for compounds other than patulin and penicillic acid is in progress on other fungal species known as non- producers of patulin and penicillic acid.

Dose–response analysis of penicillic acid and patulin

Pure penicillic acid and patulin were tested in the QSIS1 and QSIS2 systems and found to be positive (data not shown). To examine the effects of these two fungal metabolites more closely, we employed a *lasB–gfp*(ASV) fusion harboured by *Ps. aeruginosa* PAO1 as a QS monitor (Hentzer *et al.*, 2002). Being a type IV QS gene, *lasB* is induced in late exponential/early stationary phase (Whiteley *et al.*, 1999). Induction by the addition of C4 HSL and 3-oxo-C12 HSL caused a burst of GFP production. Conversely, if a QSI is present the burst of GFP expression will be reduced (Hentzer *et al.*, 2003). As the QSIS systems only provide a crude estimation of the optimal concentration of the QSI, a dilution series of the compounds was incubated with the QS monitor. Growth and green fluorescence were recorded over a period of 14 h. Upon induction, the untreated cultures increased GFP production approximately 10-fold. Addition of either penicillic acid or patulin reduced the induction of fluorescence in a concentration-dependent manner. At the highest concentrations tested (40 μM and 80 μM for patulin and penicillic acid, respectively) the induction of the QS-controlled *lasB* promoter was abolished. These concentrations did not affect growth rate of the cultures (Fig. 4). This suggests that the two compounds specifically block bacterial QS.

Definition of the QS regulon

The QS regulon was previously identified by means of DNA microarray analysis of gene expression (Hentzer *et al.*, 2003). Briefly, the QS regulon was defined as genes induced by the addition of C4 HSL and 3-oxo-C12 HSL to a *lasI rhlI* double mutant. It is possible, however, that the presence of high, exogenous AHL concentrations during the entire growth cycle could influence the expression profile of genes in a non-natural manner, which would then affect mapping of the QS regulon. To pursue this particular problem we chose to define the QS regulon as consistently down-regulated (> 5-fold) genes in both a *lasI rhlI* and a *lasR rhlR* mutant as compared to their parent wild-type (Table 2).

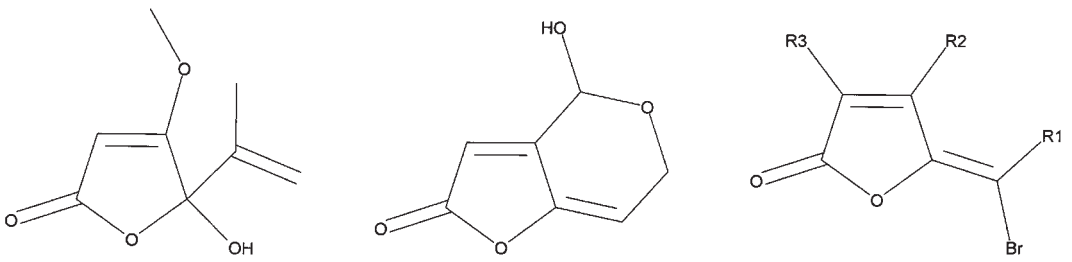

Fig. 3. Structures of penicillic acid (left), patulin (middle) and a general *Delisea pulchra* furanone structure (right).

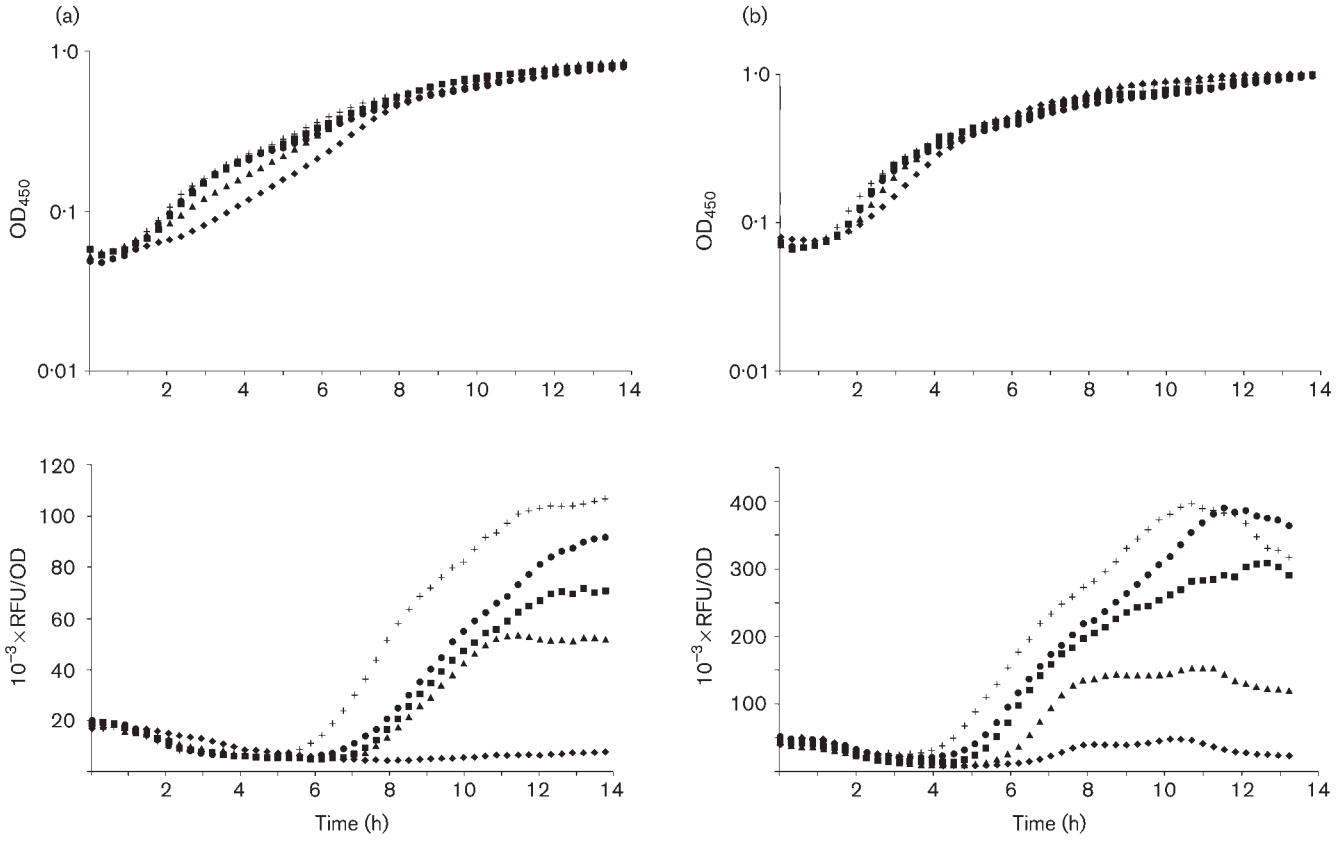

Fig. 4. Dose–response curves of patulin (a) and penicillic acid (b). Dilution series were incubated with the QS monitor PAO1 (*lasB–gfp*) (Hentzer *et al.*, 2002). Growth (top panels) and specific fluorescence (bottom panels, expressed as relative fluorescence units per OD_{450} unit) were followed over 14 h. Patulin was tested at 40 μM (◆), 10 μM (▲), 3 μM (■), 0·6 μM (●) and 0 μM (+). Penicillic acid was tested at 80 μM (◆), 20 μM (▲), 5 μM (■), 1 μM (●) and 0 μM (+).

In addition, the expression profiles of a *lasR* and a *rhlR* mutant were determined (Table 2). The 172 genes of this alternative QS regulon can be divided into four groups on the basis of their expression pattern within the four mutants. Group A contains 34 genes which require *lasR* for expression, i.e. they are downregulated in both the *lasR* and the *lasR rhlR* mutants. The 23 genes responsive to *rhlR* are located in group B. Group C consists of 92 genes which are downregulated in all three mutants. Finally, group D comprises the 23 genes that are downregulated only in the *lasR rhlR* double mutant; if only one of *lasR* or *rhlR* was mutated, no effect was observed. Remarkably, the genes in group D generally exhibited a lower degree of regulation (less average fold change) than those in group C. We speculate that the group D genes are only partially regulated by QS. Of the 172 genes in the alternative QS regulon, 99 are in common with the previous QS regulon defined by Hentzer *et al.* (2003). It must be emphasized that the previous QS regulon was based on several sample points throughout the growth cycle whereas the present QS regulon was based on a single sample point at OD_{600} 2, where the majority of QS genes are being expressed (Hentzer *et al.*, 2003).

Target specificity

Next, DNA microarray analysis was performed to determine the target specificity of the two QSI compounds towards the QS-regulated genes in *Ps. aeruginosa*. Cultures of *Ps. aeruginosa* PAO1 were grown to exponential phase, then split into subcultures at OD_{600} 0·5. These cultures were either not treated or treated with 8 μM patulin and 147 μM penicillic acid, respectively (concentrations at which growth was not affected). Samples for DNA microarray analysis were taken at OD_{600} 2·0. Absolute expression values from treated cultures or QS mutant cultures were compared with values from untreated cultures; changes in expression are reported as simple fold changes (Table 2). Genes found 'not present' by the Affymetrix Micro Array Suite software were excluded from the calculations. According to Hentzer *et al.* (2003) and Wagner *et al.* (2003) changes in expression below 5-fold are disregarded.

In total, patulin and penicillic acid targeted 157 and 300 genes, respectively. According to the above QS definition, 49 % of the 157 genes targeted by patulin and 34 % of the 300 targeted by penicillic acid were QS controlled. Since the number of chromosomal genes is approximately 5600,

Table 2. Genes downregulated in the four QS mutants and by treatment with patulin (Pat) and penicillic acid (PA)

Pa gene	Gene	Regulation*	Product name†	Change in expression (fold)‡					
				Pat	PA	*lasR*	*rhlR*	*lasR rhlR*	*lasI rhlI*§
PA0059	*osmC*	*las*+*rhl*	Osmotically inducible protein OsmC	**–8**	**–18**	**–23**	**–26**	**–32**	**–22**
PA0105	*coxB*	*las*+*rhl*	Cytochrome *c* oxidase, subunit II	**–9**	**–86**	**–10**	**–8**	**–14**	**–12**
PA0107		*las*+*rhl*	Conserved hypothetical protein	**–13**	**–18**	**–6**	**–11**	**–14**	**–17**
PA0108	*coIII*	*las*+*rhl*	Cytochrome *c* oxidase, subunit III	**–12**	**–14**	**–5**	**–6**	**–8**	**–28**
PA0122		*las*+*rhl*	Conserved hypothetical protein	–4	**–11**	**–6**	**–6**	**–26**	**–45**
PA0188		*las*+*rhl*	Hypothetical protein	–3	–3	**–13**	**–9**	**–21**	**–8**
PA0355	*pfpI*	*las*+*rhl*	Protease PfpI	–2	–4	**–14**	**–12**	**–22**	**–12**
PA0567		*las*+*rhl*	Conserved hypothetical protein	–4	**–8**	**–17**	**–10**	**–25**	**–250**
PA0572		*las*	Hypothetical protein	–1	–2	**–8**	–1	**–14**	**–10**
PA0737		*las*+*rhl*	Hypothetical protein	–2	–2	**–53**	**–6**	**–8**	**–9**
PA0843	*plcR*	*las*	Phospholipase protein PlcR precursor	–3	**–14**	**–14**	–5	**–6**	**–7**
PA0852	*cpbD*	*las*+*rhl*	Chitin-binding protein CbpD precursor	**–12**	**–27**	**–28**	**–8**	**–74**	**–64**
PA0990		*lasrhl*	Conserved hypothetical protein	–5	–1	–4	–4	**–20**	**–6**
PA0996	*pqsA*	*las*	Prob. coenzyme A ligase	2	–1	**–36**	3	**–21**	**–7**
PA0997	*pqsB*	*las*	Homologous to β-keto–AACP synthase	2	1	**–62**	3	**–15**	**–13**
PA0998	*pqsC*	*las*	Homologous to β-keto-AACP synthase	3	1	**–32**	3	**–14**	**–8**
PA0999	*pqsD*	*las*	3-Oxoacyl-[ACP] synthase III	2	–1	**–21**	3	**–10**	**–8**
PA1000	*pqsE*	*las*	Quinolone signal response protein	3	2	**–33**	4	**–12**	**–8**
PA1001	*phnA*	*las*	Anthranilate synthase component I	2	–1	**–21**	3	**–10**	**–7**
PA1002	*phnB*	*las*	Anthranilate synthase component II	3	1	**–7**	5	**–5**	**–7**
PA1131		*rhl*	Prob. MFS transporter	–3	–3	–2	**–7**	**–11**	**–6**
PA1168		*las*	Hypothetical protein	–2	1	**–28**	–4	**–19**	**–22**
PA1190		*las*+*rhl*	Conserved hypothetical protein	**–12**	**–43**	**–8**	**–10**	**–12**	**–15**
PA1242		*rhl*	Hypothetical protein	–5	**–16**	–3	**–9**	**–21**	**–20**
PA1249	*aprA*	*lasrhl*	Alkaline metalloproteinase precursor	–3	–4	–4	–3	**–8**	**–7**
PA1250	*aprI*	*lasrhl*	Alkaline proteinase inhibitor AprI	–1	–1	–3	–1	**–6**	**–10**
PA1323		*las*+*rhl*	Hypothetical protein	–3	**–12**	**–44**	**–15**	**–39**	**–25**
PA1324		*las*+*rhl*	Hypothetical protein	–4	**–16**	**–30**	**–17**	**–27**	**–22**
PA1431	*rsaL*	*las*	Regulatory protein RsaL	–2	–2	**–2766**	–2	**–3394**	**–3646**
PA1432	*lasI*	*las*	Autoinducer synthesis protein LasI	1	2	**–37**	–1	**–133**	**–97**
PA1471		*las*+*rhl*	Hypothetical protein	–3	–4	**–8**	**–10**	**–6**	**–8**
PA1625		*rhl*	Conserved hypothetical protein	**–6**	–3	–3	**–6**	**–9**	**–7**
PA1657		*las*+*rhl*	Conserved hypothetical protein	–2	–2	**–17**	**–5**	**–15**	**–13**
PA1662		*lasrhl*	Prob. ClpA/B-type protease	–4	**–5**	–2	–4	**–9**	**–6**
PA1664		*las*+*rhl*	Hypothetical protein	**–7**	–4	**–18**	**–9**	**–11**	**–5**
PA1665		*las*	Hypothetical protein	**–6**	–2	**–17**	–3	**–47**	**–6**
PA1667		*lasrhl*	Hypothetical protein	–2	–1	–5	–3	**–5**	**–6**
PA1669		*rhl*	Hypothetical protein	**–6**	**–52**	–2	**–10**	**–13**	**–29**
PA1784		*las*	Hypothetical protein	–3	**–5**	**–8**	–1	**–11**	**–9**
PA1869		*las*+*rhl*	Prob. acyl carrier protein	**–6**	–3	**–27**	**–25**	**–288**	**–137**
PA1870		*rhl*	Hypothetical protein	**–7**	**–77**	–5	**–14**	**–13**	**–22**
PA1871	*lasA*	*las*+*rhl*	LasA protease precursor	**–9**	**–17**	**–25**	**–7**	**–148**	**–105**
PA1874		*las*	Hypothetical protein	**–8**	**–15**	**–7**	2	**–6**	**–8**
PA1875		*lasrhl*	Prob. outer-membrane protein precursor	**–7**	**–29**	–3	1	**–9**	**–34**
PA1901	*phzC2*	*las*+*rhl*	Phenazine biosynthesis protein PhzC	**–7**	**–9**	**–19**	**–51**	**–25**	**–22**
PA1902	*phzD2*	*las*+*rhl*	Phenazine biosynthesis protein PhzD	**–7**	**–77**	**–105**	**–91**	**–132**	**–134**
PA1903	*phzE2*	*las*+*rhl*	Phenazine biosynthesis protein PhzE	**–6**	**–46**	**–20**	**–20**	**–24**	**–17**
PA1904	*phzF2*	*las*+*rhl*	Prob. phenazine biosynthesis protein	**–6**	**–13**	**–82**	**–45**	**–62**	**–134**
PA1905	*phzG2*	*las*+*rhl*	Prob. pyridoxamine 5′-phosphate oxidase	**–6**	**–19**	**–22**	**–30**	**–33**	**–14**
PA1914		*las*	Conserved hypothetical protein	**–17**	**–6**	**–84**	–1	**–15**	**–93**
PA2021		*las*+*rhl*	Hypothetical protein	**–8**	–5	**–9**	**–13**	**–12**	**–9**
PA2046		*las*+*rhl*	Hypothetical protein	–3	**–11**	**–20**	**–46**	**–22**	**–32**

Table 2. cont.

Pa gene	Gene	Regulation*	Product name†	Change in expression (fold)‡					
				Pat	PA	*lasR*	*rhlR*	*lasR rhlR*	*lasI rhlI*§
PA2067		*lasrhl*	Prob. hydrolase	**–6**	**–18**	–5	–4	**–6**	**–6**
PA2068		*las*+*rhl*	Prob. MFS transporter	**–7**	**–8**	**–18**	**–25**	**–19**	**–9**
PA2069		*las*+*rhl*	Prob. carbamoyltransferase	**–22**	**–529**	**–25**	**–27**	**–73**	**–29**
PA2137		*las*+*rhl*	Hypothetical protein	**–114**	**–9**	**–16**	**–71**	**–16**	**–11**
PA2139		*rhl*	Hypothetical protein	**–32**	**–25**	–3	**–24**	**–10**	**–9**
PA2141		*las*+*rhl*	Hypothetical protein	**–42**	**–61**	**–33**	**–69**	**–46**	**–6**
PA2142		*las*+*rhl*	Prob. short-chain dehydrogenase	**–9**	**–15**	**–6**	**–27**	**–16**	**–13**
PA2143		*las*+*rhl*	Hypothetical protein	**–15**	**–11**	**–101**	**–84**	**–60**	**–67**
PA2144	*glgP*	*lasrhl*	Glycogen phosphorylase	**–10**	–4	–5	–5	**–21**	**–7**
PA2146		*las*+*rhl*	Conserved hypothetical protein	**–9**	**–147**	**–12**	**–10**	**–11**	**–7**
PA2148		*lasrhl*	Conserved hypothetical protein	–3	–3	–4	–4	**–8**	**–8**
PA2149		*las*+*rhl*	Hypothetical protein	**–6**	**–9**	**–25**	**–11**	**–7**	**–6**
PA2151		*las*+*rhl*	Conserved hypothetical protein	**–6**	**–64**	**–23**	**–31**	**–125**	**–39**
PA2153	*glgB*	*las*+*rhl*	1,4-α-Glucan branching enzyme	**–14**	**–18**	**–10**	**–9**	**–11**	**–7**
PA2158		*las*+*rhl*	Prob. alcohol dehydrogenase (Zn-dependent)	–5	**–17**	**–19**	**–52**	**–10**	**–65**
PA2163		*rhl*	Hypothetical protein	–4	**–54**	–4	**–5**	**–8**	**–8**
PA2165		*rhl*	Prob. glycogen synthase	**–6**	**–12**	–4	**–8**	**–5**	**–9**
PA2166		*las*+*rhl*	Hypothetical protein	**–16**	**–8**	**–19**	**–19**	**–38**	**–15**
PA2167		*rhl*	Hypothetical protein	**–6**	**–12**	–4	**–15**	**–8**	**–9**
PA2170		*rhl*	Hypothetical protein	**–7**	**–18**	–3	**–6**	**–14**	**–8**
PA2171		*las*+*rhl*	Hypothetical protein	**–6**	**–86**	**–31**	**–22**	**–110**	**–28**
PA2176		*las*+*rhl*	Hypothetical protein	**–5**	**–103**	**–16**	**–12**	**–25**	**–19**
PA2178		*lasrhl*	Hypothetical protein	**–7**	**–37**	–2	–5	**–6**	**–11**
PA2184		*las*+*rhl*	Conserved hypothetical protein	**–6**	**–250**	**–10**	**–21**	**–14**	**–16**
PA2190		*las*+*rhl*	Conserved hypothetical protein	**–13**	**–10**	**–5**	**–7**	**–15**	**–17**
PA2193	*hcnA*	*rhl*	Hydrogen cyanide synthase HcnA	–1	–3	–4	**–9**	**–254**	**–260**
PA2194	*hcnB*	*rhl*	Hydrogen cyanide synthase HcnB	–1	–3	–3	**–8**	**–43**	**–123**
PA2195	*hcnC*	*rhl*	Hydrogen cyanide synthase HcnC	–1	**–6**	–3	**–7**	**–39**	**–21**
PA2300	*chiC*	*las*+*rhl*	Chitinase	**–39**	**–133**	**–66**	**–34**	**–67**	**–34**
PA2302		*las*	Prob. non-ribosomal peptide synthetase	–2	–2	**–10**	–1	**–18**	**–13**
PA2303		*las*	Hypothetical protein	–1	–3	**–57**	–1	**–214**	**–88**
PA2304		*las*	Hypothetical protein	–1	–2	**–9**	1	**–23**	**–18**
PA2305		*las*	Prob. non-ribosomal peptide synthetase	–1	–2	**–9**	1	**–17**	**–6**
PA2414		*las*+*rhl*	L-Sorbosone dehydrogenase	**–7**	**–11**	**–9**	**–8**	**–18**	**–32**
PA2415		*las*+*rhl*	Hypothetical protein	–4	**–10**	**–7**	**–5**	**–9**	**–14**
PA2423		*las*	Hypothetical protein	–1	–1	**–7**	2	**–6**	**–6**
PA2433		*las*+*rhl*	Hypothetical protein	**–9**	**–43**	**–53**	**–33**	**–67**	**–26**
PA2485		*las*+*rhl*	Hypothetical protein	–3	–4	**–12**	**–12**	**–18**	**–12**
PA2486		*rhl*	Hypothetical protein	1	–2	–5	**–6**	**–11**	**–9**
PA2566		*lasrhl*	Conserved hypothetical protein	–2	–2	–4	–2	**–7**	**–10**
PA2570	*pa1L*	*las*+*rhl*	PA-I galactophilic lectin	**–19**	**–9**	**–21**	**–30**	**–49**	**–45**
PA2587	*pqsH*	*las*	Prob. FAD-dependent monooxygenase	–2	–1	**–20**	1	**–36**	**–46**
PA2588		*las*	Prob. transcriptional regulator	–5	–3	**–77**	–2	**–34**	**–7**
PA2591		*lasrhl*	Prob. transcriptional regulator	–2	–1	–2	1	**–15**	**–7**
PA2592		*lasrhl*	Prob. periplasmic spermidine/putrescine-binding protein	–2	–1	–4	–2	**–18**	**–7**
PA2708		*las*+*rhl*	Hypothetical protein	–3	–4	**–5**	**–13**	**–8**	**–6**
PA2747		*las*+*rhl*	Hypothetical protein	**–6**	**–7**	**–29**	**–10**	**–37**	**–33**
PA2751		*las*+*rhl*	Conserved hypothetical protein	–3	–5	**–8**	**–6**	**–7**	**–8**
PA2754		*las*+*rhl*	Conserved hypothetical protein	–2	–3	**–7**	**–5**	**–7**	**–12**
PA2777		*las*+*rhl*	Conserved hypothetical protein	–5	**–79**	**–6**	**–9**	**–6**	**–8**
PA2873		*lasrhl*	Hypothetical protein	–2	2	–1	–1	**–6**	**–13**

Table 2. cont.

Pa gene	Gene	Regulation*	Product name†	Change in expression (fold)‡					
				Pat	PA	*lasR*	*rhlR*	*lasR rhlR*	*lasI rhlI*§
PA2937		*las*+*rhl*	Hypothetical protein	–5	**–17**	**–7**	**–5**	**–9**	**–7**
PA2939		*las*	Prob. aminopeptidase	–4	**–6**	**–12**	2	**–9**	**–40**
PA3231		*las*+*rhl*	Hypothetical protein	**–11**	**–17**	**–9**	**–10**	**–13**	**–34**
PA3273		*las*+*rhl*	Hypothetical protein	**–11**	**–13**	**–7**	**–27**	**–13**	**–13**
PA3274		*las*+*rhl*	Hypothetical protein	**–6**	**–18**	**–58**	**–17**	**–37**	**–45**
PA3326		*lasrhl*	Prob. Clp-family ATP-dependent protease	–2	–2	–3	–4	**–11**	**–12**
PA3327		*rhl*	Prob. non-ribosomal peptide synthetase	–2	**–6**	–4	**–10**	**–13**	**–5**
PA3328		*rhl*	Prob. FAD-dependent monooxygenase	–1	**–13**	–4	**–12**	**–26**	**–7**
PA3329		*las*+*rhl*	Hypothetical protein	–1	–4	**–10**	**–69**	**–174**	**–55**
PA3330		*las*+*rhl*	Prob. short chain dehydrogenase	–2	–4	**–7**	**–19**	**–178**	**–62**
PA3331		*las*+*rhl*	Cytochrome P450	–2	**–7**	**–5**	**–24**	**–62**	**–20**
PA3332		*las*+*rhl*	Conserved hypothetical protein	–2	**–18**	**–6**	**–18**	**–19**	**–10**
PA3333	*fabH2*	*las*+*rhl*	3-Oxoacyl-[ACP] synthase III	–2	**–5**	**–5**	**–10**	**–33**	**–17**
PA3334		*las*+*rhl*	Prob. acyl carrier protein	–3	–3	**–7**	**–11**	**–17**	**–43**
PA3335		*lasrhl*	Hypothetical protein	–2	–3	–3	–4	**–9**	**–18**
PA3336		*rhl*	Prob. MFS transporter	**–7**	–3	–4	**–23**	**–9**	**–8**
PA3361	*lecB*	*las*+*rhl*	Fucose-binding lectin PA-IIL	**–7**	**–11**	**–11**	**–15**	**–29**	**–22**
PA3369		*las*+*rhl*	Hypothetical protein	**–8**	**–9**	**–28**	**–14**	**–42**	**–20**
PA3370		*las*+*rhl*	Hypothetical protein	**–17**	**–19**	**–45**	**–31**	**–66**	**–39**
PA3371		*las*+*rhl*	Hypothetical protein	**–12**	**–18**	**–36**	**–26**	**–52**	**–24**
PA3460		*las*	Prob. acetyltransferase	–2	**–11**	**–6**	–5	**–6**	**–5**
PA3476	*rhlI*	*lasrhl*	Autoinducer synthesis protein RhlI	–3	–2	–2	–2	**–41**	**–19**
PA3478	*rhlB*	*las*+*rhl*	Rhamnosyltransferase chain B	**–13**	**–17**	**–17**	**–53**	**–123**	**–120**
PA3479	*rhlA*	*las*+*rhl*	Rhamnosyltransferase chain A	**–14**	**–27**	**–13**	**–55**	**–196**	**–320**
PA3520		*las*+*rhl*	Hypothetical protein	**–10**	**–6**	**–12**	**–7**	**–18**	**–28**
PA3581	*glpF*	*las*+*rhl*	Glycerol uptake facilitator protein	**–19**	**–39**	**–6**	**–35**	**–89**	**–61**
PA3584	*glpD*	*las*+*rhl*	Glycerol 3-phosphate dehydrogenase	**–9**	**–10**	**–7**	**–8**	**–20**	**–6**
PA3691		*las*+*rhl*	Hypothetical protein	–2	**–7**	**–23**	**–12**	**–19**	**–12**
PA3692		*las*+*rhl*	Prob. outer-membrane protein precursor	–3	**–9**	**–29**	**–12**	**–17**	**–31**
PA3724	*lasB*	*las*+*rhl*	Elastase LasB	**–7**	**–12**	**–13**	**–5**	**–224**	**–167**
PA3734		*rhl*	Hypothetical protein	–5	**–31**	–4	**–9**	**–21**	**–7**
PA3788		*lasrhl*	Hypothetical protein	–3	**–8**	–5	–4	**–6**	**–9**
PA3819		*las*+*rhl*	Conserved Hypothetical Protein	–2	–4	**–8**	**–7**	**–7**	**–6**
PA3888		*las*	Prob. permease of ABC transporter	–2	–2	**–8**	–5	**–10**	**–6**
PA3890		*las*+*rhl*	Prob. permease of ABC transporter	–2	**–34**	**–36**	**–32**	**–88**	**–18**
PA3904		*las*	Hypothetical protein	–3	1	**–212**	–1	**–41**	**–68**
PA3906		*las*	Hypothetical protein	–3	–2	**–9**	–1	**–177**	**–7**
PA3907		*las*	Hypothetical protein	–1	1	**–7**	1	**–13**	**–6**
PA3908		*lasrhl*	Hypothetical protein	–2	1	–4	1	**–50**	**–6**
PA4078		*lasrhl*	Prob. nonribosomal peptide synthetase	**–6**	**–44**	–3	–5	**–11**	**–6**
PA4130		*lasrhl*	Prob. sulfite or nitrite reductase	–2	1	–3	2	**–6**	**–10**
PA4133		*las*	Cytochrome *c* oxidase subunit (cbb_3-type)	–5	1	**–6**	2	**–13**	**–57**
PA4134		*las*	Hypothetical protein	**–27**	–1	**–12**	–1	**–6**	**–12**
PA4139		*las*	Hypothetical protein	–1	1	**–6**	–4	**–10**	**–6**
PA4141		*las*+*rhl*	Hypothetical protein	–5	**–6**	**–25**	**–58**	**–105**	**–73**
PA4142		*las*+*rhl*	Prob. secretion protein	**–5**	**–12**	**–24**	**–26**	**–72**	**–27**
PA4143		*las*+*rhl*	Prob. toxin transporter	**–9**	**–6**	**–8**	**–9**	**–15**	**–8**
PA4171		*las*+*rhl*	Prob. protease	–3	**–6**	**–11**	**–9**	**–15**	**–5**
PA4175	*prpL*	*las*	Pvds-regulated endoprotease, lysyl class	**–6**	**–9**	**–28**	–2	**–47**	**–28**
PA4209	*phzM*	*las*+*rhl*	Prob. phenazine-specific methyltransferase	–3	–4	**–13**	**–7**	**–19**	**–10**
PA4210	*phzA1*	*las*+*rhl*	Prob. phenazine biosynthesis protein	**–115**	**-50**	**–89**	**–20**	**–123**	**–65**
PA4211	*phzB1*	*las*+*rhl*	Prob. phenazine biosynthesis protein	**–9**	**-226**	**–62**	**–46**	**–61**	**–51**

Table 2. cont.

Pa gene	Gene	Regulation*	Product name†	Change in expression (fold)‡					
				Pat	PA	*lasR*	*rhlR*	*lasR rhlR*	*lasI rhlI*§
PA4217	*phzS*	*las*+*rhl*	Flavin-containing monooxygenase	**–6**	**–22**	**–9**	**–11**	**–13**	**–11**
PA4290		*rhl*	Prob. chemotaxis transducer	**–20**	**–24**	–4	**–16**	**–6**	**–7**
PA4345		*rhl*	Hypothetical protein	–4	–4	–4	**–7**	**–9**	**–11**
PA4377		*lasrhl*	Hypothetical protein	**–13**	**–7**	–4	–3	**–7**	**–10**
PA4677		*las*	Hypothetical protein	–2	–4	**–15**	–1	**–40**	**–17**
PA4738		*las*+*rhl*	Conserved hypothetical protein	**–6**	**–16**	**–45**	**–17**	**–54**	**–40**
PA4739		*las*+*rhl*	Conserved hypothetical protein	–5	**–12**	**–34**	**–17**	**–34**	**–34**
PA4876	*osmE*	*las*+*rhl*	Osmotically inducible lipoprotein OsmE	–3	**–7**	**–8**	**–7**	**–9**	**–18**
PA4880		*las*+*rhl*	Prob. bacterioferritin	–2	**–7**	**–8**	**–13**	**–30**	**–6**
PA5099		*las*+*rhl*	Prob. transporter	**–252**	**–66**	**–10**	**–40**	**–15**	**–16**
PA5212		*las*+*rhl*	Hypothetical protein	–1	–4	**–9**	**–6**	**–9**	**–9**
PA5220		*rhl*	Hypothetical protein	–3	**–23**	–4	**–7**	**–9**	**–8**
PA5235	*glpT*	*rhl*	Glycerol-3-phosphate transporter	**–5**	–3	–2	**–14**	**–32**	**–15**
PA5297	*poxB*	*lasrhl*	Pyruvate dehydrogenase (cytochrome)	–2	–1	–3	–3	**–6**	**–18**
PA5480		*rhl*	Hypothetical protein	–2	**–16**	–1	**–6**	**–20**	**–14**
PA5481		*las*+*rhl*	Hypothetical protein	**–8**	**–16**	**–74**	**–23**	**–51**	**–46**
PA5482		*las*+*rhl*	Hypothetical protein	–5	**–16**	**–62**	**–12**	**–43**	**–60**

**las*+*rhl*, downregulated in both single mutants (*lasR* and *rhlR*); *lasrhl*, downregulated only in the double *lasR rhlR* mutant.

†Prob., probable.

‡These data are included in those presented by Hentzer *et al.* (2003).

§Downregulation values >5 are shown in bold.

patulin and penicillic acid affected the expression of 3–5 % of all genes. QS-controlled genes are overrepresented among the genes downregulated by the compounds, indicating that they have specificity for QS-regulated genes. Of the QS regulated genes, 45 % and 60 % were affected by patulin and penicillic acid respectively. Interestingly, patulin downregulated 43 % of the group B genes, 58 % of the group C genes and 26 % of the group D genes, but only 15 % of the group A genes. This suggests that RhlR-controlled genes are preferentially targeted by patulin. Similarly, penicillic acid downregulated 65 %, 80 % and 30 % of groups B, C and D, respectively, whereas only 21 % of group A genes were downregulated. Once again this suggests that RhlR-controlled genes are preferentially targeted by penicillic acid.

Hentzer *et al.* (2003) found an 80 % target specificity of furanone C-30 to QS-regulated genes. However, when the same analysis was performed according to the QS regulon described above, it was found that C-30 exhibits a target specificity of 64 %. Thus, the QS target specificity of the QSIs identified in the present study is comparable to that of C-30.

Among the genes repressed by patulin and penicillic acid treatment, many encode virulence factors which had previously been reported to be controlled by QS (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003; Whiteley *et al.*, 1999). These include the genes encoding LasA protease (*lasA*, PA1871), LasB elastase (*lasB*, PA3724), chitinase (*chiC*, PA2300) and chitin-binding protein (*chiD*, PA0852), and the genes encoding phenazine biosynthesis proteins (*phzC–G*, PA1901–PA1905), the rhamnosyl transferase AB operon (*rhlAB*, PA3478–PA3479), fucose-binding lectin (*lecB*, PA3361) and PA-I galactophilic lectin (*pa1L*, PA2570). Pyoverdine production seems to be unaffected by patulin and penicillic acid, and the expression level of *pvd* genes was unaltered in the QS mutants; however, the PvdS-regulated endoprotease gene encoding PrpL (PA4175) was downregulated by both patulin and penicillic acid as well as in the QS mutants. PrpL is able to cleave lactoferrin, transferrin, elastin and casein, and may potentially play a role in virulence (Wilderman *et al.*, 2001). Nouwens *et al.* (2003) found reduced levels of PrpL in culture supernatants of *Ps. aeruginosa* strains containing a *las* mutation, but not in *rhl* mutants (Nouwens *et al.*, 2003). The expression of *prpL* in a *lasI rhlI* mutant is restored by the addition of 3-oxo-C12 HSL, which further supports the idea that expression of *prpL* is primarily LasR regulated (Hentzer *et al.*, 2003; Schuster *et al.*, 2003). It is not known if this is by direct regulation by the *las* QS system or via an intermediary regulator such as PvdS. The significant downregulation of *chiC* by patulin and penicillic acid was not unexpected considering that the major structural component of fungal cells is chitin. This suggests that the QSI properties of some mycotoxins are not just coincidental, but are instead a strategy used by some fungi to minimize damage caused by bacteria producing chitinase. A number

of other genes, including *osmC* (PA0052, osmotically inducible protein) *coxA* and *coxB* (PA0105 and PA106, cytochrome *c* oxidase subunit I and II) and several genes encoding hypothetical proteins previously identified as being QS regulated (such as the operon at PA3326–PA3336) were also found to be downregulated in the presence of patulin and penicillic acid. One of these genes, *fabH2*, encodes a 3-oxoacyl-ACP synthase, probably involved in synthesis of the 3-oxo-C12 HSL signal molecule. This suggests that one of the modes of action by which penicillic acid inhibits QS is by preventing signal molecule synthesis. It should be noted that the QSIS1 and 2 screens select molecules which interfere with the LuxR homologue proteins; thus inhibition of AHL synthesis is just an additional feature of penicillic acid as QSI rather than its primary activity.

Genes encoding central parts of the QS circuit, such as *lasR*, *rsaL*, *lasI*, *rhlR* and *rhlI* (PA1430–PA1432, PA3437 and PA3476), and genes involved in the synthesis of PQS (*pqsA–E*, *pqsH*, PA0996–PA1000, PA2587) were almost unaffected by treatment with the two QSI compounds. This suggests that patulin and penicillic acid do not directly interfere with the regulatory systems controlling transcription of the *lasRI* and *rhlRI* genes, but instead act on these QS regulators at the post-transcriptional level. This suggests that the two QSI compounds interfere with the RhlR and LasR proteins.

Patulin accelerates LuxR turnover

Patulin was selected for more thorough analysis of its effect on bacterial phenotypes related to pathogenicity since it was found to actively inhibit *Ps. aeruginosa* QS at a concentration substantially lower than the LD_{50} (Hayes, 1981). Manefield *et al.* (2002) demonstrated the ability of furanone compounds to cause instability of the LuxR QS regulatory protein.

LuxR was overexpressed from an IPTG-inducible promoter in the presence of high concentrations of GroESL in accordance with Manefield *et al.* (2002). After cells were washed free of the inducer, they were treated with 3·5 μM patulin (this concentration does not affect growth). The amount of LuxR protein present in the samples was visualized after 1 h of treatment by Western blotting with a chemiluminescent LuxR antibody. Digital images of the gel reported chemoluminescence with increasingly lighter colours indicating higher band intensity. Treatment with patulin markedly decreased the amount of LuxR present in the samples (Fig. 5). As a reference, furanone C-2 was included as a control (Manefield *et al.*, 2002). The reduced amount of LuxR correlated with the strong signal obtained in the QSIS systems and with downregulation of the fluorescent signal from a LuxR-controlled *luxI–gfp* fusion (data not shown). This is in accordance with a model where patulin interacts directly with QS regulators. Interestingly, a third LuxR homologue, designated QscR, has been identified in the *Ps. aeruginosa* chromosome (Chugani *et al.*, 2001). This protein is a negative regulator of several

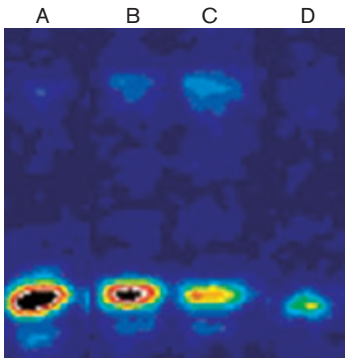

Fig. 5. Western blot analysis of LuxR content using a chemiluminescent LuxA antibody. LuxR-overproducing *E. coli* cells were either untreated (A), or treated with 20 μM furanone C-2 (B), 100 μM furanone C-2 (C) or 3·5 μM patulin (D). Lighter colours indicate higher band density.

QS-controlled virulence factors and is able to form multimers and heterodimers with both LasR and RhlR (Ledgham *et al.*, 2003). No effect of the QSIs on QscR transcription was observed in our DNA array experiments (Table 2). Whether the inhibitors interact directly with QscR, as is the case for LuxR, is presently unknown. Another member of the LuxR family of transcriptional regulators, VqsR, has also been found in *Ps. aeruginosa*. This protein is a major virulence factor regulator and has been found to positively affect expression of QS-controlled genes (Juhas *et al.*, 2004). Again, we have not investigated whether the QSIs interact directly with this regulator, but there is no effect on transcription of *vqsR* when *Ps. aeruginosa* is treated with penicillic acid or patulin. Interestingly, *vqsR* is significantly downregulated in both of the double mutants (Table 2). As we speculate that the inhibitors mainly target the RhlR protein, the lack of effect on *vqsR* transcription from the QSIs is not a surprising result.

QSI effect on biofilm tolerance to tobramycin

It has previously been shown that *Ps. aeruginosa* biofilm cells are highly tolerant to antibiotic treatments (Anwar *et al.*, 1990). Davies *et al.* (1998) demonstrated that a QS mutant of PAO1 is more susceptible to antimicrobial treatments than the wild-type counterpart. Furthermore, Hentzer *et al.* (2003) showed that biofilms treated with the QSI compound furanone C-30 became susceptible to both SDS and tobramycin treatment. We tested whether patulin exhibited a similar effect. Two sets of PAO1 biofilms were allowed to form in flow chambers in which the medium contained either no patulin or 8 μM patulin for 3 days. The biofilms were then challenged with tobramycin (340 μg ml^{-1}) for 24 h. The effect of the antibiotic treatment was assessed by means of live/dead staining (Fig. 6). In the biofilm treated with tobramycin only a few cells, mainly localized in the top layer, were dead, whereas almost all the cells in the biofilm treated with both tobramycin and patulin were

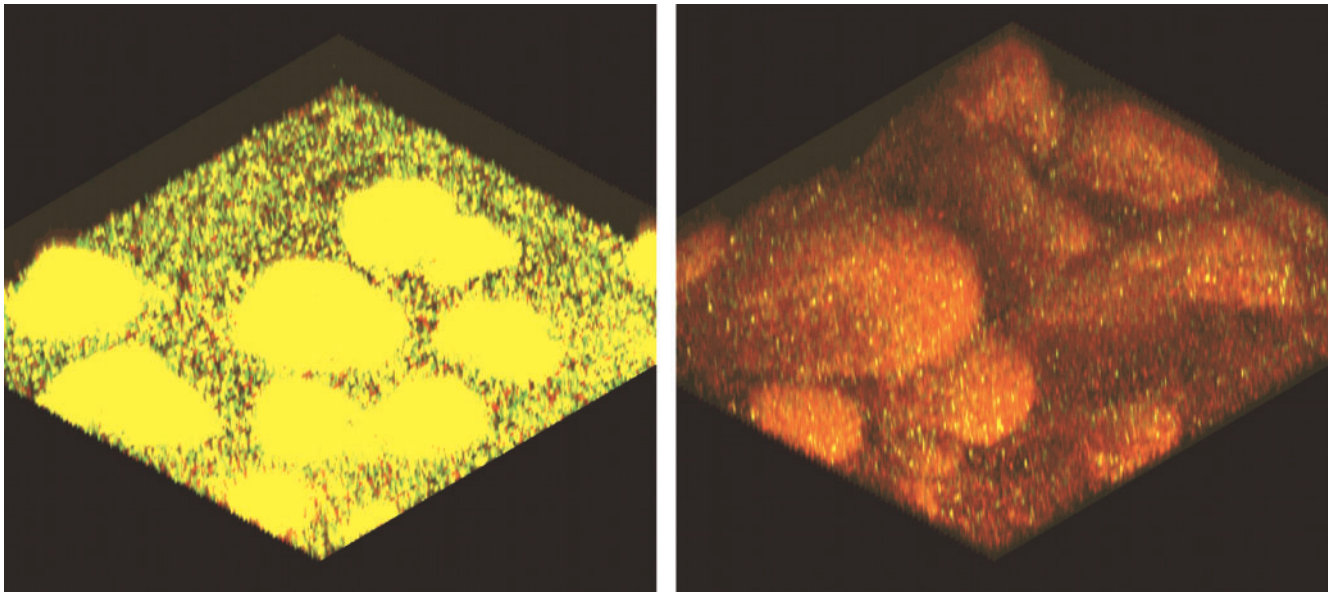

Fig. 6. Biofilms formed by *Ps. aeruginosa* in the absence (left) or presence (right) of 1·2 μg patulin ml^{-1} and treated with tobramycin for 24 h. The cells are stained with the Baclight live/dead kit, which stains live cells green and dead cells red.

dead. Treatment with patulin alone did not affect development of the biofilm (data not shown).

PMN activation is affected by a QS-controlled mechanism

In recent work by Bjarnsholt *et al.* (2005) it was found that the activation of neutrophile PMNs by *Ps. aeruginosa* is controlled by QS. A *lasR rhlR* mutant activated PMNs whereas the wild-type *Ps. aeruginosa* PAO1 failed to do so. In addition, when the wild-type was treated with furanone C-30, activation was observed. We wanted to investigate if the QS inhibitory effect observed by patulin and penicillic acid created a similar increase in activation of PMNs. Biofilms of wild-type *Ps. aeruginosa* PAO1 were grown for 3 days with and without 1·2 μg ml^{-1} patulin and penicillic acid. PMNs were injected into the biofilm flow chambers and development of oxidative burst was recorded after 2 h by 123-DHR staining (Fig. 7). A marked difference in green fluorescence signal intensity was observed for the PMNs incubated on the wild-type biofilms compared to the QSI-treated biofilms. These experiments suggest that a QSI-treated biofilm, in contrast to its untreated counterpart, fully activates the PMNs to produce H_2O_2.

Ps. aeruginosa in mouse lungs is cleared rapidly when treated with patulin

We have shown that a functional QS system plays a major role in the persistence of a pulmonary infection in mice with respect to both clearance of the bacteria and the onset of the innate immune defence (Bjarnsholt *et al.*, 2005). We investigated whether patulin could promote a faster clearing of bacteria in mice, as seen with other QSIs (Hentzer *et al.*, 2003; Wu *et al.*, 2004; Rasmussen *et al.*, 2005). BALB/c mice were used to establish a pulmonary model of chronic lung infection. The immune response of the BALB/c mice is Th-2 dominated (Moser *et al.*, 1999), resembling the immune response of cystic fibrosis patients to *Ps. aeruginosa* pulmonary infection (Moser *et al.*, 2000). In order to study the effect of patulin in a pulmonary infection model two groups of 72 mice were treated with either patulin or placebo (saline). The treatments were given as a subcutaneous injection every 24 h for 7 days, administering 2·5 μg per g body weight ($\sim$16 μM; the average mouse volume is taken as 20 ml). After 1 day of prophylactic treatment, the mice were intratracheally challenged with alginate beads containing *Ps. aeruginosa* (Pedersen *et al.*, 1990). After the challenge, a higher mortality was observed for the placebo-treated group on day 1: 12 died compared to 5 in the patulin-treated group; however, the difference was not found to be statistically significant.

Clearance of bacteria from the lungs was assessed on day 1 and day 3 after bacterial challenge. On day 1 there was only a slight difference between the two groups, with the patulin-treated group showing faster clearance. On day 3, a 20-fold lower bacterial content was observed with the patulin-treated group, suggesting that the treatment promoted clearing compared to the placebo ($P<0·04$) This is in accordance with the results of Rasmussen *et al.* (2005), Hentzer *et al.* (2003) and Wu *et al.* (2004) using other QSI compounds.

Conclusions

A recently published screening system, the QSI selector, was used to screen extracts prepared from 50 *Penicillium*

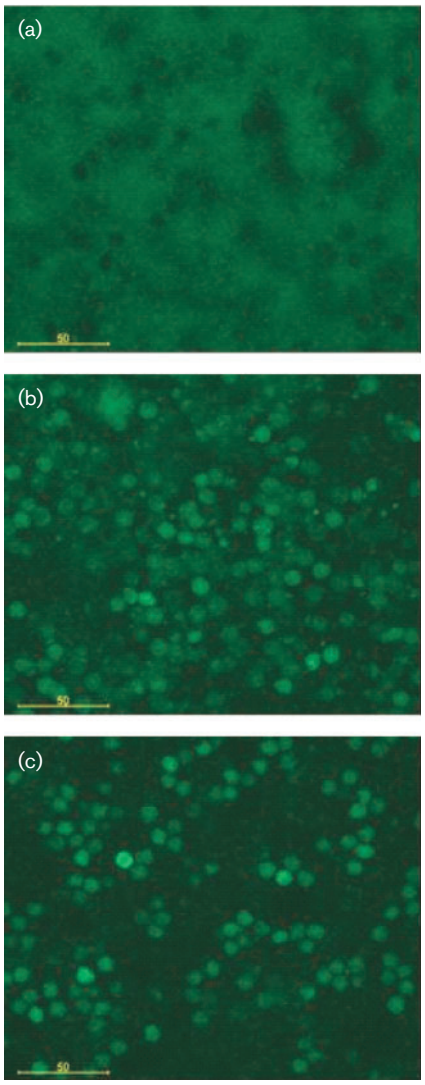

Fig. 7. Biofilms formed by *Ps. aeruginosa* PAO1 in the absence of QSI (a), and in the presence of 1·2 μg patulin ml^{-1} (b) or 1·2 μg penicillic acid ml^{-1} (c). Freshly isolated PMNs were exposed to biofilm bacteria in the biofilm flow chambers and stained with 123-DHR. PMNs developing oxidative burst appear green fluorescent. Bars, 50 μm.

species. Approximately 33 % of the extracts contained potential QSIs. The extracts from *P. radicicola* and *P. coprobium* were subjected to further analysis, which showed that the QSI produced by *P. radicicola* was penicillic acid and the QSI produced by *P. coprobium* was patulin. The ability to inhibit QS was verified by DNA array analysis, which suggested that penicillic acid and patulin targeted the RhlR and the LasR proteins. *Ps. aeruginosa* biofilms formed in the presence of patulin were susceptible to tobramycin treatment, whereas control biofilms were tolerant. Furthermore, the wild-type *Ps. aeruginosa* biofilms formed in the presence of patulin induced oxidative burst in PMNs that settled on top of the biofilm bacteria. The reduction in virulence factor expression and activation of PMN oxidative burst may explain why patulin accelerated the clearance of *Ps. aeruginosa* from the lungs of infected mice. Even though the two mycotoxins are not currently drug lead compounds, this work demonstrates that fungi produce QSIs. Ongoing work in our laboratories is aimed at identifying non-toxin QSIs produced by fungi.

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Article 2

Skindersoe, M. E., Epstein-Ettinger, P., Rasmussen, T. B., Bjarnsholt, T., de Nys R., & Givskov M.

Quorum sensing antagonism from marine organisms

Marine Biotech. In press

Photo collage (by Peter Vennestrøm):
Ascidian selected for sampling
The sponge *Luffariella variabilis*
Extraction of samples

Original Article

MARINE BIOTECHNOLOGY

Quorum Sensing Antagonism from Marine Organisms

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Abstract

With the global emergence of multiresistant bacteria there is an increasing demand for development of new treatments to combat pathogens. Bacterial cell–cell communication [quorum sensing (QS)] regulates expression of virulence factors in a number of bacterial pathogens and is a new promising target for the control of infectious bacteria. We present the results of screening of 284 extracts of marine organisms from the Great Barrier Reef, Australia, for their inhibition of QS. Of the 284 extracts, 64 (23%) were active in a general, LuxR-derived QS screen, and of these 36 (56%) were also active in a specific *Pseudomonas aeruginosa* QS screen. Extracts of the marine sponge *Luffariella variabilis* proved active in both systems. The secondary metabolites manoalide, manoalide monoacetate, and secomanoalide isolated from the sponge showed strong QS inhibition of a *lasB::gfp*(ASV) fusion, demonstrating the potential for further identification of specific QS antagonists from marine organisms.

Keywords: *Luffariella variabilis* — manoalide — marine natural products — quorum sensing — quorum sensing inhibitors

Introduction

More than half of all prescribed drugs have their origin in natural sources (Newman and Cragg 2004a, 2004b), and the diversity of chemical structures produced by marine organisms provides a potential source of novel pharmaceuticals (Harper et al. 2001; Paterson and Anderson 2005; Marris 2006). While there are few natural products derived from marine organisms in clinical use, the potential applications of these compounds are broad (Proksch et al. 2002; Marris 2006). Marine natural products are in clinical or preclinical evaluation against severe pain, cancers, allergy/asthma, inflammation, Alzheimer's disease, HIV, and tuberculosis (reviewed in Proksch et al. 2002; Newman and Cragg 2004a, 2004b), with the majority of these natural products being sponge or tunicate derived (Newman and Cragg 2004a, 2004b). They also have potential application as anti-infective therapies (Fenical and Jensen 2006). The development of novel anti-infectives is particularly critical because of the increasing number of multidrug resistant bacteria (Tenover and Hughes 1996; Reacher et al. 2000). One strategy to develop new mechanisms to combat bacterial infections is through the development of antipathogenic drugs that function by attenuating the bacteria with respect to virulence. Because this can be achieved without affecting the growth of bacteria, this principle is less likely to impose a selective pressure for resistance (Hentzer and Givskov 2003; Hentzer et al. 2003). Many pathogenic bacteria synchronize their expression of virulence genes by cell-to-cell communication systems. This phenomenon, quorum sensing (QS), enables bacteria to monitor cell density. QS relies on low molecular weight signal molecules that are capable of activating transcriptional regulators in order to couple gene expression with population density. In gram-negative bacteria, QS is mediated mostly by *N*-acyl homoserine lactones (AHLs). QS systems function by means of two proteins: the AHL synthetase (the LuxI-homologue) responsible for the AHL signal generation and the LuxR

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homologue, which is a dual-function receptor-response protein recognizing and binding the AHL molecules, allowing interactions with QS-controlled promoter sequences. The signal molecules are produced constitutively at a basal low level by the LuxI homologue and are distributed in the cell and the surrounding environment either actively or by diffusion. The concentration of AHL molecules increases with increasing cell density, and reaching a certain critical level (when the populations are "quorate") they bind to the LuxR homologues, creating active LuxR–AHL complexes. This complex functions as a transcription factor that binds to the promoter region of the QS-regulated genes, where it triggers gene expression (Fuqua et al., 1994), including virulence traits (Toder et al. 1991; Gambello et al. 1993) It has been proposed that for some opportunistic pathogens, such as *Pseudomonas aeruginosa*, it is essential to stay "silent" and delay production of virulence factors until the cell population has reached a level sufficient to overwhelm the host's defenses. Therefore, QS offers the invading bacteria with an advantage during the establishment of infections. A strategy for the development of novel anti-infectives is to block the bacterial communication pathway. In contrast to the sophisticated mammalian immune system plants, fungi and marine invertebrates rely on physical and chemical defense strategies to protect them against bacterial pathogens. This provides a logical source to search for natural products with the ability to inhibit QS in natural libraries of chemically rich organisms. A proof of this concept is the halogenated furanones derived from the marine macro alga *Delisea pulchra* (Givskov et al. 1996), which inhibits QS *in vitro* as well as *in vivo* experimental settings (Manefield et al. 2000; Hentzer et al. 2003; de Nys et al. 2006). The treatment of the opportunistic pathogen *P. aeruginosa* in a mouse pulmonary infectious model with furanone-derived drugs greatly accelerates eradication of the pathogen (Hentzer et al. 2003). Furanones also reduce the mortality of vibriosis in rainbow trout challenged with pathogenic *Vibrio anguillarum* (Rasch et al. 2004). The use of QS inhibitors (QSIs) has a wide range of applications, from veterinary treatment in aquaculture to medical treatments in humans. In this study we present the results from a screening of 284 marine samples from the Great Barrier Reef for the inhibition of QS using high-throughput QSI screens demonstrating the broad scale presence of QSI activity in this marine habitat. We also demonstrate specific QS inhibition for the marine natural products manoalide, secomanoalide, and manoalide monoacetate from the active extract of the sponge *Luffarilla variabilis*.

Materials and Methods

Sample Collection: Identification of Marine Organisms. Samples were collected in duplicate on SCUBA at depths of 1 to 10 m from several locations at the inner and mid-shelf reefs of the Central Great Barrier Reef in the vicinity of (18° 35′ 37″ S 146° 29′ 07″ E) in the Palm Islands, Queensland, Australia (Collection permit number G03.8695.1).

Organisms were identified to the lowest taxonomic level using field guides and specific taxonomic keys for sponges (Hooper and Van Soest 2002), ascidians (Goslinger et al. 1996), corals (Veron 1993; Fabricius and Aldersdale 2001; Erhardt and Knop 2005), and algae (Huisman 2000).

Extract Preparation. After collection, samples (n=2 for each species collected) were crushed or cut into small pieces and extracted with ethyl acetate (1 ml per gram of sample) for 24 h. Subsequently, the ethyl acetate fraction was removed and evaporated to almost complete dryness and samples were redissolved in 96% ethanol (in 10% of the volume ethyl acetate used) for use in QSI assays.

Purification of Manoalides from *Luffariella variabilis.* A frozen sample of the sponge *Luffariella variabilis* was freeze-dried (65 g dry weight) and extracted with dichloromethane (3×200 ml) at room temperature. The combined dried extracts (358 mg) were dissolved in methanol and chromatographed using preparative reversed-phase HPLC [Phenomenex, Luna C18 (2), 5 μm, 250×21 mm; gradient of acetonitrile–water 70:30 to 100:0 over 60 min as eluent, flow rate 10 ml/min; UV detection at 254 nm]. Fractions containing secomanoalide and manoalide and manoalide monoacetate were further purified using semipreparative HPLC [Phenomenex, Luna C18 (2), 5 μm, 250×10 mm; gradient of acetonitrile-water. 73:27 to 81:21 over 25 min as eluent, flow rate 4 ml/min; UV detection at 254 nm]. Manoalide, manoalide monoacetate, and secomanoalide (see Fig. 1 for structures) were identified by comparison of their nuclear magnetic resonance (NMR) data with literature data (Tsuda et al. 2002).

Bacterial Screens for QS Inhibition. As an initial, promiscuous screen for QSI activity we employed the screening system QSIS1. The quorum sensor element is derived from the *Vibrio fischeri* LuxR-regulated QS system and cloned in *E. coli,* where it responds to a broad range of AHLs and QS inhibitors (Andersen et al. 2001; Rasmussen et al. 2005a, 2005b). Samples that screened positive with

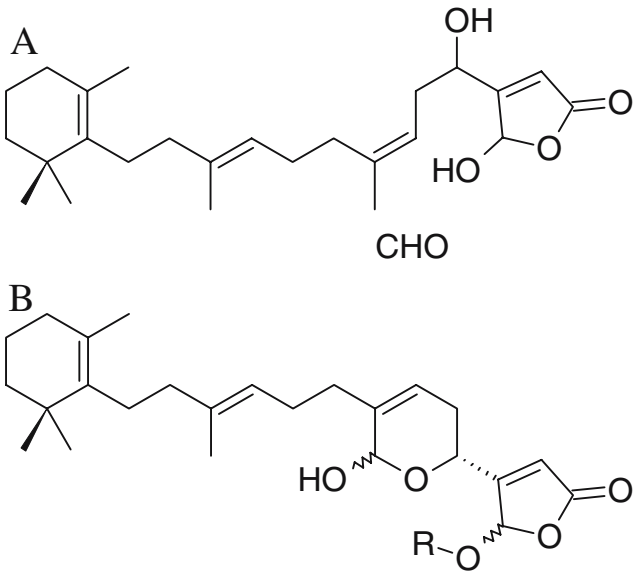

Figure 1. Structures of manoalide, manoalide monoacetate and secomanoalide. (**A**) secomanoalide. (**B**) R=H; manoalide, R=Ac; manoalide monoacetate.

the QSIS1 were further screened by means of QSIS2, a *P. aeruginosa* QS specific screen. The general concept of both selector systems implies a QS regulator (*luxR* homologue) gene and a QS-controlled promoter fused to a suicide gene. In the presence of signal molecules, the gene under QS control is transcribed, causing cell death. However, in the presence of a QSI compound production of the toxic gene product will be switched off, and the bacteria will be rescued and able to grow normally. The suicide gene product of the QSIS1 selector system is the cytolytic phospholipase A originating from *Serratia liquefaciens* MG1 (Givskov et al. 1988; Givskov and Molin 1993). QSIS2 bacteria harbor the QS regulated promoter for elastase B fused to *sacB*, which encodes the secretory levansucrase of *Bacillus subtilis*. SacB catalyses a transfructosylation reaction by which sucrose is hydrolyzed, thereby contributing to the production of the bactericidal polymer levan (Gay et al. 1983).

The quorum sensing inhibitor selectors 1 and 2 (QSIS1 and QSIS2) have been described in detail by (Rasmussen et al. 2005a). QSIS1 harbors the plasmid pUC18Not-*luxR*-P_{luxI}-RBSII-*phlA* T_0-T_1, Ap^r maintained in CSH37 (Miller 1972), an *Escherichia coli* strain constitutively expressing *β*-galactosidase. QSIS2 is maintained in *P. aeruginosa* JP2 (a *lasI, rhlI* mutant unable to produce AHL signals) and contains the plasmid pUCP22NotI-P_{lac}*lasR* P_{lasB}-*sacB* Gm^R, Car^R. The QS reporter strain Tn5-LAS, which harbors the fragment P_{lasB}-*gfp*(ASV) along with P_{lac}-*lasR* as a mini-Tn5-Gm chromosomal insert in the wild type *P. aeruginosa* strain PAO1-ATCC (Hentzer et al. 2002), was also used.

In brief, preparation of QSIS screens were performed as follows: QSIS1: BT medium [B medium (Clark and Maaloe, 1967) plus 2.5 mg of thiamine per liter] containing 2% agar (wt/vol) was melted, 10% A10 was added (Clark and Maaloe, 1967), and the mixture was cooled to 45°C. *N*-3-oxo-hexanoyl-L-homoserine lactone (Sigma-Aldrich, Germany), ampicillin (Ap, VepiDan, Denmark), X-Gal (5-bromo-4-chloro-3-indoxyl-*β*-D-galactopyranoside, Apollo Scientific, UK), and 1-methyl-2-pyrrelidone (Merck, Germany) were added in final concentrations of 100 nM, 100 *μ*g/ml, 40 *μ*g/ml, and 0.2% (vol/vol) respectively and the medium was supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) casamino acid. QSIS1 overnight culture [grown in ABT media supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) casamino acid and 100 μg/ml ampicillin] was added to a final concentration of 0.4%.

Preparation of QSIS2 media. LB agar (Bertani, 1951) containing 2% agar (wt/vol) was melted, 10 % A10 was added, and the mixture was cooled to 43°C, whereupon sucrose, sodium citrate, OdDHL (*N*-3-oxododecanoyl-L-homoserinlactone), BHL (*N*-butanoyl-L-homoserine lactone, Sigma-Aldrich), gentamicin (Gm; gentamicin sulfate, Biochrom AG, Berlin, Germany) were added to final concentrations of 60 mg/ml, 40 mM, 200 nM, 200 nM, and 80 μg/ml, respectively. QSIS2 overnight culture [grown in ABT media supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) casamino acid and 80 μg/ml of gentamicin] was added to a final concentration of 2.0%.

The QSIS media were prepared as described above and plates for the screening assay were subsequently made by pouring the mixture into boxes, giving an agar thickness of 0.5 cm. Wells with a diameter of 5 mm were made in the agar plates using a mould. On solidification, 50 μl of the redissolved extracts (see *Extract preparation*) or solutions of pure compounds dissolved in ethanol (10 mg/ml) were added to the wells. The plates were then incubated at 30°C for 16 h. Appearance of a circular zone of growth around the well indicates the presence of QSI-active compounds in the sample added. In case of QSIS1, a blue zone emerged along with growth due to the presence of hydrolyzed X-Gal in the plate. To visualize growth of QSIS2, a 0.1% solution of tetrazolium red (2,3,5-triphenyltetrazolium chloride, Sigma) dissolved in 0.9% NaCl was poured on top of the plates (0.1 ml/cm^2) and the plates were incubated for 30 min at 30°C. After incubation, non-bound color was washed off. Most

Table 1. Marine organisms extracted and tested positive in the QSIS screen(s)

QSIS1	*QSIS2*	*Source of extract – identification of marine organisms*
+	–	Algae – *Caulerpa* sp. (Caulerpaceae)
++	–	Algae – filamentous red – pearl line (Rhodomelaceae)
+	+	Algae – *Laurencia* sp I – pearl line (Rhodomelaceae)
+	+	Algae – *Liagara* sp. or *Galaxaura* sp. lightly calcified (Galaxauraxeae)
++	–	Ascidian – colonial – encrusting – dark purple (Polycitoridae)
++	++	Ascidian – colonial – encrusting – dark purple *Polysyncraton* sp. (Didemnidae)
+	–	Ascidian – colonial – encrusting – dark red (Didemnidae)
++	–	Ascidian – colonial – encrusting – green *Didemnum* sp.(Didemnidae)
++	+	Ascidian – colonial – encrusting (Didemnidae)
+	–	Ascidian – *Didemnum* sp. (Didemnidae)
+	+	Ascidian – solitary – crimson – *Acropora* (Didemnidae)
++	+	Ascidian – solitary – *Polycarpa aurata* (Polycitoridae)
+	+	*Chlorodesmis fastigiata* (Turtle weed) (Udotaceae)
+	–	Fan – gorgonian – white – fine branching (Gorgoniidae)
+	+	Hard coral – *Acropora* sp. (Acroporidae)
+	–	Hard coral – *Acropora* sp.(Acroporidae)
+	–	Hard coral – *Favites* sp.(Faviidae)
+	–	Hard coral – *Fungia* simplex (Fungiidae)
++	+	Hard coral – *Fungia* sp.(Fungiidae)
+	–	Hard coral – massive – *Barbattoia* sp.(Poritidae)
+	–	Hard coral – massive (Mussidae)
+	+	Hard coral – *Millipora* sp.(Milleporidae)
++	+	Hard coral – *Turbinaria* sp. (Dendrophyllidae)
++	++	Soft coral – encrusting – yellow *Dampia* sp. (Alcyoniidae)
+	++	Soft coral – hard skeleton – *Goniopora* sp. (Poritidae)
+	++	Soft coral – *Sarcophyton* sp. – small individual – yellow (Alcyoniidae)
+	–	Soft coral – *Sarcophyton* sp. (Alcyoniidae)
+++	+	Soft coral – *Sarcophyton* sp.– solitary (Alcyoniidae)
++	++	Soft coral – *Sinularia* sp. – (Alcyoniidae)
+	+	Soft coral – *Sinularia* sp. – cream (Alcyoniidae)
+++	+++	Soft coral – *Sinularia* sp. – yellow branches – weeping (Alcyoniidae)
+	–	Soft coral – *Sinularia* sp. (Alcyoniidae)
++	+	Soft coral – solitary – *Sinularia* sp. (Alcyoniidae)
+++	+	Soft coral – solitary – small *Sarcophyton* (Alcyoniidae)
+	+	Sponge – brown – intertidal
++	++	Sponge – *Coscinoderma* n.sp. (Thorectidae)
++	–	Sponge – encrusting – grey *Dysidea* sp. (Dysideidae)
+	–	Sponge – encrusting – light yellow – *Ircinia ramosa* (Irciniidae)
+	–	Sponge – encrusting – yellow – brittle
+	+	Sponge – encrusting orange – large – unique *Agelas* sp. (Agelasidae)
++	′+	Sponge – encrusting orange *Agelas* sp. (Agelasidae)
++	–	Sponge – erect – *Ianthella* sp. (Ianthellidae)
+	+	Sponge – erect – lattice forming – yellow *Ircinia* sp. (Irciniidae)
+	–	Sponge – erect – orange – unique shape (Axinellidae)
+	–	Sponge – erect – tubes – yellow – (Callyspongidae).
+	+	Sponge – erect – white – small – *Dysidea* sp. (Dysideidae)
+	+	Sponge – Golf ball – purple *Cinachrya* sp. (Tetillidae)
+	+	Sponge – grey solitary *Hyrtios* sp. (Thorectidae)
+	–	Sponge – *Ircinia gigantea* (Irciniidae)
+	–	Sponge – lilac solitary – soft (possibly Niphatidae)
+	+	Sponge – massive – black – (Thorectidae)
+	+++	Sponge – massive – erect – red–brown – *Jaspis* sp. (Jaspidae)
+++	+	Sponge – massive –black – *Luffariella variabilis* (Thorectidae)
+++	++	Sponge – orange (lobate)
++	+	Sponge – *Rhopaloeides odorabile* (Spongiidae)
+	–	Sponge – solitary – erect – *Acanthella* sp. (Axinellidae)
+	–	Sponge – solitary – erect – yellow
+	–	Sponge – solitary – erect
++	–	Sponge – solitary – erect – light pink *Dysidea* sp. (Dysideidae)
+	+	Sponge – solitary – orange – *Acanthella* sp.(Axinellidae)
++	+++	Sponge – Tetillidae
++	++	Sponge *Ircinia* n. sp. (Irciniidae)
+	–	Sponge– lobed – *Carteriospongia* (Thorectidae)
+	–	Sponge– lobed – *Carteriospongia* or *Phyllospongia* (Thorectidae)

+, Low activity; ++, medium activity; +++, strong activity; –, no activity.

Table 2. Percentage (and number) of active extracts in QSIS1 (n=64) and QSIS2 (n=36) reporter systems for each major taxonomic group

	QSIS1	*QSIS2*
Algae	7.8 (5)	8.3 (3)
Ascidians	12.5 (8)	11.1 (4)
Hard corals	14.1 (9)	11.1 (4)
Soft corals	17.2 (11)	25 (9)
Sponges	46.9 (30)	44.4 (16)
Others	1.6 (1)	0 (0)

reduce tetrazolium red to the red-colored triphenyl formazan (TPF) by using tetrazolium red as an electron acceptor. Thus growth of QSIS2 was seen as a red zone of triphenyl formazan around the wells containing QSI-active compounds.

Dose–Response Relationship. To investigate the efficiency of a putative inhibitor of QS in *P. aeruginosa* the QS reporter strain Tn5-LAS was applied as follows: 150 µl of ABT medium supplemented with 0.5% casamino acids were added to wells in a microtiter dish (Black Isoplate®, Perkin Elmer). A twofold serial dilution series of the extract or pure compound was made, leaving the last well for reference (concentration of the tested agent=zero). Finally 150 µl 50× diluted o.n. culture of Tn5-LAS (OD_{450} of diluted culture=0.1) was added to the wells. The Gfp(ASV)-expression (measured as fluorescence; excitation and emission wavelength 485 and 535 nm, respectively) was measured every 15 min during the following 18 h by the use of the multilabel plate reader Wallac 1420 VICTOR^2 ™ (Perkin Elmer). The OD_{450} was also determined as a measure for growth. During the assay the temperature was held at a constant 34°C.

Results and Discussion

Screening of Marine Organisms. All specimens collected were sampled, extracted, and screened with the QSIS1 and QSIS2 screens. A high proportion of the samples demonstrated QSI activity, with 23% (64 out of 284) of the samples testing positive in the QSIS1 screen. A further 56% of these (36 out of 64) were also active in the specific *P. aeruginosa* based selector system QSIS2 (Table 1). The high frequency of organisms that produced QSI compounds may reflect that soft-bodied, sessile, or slow-moving marine invertebrates are rich in bioactive metabolites that might serve as defense against potential predators, competitors, and infectious microorganisms (reviewed in Paul et al. 2006). Sponge extracts provided the greatest number of active extracts (47%; Table 2), however, they were also the most common source of extracts (38.3%; Table 3). In terms of proportion of active extracts, soft corals provided the highest levels of relative activity, with 31.4% of soft coral extracts being active in QSIS1 and 25.7% in QSIS2 (Table 3). Sponges were the next most active group, with 28.8% of sponge extracts being active in QSIS1 and 15.4% in QSIS2 (Table 3). However, all major groups including the algae had some selective QSI activity (Table 3). This suggests that the ability to produce inhibitors of bacterial signaling is either inherited from a common ancestor or that this mechanism has evolved convergently.

Natural Products from Luffariella variabilis. The sponge *Luffariella variabilis* showed strong positive activity in both selector systems and was selected for a further study of a dose–response relationship using the QS reporter strain Tn5-LAS. *L. variabilis* produces the sesterterpenoids manoalide and secomanoalide as its major metabolites (Ettinger-Epstein et al. 2007). Manoalide and its related compounds are potent anti-inflammatory agents and irreversibly inactivate vertebral phospholipase A_2 (Bianco et al. 1995; Potts et al. 1992). Because the strong activity of the *L. variabilis* extract could be caused by inhibition of bacterial phospholipase in the QSIS1 system, secomanoalide, manoalide, and manoalide monoacetate were also tested in QSIS2. A phospholipase inhibitor may provide a false positive in QSIS1, but not in QSIS2. All three compounds were positive in both selector systems. To further investigate the activity of secomanoalide, manoa-

Table 3. (A) Proportion (%, and number) of the total number of extracts for each major taxonomic group, (B) percentage (and number) of QSIS1 active extracts relative to the number in each taxonomic group, and (C) percentage (and number) of QSIS2 active extracts relative to the number in each taxonomic group

	(A) Total extracts	*(B) QSIS1*	*(C) QSIS2*
Algae	12.6 (35 of 284)	14.3 (5 of 35)	8.6 (3 of 35)
Ascidians	16.6 (45 of 284)	17.8 (8 of 45)	8.7 (4 of 45)
Hard corals	15.2 (42 of 284)	21.4 (9 of 42)	9.5 (4 of 42)
Soft corals	7.6 (35 of 284)	31.4 (11 of 35)	25.7 (9 of 35)
Sponges	38.3 (104 of 284)	28.8 (30 of 104)	15.4 (16 of 104)
Others	12.6 (23 of 284)	4.3 (1 of 23)	0 (0 of 23)

lide, and manoalide monoacetate, we employed a QS reporter system harboring the *lasB-gfp*(ASV) fusion Tn5-Las in a *P. aeruginosa* PAO1 background (Hentzer et al. 2002). In this construction, the expression of unstable Gfp(ASV) is regulated by the QS-controlled *lasB*-promoter. This reporter strain produces signal molecules that during growth accumulate in the medium. Expression of *lasB* will be induced, leading to increased fluorescence after 8 h, demonstrating that "quorate" conditions were reached. The presence of an exogenous QSI compound will decrease expression of fluorescence proportional to the concentration and efficacy of the inhibitor. To establish a dose–response relationship, dilution series of the manoalide, secomanoalide, and manoalide monoacetate were incubated with the QS reporter, and growth and fluorescence were recorded over 18 h. The OD_{450} and fluorescence expressed by the QS reporter

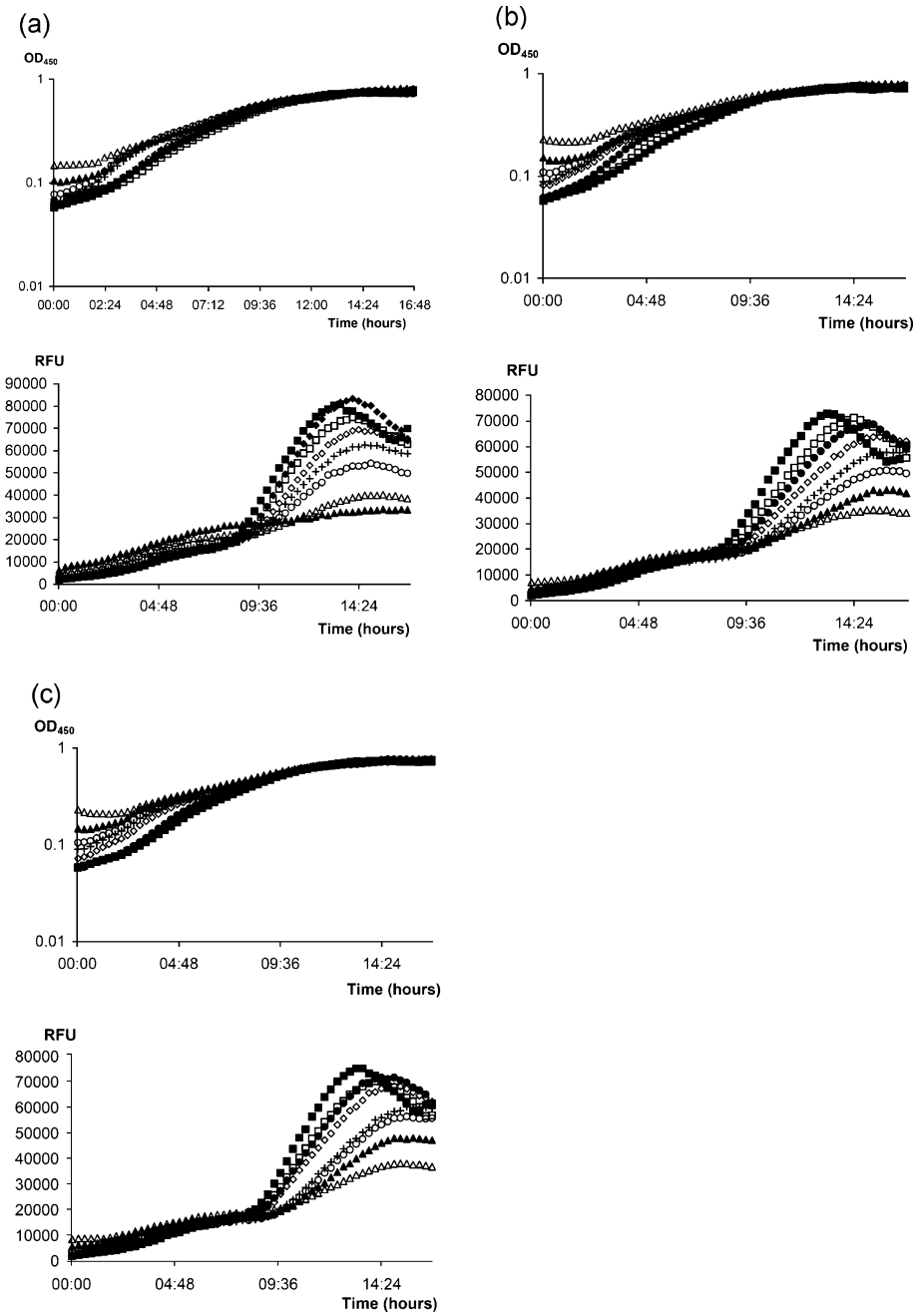

Figure 2. OD and relative fluorescence units (RFU). The QS reporter strain PAO1 TN5_LAS was incubated with different concentrations of (**a**) manoalide monoacetate and (**b**) secomanoalide: Δ, 20 μM; ▲, 10 μM; ○, 5 μM; +2.5 μM; ◇, 1.3 μM; •, 0.7 μM; □, 0.4 μM; ▪, reference, no compound added and (**c**) manoalide: Δ, 10 μM; ▲, 5 μM; ○, 2.5 μM+1.3 μM; ◇, 0.7 μM; •, 0.4 μM; □, 0.2 μM; ▪, reference, no compound added. These graphs represent the results of one out of several similar experiments.

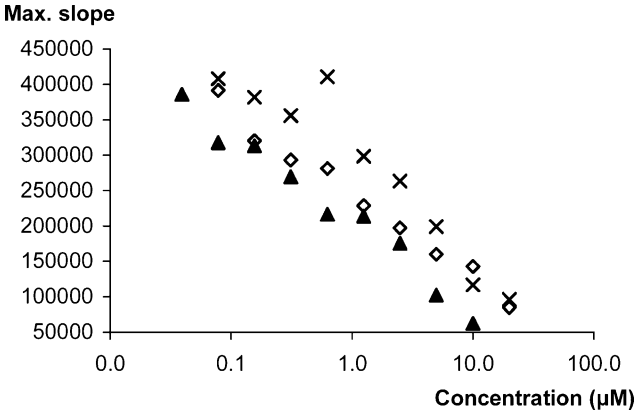

Figure 3. Maximal slope (ΔRFU/Δtime). The QS reporter strain PAO1 TN5_LAS was incubated with different concentrations of (▲) manoalide, (◇) secomanoalide, and (×) manoalide monoacetate. The slope for every time point has been calculated and the maximal slope is plotted as a function of the concentration.

in response to manoalide monoacetate, manoalide, and secomanoalide ranging from 0.2 μM to 20 μM are shown in Fig. 2. The slope of the resulting curves represents the synthesis rates (ΔRFU/Δtime) and is therefore a measure of gene expression of the QS controlled *lasB::gfp* fusion. The maximal slope for each concentration of the compounds is plotted as a function of the concentration of manoalide, manoalide monoacetate, and secomanoalide. The slope is calculated for each time point as $\frac{\sum(x_{a-c}-\overline{x})\cdot(y_{a-c}-\overline{y})}{\sum(x_{a-c}-\overline{x})^2}$ where x_a is the time before x_b, x_b is the time for which the slope is calculated and x_c is the time after x_b. Likewise y_a is the RFU (relative fluorescence unit) at time x_a, y_b is the RFU at the time x_b, and y_c is the RFU at time x_c. $\overline{x}$ is the average of x_a, x_b, and x_c and $\overline{y}$ is the average of y_a, y_b , and y_c. Reduction in synthesis rate (slope) is a measure of QS inhibition. As seen in Fig. 3, addition of manoalide monoacetate, manoalide, and secomanoalide reduced the slope in a dose-dependent manner. The efficacy of manoalide, secomanoalide, and manoalide monoacetate as QSIs can be calculated as IC_{50} [the concentration needed to reduce expression of the *lasB::gfp* (ASV) fusion to 50%] based on the logarithmic equation for synthesis rate as a function of the concentration of compound. The IC_{50} values are 0.658 μM, 1.110 μM, and 1.123 μM for manoalide, secomanoalide, and manoalide monoacetate, respectively.

The 2(5*H*)-furanone moiety, which is present in all three manoalide derivatives (Fig. 1), is conserved among many QSIs of natural origin such as the furanones, patulin, and penicillic acid (Givskov et al. 1996; Rasmussen et al. 2005b). Strawberries and peas (*Medicago truncatula*), which also possess QS inhibitory activity, produce compounds with a furanone moiety (Gao et al. 2003). Slaughter (1999) raised the hypothesis that over an evolutionary time scale some furanones, due to their properties, were utilized as interorganism signaling molecules, inducing a range of responses. The QSI activities of furanones produced by eukaryotes may represent a conserved mechanism for communication between eukaryotes and prokaryotes.

Conclusion

Two QSIS systems were used to screen 284 extracts of marine organisms collected on the central Great Barrier Reef, Australia. QS inhibitory activity was exhibited by 64 extracts (23%) from a range of hard and soft corals, algae, sponges, and seaweed. The presence of QSIs in all of the major groups of organisms collected indicates the importance of QS among marine living organisms and supports further investigations of the potential of marine derived QS inhibitors. This was further demonstrated by the QSI specific activity of the three related C_{25} sesterterpene metabolites—manoalide, manoalide monoacetate, and secomanolaide—from the sponge *Luffariella variabilis*. QSI active compounds have broad potential veterinary and biomedical applications (Rasmussen and Givskov 2006), and the screening of natural libraries with maximum diversity will increase the probability for providing new candidates for drug development.

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Article 3

Skindersoe, M. E., Phipps, R., Yang, L., Jensen, P.O., Rasmussen, T. B., Bjarnsholt, T., Høiby, N. and Givskov, M.

Effects of antibiotics on quorum sensing in Pseudomonas aeruginosa

Submitted to
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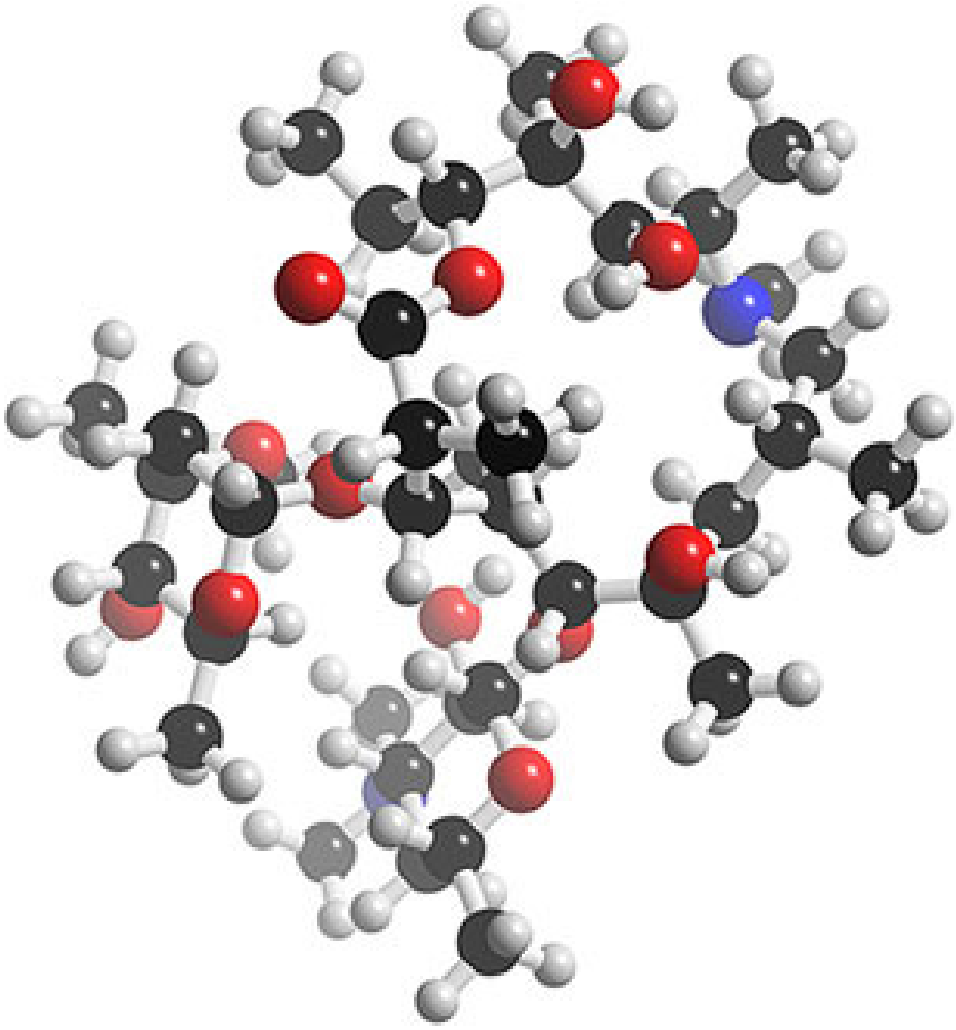

3D model of azithromycin

EFFECTS OF ANTIBIOTICS ON QUORUM SENSING IN PSEUDOMONAS AERUGINOSA

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Abstract

During the infectious process *Pseudomonas aeruginosa* employs bacterial communication (quorum sensing, QS) to coordinate expression of tissue damaging factors. QS controlled gene expression plays a pivotal role in the virulence of *P. aeruginosa* and QS deficient mutants cause less severe infections in animal infection models. Treatment of chronically *P. aeruginosa* infected Cystic Fibrosis (CF) patients with the macrolide antibiotic azithromycin (AZM) has been demonstrated to improve clinical outcome. Several studies indicate that AZM may accomplish its beneficial action in CF patients by impeding QS, thereby reducing pathogenicity of *P. aeruginosa.* This led us to investigate whether QS inhibition is a common feature of antibiotics. Here we present the results of a screening of 12 antibiotics for QS inhibitory (QSI) activity using the previously published QS Inhibitor Selector 1 (QSIS1). Three tested antibiotics: AZM, ceftazidime (CFT) and ciprofloxacin (CPR) were very active in the assay and were further examined for their effects on QS regulated virulence factor production in *P. aeruginosa*. The three antibiotics administered in sub-inhibitory concentrations were investigated by use of DNA microarrays. Although all three antibiotics down-regulate a significant proportion of QS regulated genes, the target specificities for QS controlled gene expression are lower than the QS inhibitor furanone C-30. However, consistent results from the virulence factor assays and DNA microarray support that AZM, CFT and CPR decrease expression of a range of QS regulated virulence factors. Our data suggest that these three antibiotics may, beside their traditional bactericidal and bacteriostatic applications, find use as quorum sensing inhibitors.

Key words

Azithromycin (macrolide), ciprofloxacin (fluoroquinolone), ceftazidime (cephalosporin), quorum sensing, quorum sensing inhibition, subMIC antibiotics

Abbreviations

AZM; azithromycin, CFT; ceftazidime, CPR; ciprofloxacin, LBD; Ligand Binding Domain, QS; quorum sensing, QSI; quorum sensing inhibitor(y), T3S Type III secretion

Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* causes severe infections, particularly in immunocompromised individuals and cystic fibrosis (CF) patients. The progressive deterioration of the lungs caused by chronic *P. aeruginosa* infections, and thus persistent inflammation, is currently the main reason for the morbidity and mortality in patients with CF (54). Intensive anti-pseudomonal treatment (maintenance therapy) has greatly improved the prospects for CF patients (25, 29, 71). A serious side-effect of antibiotic therapy is the development of resistance to the antibiotics used (2, 14, 28, 53). *P. aeruginosa* forms biofilms during the infection process which adds to the difficulties of eradicating infections by antibiotic intervention, since bacterial cells living as biofilms are much more tolerant to antibiotics than their planktonic counterparts (3, 13, 16). When the infection enters a chronic state the mucoid, alginate-overproducing phenotype dominate (21). Macrolides such as erythromycin, clarithromycin and the erythromycin derivative, azithromycin (AZM), have been shown to inhibit the enzymatic activity of guanosine diphospho-mannose dehydrogenate

in the alginate biosynthetic pathway of mucoid *P. aeruginosa* strains at concentrations well below the MIC (Minimal Inhibitory Concentration) value (42, 48). Alginate induces a continuous antigen–antibody reaction locally in the small airways. Thus a lowered alginate production will not only reduce the viscosity of the sputum, but may also lead to less inflammation and thus improved lung function (7, 39, 48). Several clinical studies have shown that long term treatment with AZM improves lung function and body weight in CF patients (27, 41, 75). AZM is not usually considered to exhibit anti-pseudomonal activity due to a MIC against *P. aeruginosa* in the range of 128-512 µg/mL (41). The highest concentrations of AZM found in the sputum of patients receiving high dose therapy (250 mg AZM daily) is 0.6-79.3 µg/mL, with the median AZM concentration being 9.5 µg /mL (5). It has been suggested that AZM treatment inhibits neutrophil recruitment to the lung by reducing proinflammatory cytokine expression and inhibition of neutrophil migration, resulting in a significant reduction in airway-specific inflammation (87). Macrolide antibiotics such as erythromycin, josamycin and AZM have been shown to change the cytokine profile and the phagocytic activity of splenocytes and peritoneal cells in *in vitro* experiments (63). It has been demonstrated that AZM reduces the production of the Th1 subset inducing cytokine IL-12 while slightly increasing IL-4. IL-4 induces differentiation of $CD4^+$ T cells into Th2 cells and thus it seems that AZM treatment has the capacity to modulate the Th1/Th2 balance in the Th2 direction (63). The Th2 subset is dominated by antibody mediated defense systems while the Th1 subset involve the cell mediated immunity. The Th1 type response is especially important in defending the host in the early stage of an infection, but is also associated with tissue destruction in chronic infections due to prolonged release of inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS) and elastases (26).

It has also been suggested that inhibition of cell-cell communication is the mode of action by which AZM exerts its activity in *P. aeruginosa* infections (59, 83). In Gram-negative bacteria cell-cell communication, also known as quorum sensing (QS), relies upon small diffusible signal molecules (*N*-acyl L-homoserine lactones, AHLs), which interact with transcriptional activators to couple gene expression with cell population density. The QS system of *P. aeruginosa* is organized hierarchically with the RhlI-RhlR components being subordinate to the LasI-LasR components. LasI directs synthesis of OdDHL (*N*-3-oxo-dodecanoyl-L-homoserine lactone) whereas RhlI synthesizes BHL (*N*-butanoyl-L-homoserine lactone). *P. aeruginosa* cells are permeable to BHL which freely diffuses over the cell membrane whereas active efflux is required for transportation of OdDHL (66). Besides the AHL signal molecules BHL and OdDHL, a third intercellular signal; 2-heptyl-hydroxy-4-quinolone (designated the *Pseudomonas* quinolone signal; PQS) has been found to be part of the QS regulon in *P. aeruginosa* (70). They all function in concert to control the expression of an array of genes, including genes encoding tissue damaging exoproducts (31, 50, 61, 65). Besides controlling production of virulence factors, the QS signal molecules PQS and OdDHL have been reported to possess immunomodulatory effects, and in this way contribute directly to the pathogenesis of *P. aeruginosa* (23, 40, 84). Several authors have shown the effects of AZM on expression of QS regulated virulence genes *in vitro* (59, 83). We have recently demonstrated by means of a CF-mouse model that AZM treatment, despite having no effect on growth of infecting bacteria, significantly improved clearance of pulmonary bacteria, reduced the extents of lung abscesses and decreased the severity of lung pathology and reduced alginate production *in vitro* and *in vivo* (38). Since QS seems to play a key role in the expression of virulence and interaction with host protection, inhibitors of QS have been suggested as important components of future anti-pseudomonal therapy (35). Examples of compounds that can block QS in *P. aeruginosa* are the halo-furanones (32, 36) originating from a benthic macroalgae and the fungal metabolites patulin and penicillic acid (73). Furanone C-30 inhibits QS at a concentration range from 1 to 10 µM but administered in excess *i.e.* in concentrations 10 fold higher, the compound impairs growth and exhibit antibiotic properties. In addition to QSI activity, the bioactive halo-furanones exhibit a multitude of activities including antibiotic and anti-fouling properties and can therefore be considered as multifunctional compounds engaged in communication as well as competition (for a recent review see (20)). Recently it was demonstrated that subminimum inhibitory concentrations (subMICs) of antibiotics broadly affect patterns of transcription in bacteria and seems to function as signaling mediators,

rather than targeting growth of rivals (33). This raises the question whether other compounds previously identified for their antibiotic properties may turn out to possess communication interfering properties and potentially QS inhibitory (QSI) activity. In line with that, we present the results of a screening of 12 antibiotics for QSI activity, identifying three antibiotics; AZM, ciprofloxacin (CPR) and ceftazidime (CFT) exhibiting strong QSI activity in the assay. The effect of these three antibiotics was further investigated by means of transcriptome analysis and phenotype assays.

Results and discussion

Screening for QSI activity

AZM has been shown to inhibit expression of a subfraction of QS controlled genes (59, 83). Along with AZM, we have investigated 11 other antibiotics (including one antifungal compound; griseofulvin) for the ability to interfere with bacterial QS using a simple plate diffusion screen based on the Quorum Sensing Inhibitor Selector QSIS1 (72). By employing our standard conditions (72), we found that chloramphenicol, gentamicin, griseofulvin, kanamycin, piperacillin, spectinamycin, tetracycline, tobramycin and streptomycin showed low or no QSI activities whereas AZM, in accordance with previous investigations, was very active. Also the fluoroquinolone, ciprofloxacin (CPR) and the cephalosporin, ceftazidime (CFT) showed strong QSI activities in the QSIS1 assay (Fig. 1).

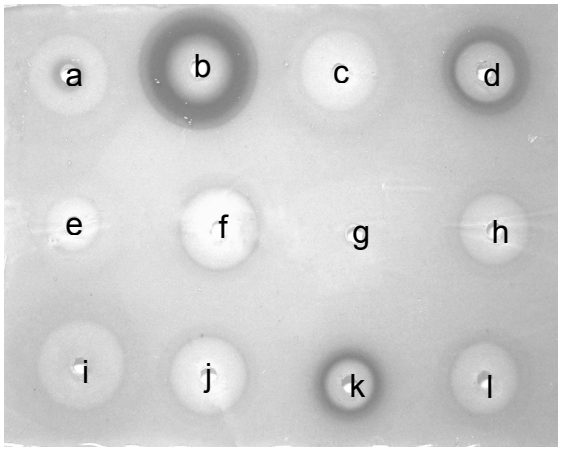

Figure 1. Screening of antibiotics by the use of the QSIS1 assay a) piperacillin b) azithromycin c) chloramphenicol d) ciprofloxacin e) gentamicin f) spectinamycin, g) griseofulvin h) kanamycin i) tetracycline j) tobramycin k) ceftazidime l) streptomycin

CPR and CFT are employed in the clinic as anti-pseudomonal treatments, whereas AZM exhibits low if any bactericidal or bacteriostatic effect on *P. aeruginosa*. CPR inhibits bacterial DNA synthesis, by targeting DNA gyrase (topoisomerase II), responsible for the supercoiling and uncoiling of the DNA. Uncoiling of the structure is the initial step for replication, transcription, and repair of the DNA, thus inhibition of this step will eventually lead to the death of the cell. CFT is a β-lactam and disrupts the synthesis of the peptidoglycan layer of bacterial cell walls. The mode of action of AZM is to bind the 50S subunit of the bacterial ribosome thereby inhibiting translation of mRNA. These three antibiotics therefore exhibit diverse mechanisms of antimicrobial action and they are structurally very different. We chose to further investigate the effects on *P. aeruginosa* QS regulated gene expression and virulence factor production with AZM, CPR and CFT as representatives for these widely diverse groups of antibiotics.

AZM, CPR and CFT interfere with production of QS controlled virulence factors.

Heat stable hemolysin

First we investigated the effect of AZM, CPR and CFT on the production of heat stable hemolysin. *P. aeruginosa* produces at least two hemolysins; the heat-labile phospholipase C (PA0844, *phlC*) and the heat-stable rhamnolipid (PA3479, PA3478, PA1130 and PA3387, *rhlA-C, G*) (8, 45, 81). Besides being a hemolysin, rhamnolipid has also been reported to immediately lyse neutrophils, macrophages and various animal cells (44, 56, 80). Expression of rhamnolipid therefore contributes significantly to the virulence of *P. aeruginosa.* Rhamnolipid synthesis is turned on in early stationary phase of *P. aeruginosa* PAO1 cultures (61, 88). After 14-18 hours of growth (under the conditions outlined in the Materials and Methods section), the concentration of rhamnolipid in the supernatant exceeds 50 μg/mL, which is sufficient to lyse erythrocytes within 20 minutes (our observation). The lysis of erythrocytes by rhamnolipid can be used as a simple assay for detection of rhamnolipid by mixing autoclaved supernatant with blood and incubate the mixture at room temperature for 20 minutes. A clearing and a non-precipitating red coloring of the blood-supernatant solution indicates rhamnolipid present at a concentration above 50 μg/mL, while lack of

hemolytic activity (characterized by the precipitation of intact blood cells) indicates that little or no rhamnolipid is present in the supernatant. Investigating supernatants from *P. aeruginosa* grown in the presence or absence of AZM, CPR and CFT and supernatants from the QS mutant Δ*lasI*-Δ*rhlI,* we found that only the untreated wild-type produced enough rhamnolipid to cause rapid hemolysis of the red blood cell. However, treatment with CFT did not fully abolish hemolytic activity (Fig. 2).

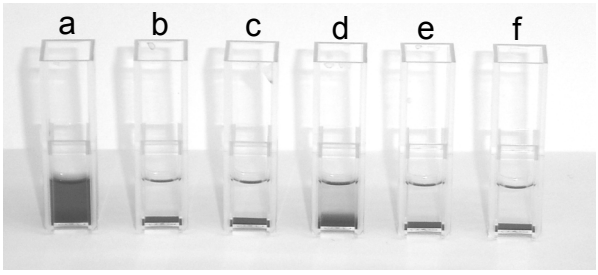

Figure 2. Hemolysis of autoclaved supernatants from *P. aeruginosa* PAO1 cultures grown for 20 h. From left to right a) PAO1 grown without antibiotics, PAO1 grown in the presence of: b) 2 µg/mL AZM, c) 8 µg/mL AZM, d) 0.25 µg/mL CFT, e) 0.04 µg/mL CPR, and f) the Δ*lasI*-Δ*rhlI* mutant of PAO1.

To quantify the effect of AZM, CFT and CPR on rhamnolipid production we determined the concentration of rhamnolipid in supernatants of treated and untreated *P. aeruginosa* by means of LC-MS. When grown in the presence of 8 µg/mL AZM, the concentration of rhamnolipid was reduced by 80% compared to untreated cultures, 2 µg/mL AZM reduced the rhamnolipid content of the supernatants to 25% compared to the untreated controls, whereas CPR (0.04 µg/ml) and CFT (0.25 µg/ml) reduced the concentration of rhamnolipid to around 60% of the untreated cultures. The QS mutant Δ*lasI*-Δ*rhlI* did not produce any measurable amount of rhamnolipid. (Fig 3).

Protease

The production of extracellular protease in *P. aeruginosa* PAO1 cultures grown in the absence or presence of AZM, CPR or CFT, as well as the Δ*lasI*-Δ*rhlI* mutant was assayed by adding sterile-filtered supernatants from overnight cultures to wells made in agar plates containing 5% skimmed milk. Protease activity was significantly lowered by AZM, CPR and CFT. (Fig. 4).

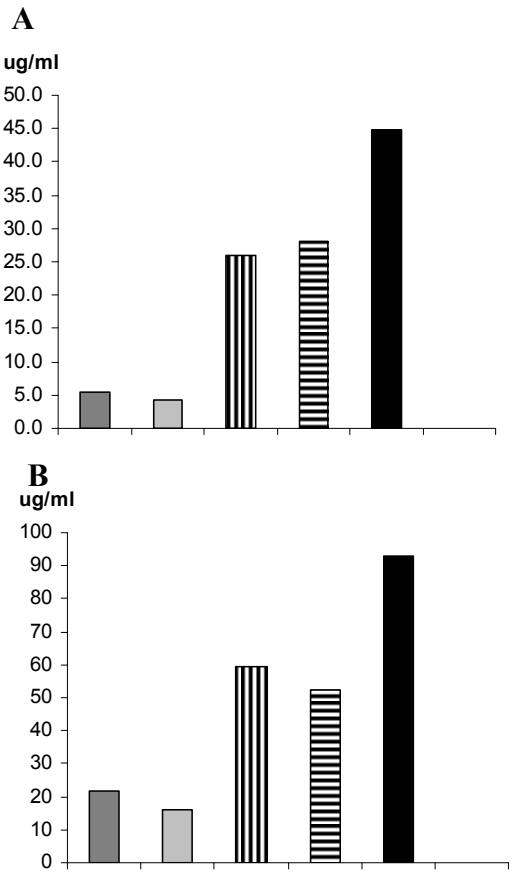

Figure 3. Determination of the concentrations of rhamnolipid in supernatants from *P. aeruginosa* cultures grown for 16 h (A) and 20 h (B) in the presence of: 2 µg/mL AZM (dark grey bar), 8 µg/mL AZM (light grey bar), 0.25 µg/mL CFT (bar w. vertical lines), 0.04 µg/mL CPR (bar w. horizontal lines), PAO1 grown without antibiotics (black bar) and Δ*lasI*-Δ*rhlI* mutant of PAO1 (no measurable rhamnolipid production, thus not visible).

Elastase

Extracellular elastase activity in the supernatants of *P. aeruginosa* PAO1 cultures (untreated or grown with AZM, CPR or CFT) was quantified by the use of the procedure from (62) (slightly modified). The QS mutant Δ*lasI*-Δ*rhlI* was assayed by the same procedure. AZM treatment reduced the elastase activity almost to the level of the Δ*lasI*-Δ*rhlI* mutant. CPR and CFT also reduced the elastase activity. (Fig. 5).

Chitinase

Chitinase activity in the supernatants of *P. aeruginosa* PAO1 cultures (untreated or grown with AZM, CPR or CFT, or the QS mutant Δ*lasI*-Δ*rhlI*) was quantified by measuring degradation of

chitin azure. AZM and CPR reduced the chitinolytic activity almost to the level of the Δ*lasI*-Δ*rhlI* mutant. CFT did not decrease chitinase activity as much as AZM and CPR, although still having a significant effect. (Fig. 6).

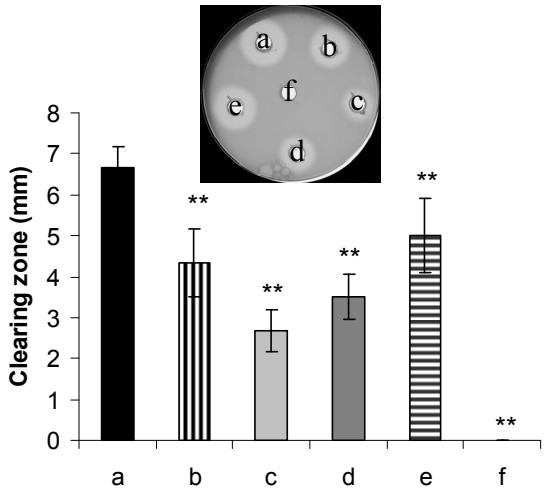

Figure 4. Protease activity of supernatant grown without antibiotics a) or in the presence of b) 0.04 µg/mL CPR, c) 8 µg/mL AZM, d) 2 µg/mL AZM e) 0.25 µg/mL CFT and f) supernatant from the Δ*lasI*-Δ*rhlI* mutant of PAO1. (** $P<0.01$, Dunnett's test). The experiment was performed as four replicates. Data are representative of two independent experiments.

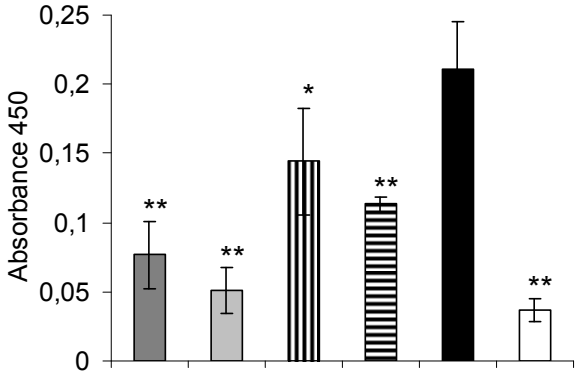

Figure 5. Elastolytic activity of supernatants from PAO1 cultures grown to OD_{600} 2.0 in the presence of: 2 µg/mL AZM (dark grey bar), 8 µg/mL AZM (light grey bar), 0.25 µg/mL CFT (bar w. vertical lines), 0.04 µg/mL CPR (bar w. horizontal lines), PAO1 grown without antibiotics (black bar) and Δ*lasI*-Δ*rhlI* mutant of PAO1 (white bar). (* $P<0.05$, ** $P<0.01$, Dunnett's test). The experiment was performed as triplicates. Data are representative of two independent experiments.

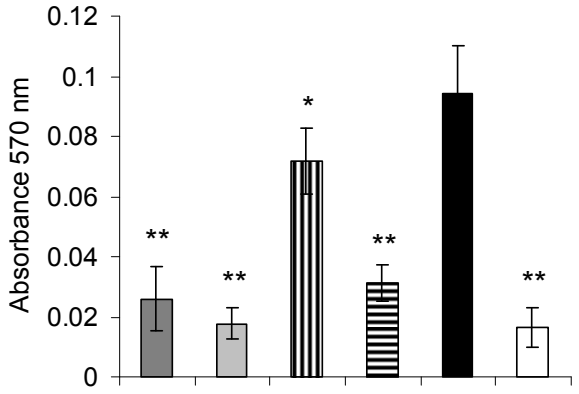

Figure 6 Chitinase activity of supernatants from PAO1 cultures grown to OD_{600} 2.0 in the presence of: 2 µg/mL AZM (dark grey bar), 8 µg/mL AZM (light grey bar), 0.25 µg/mL CFT (bar w. vertical lines), 0.04 µg/mL CPR (bar w. horizontal lines), PAO1 grown without antibiotics (black bar) and Δ*lasI*-Δ*rhlI* mutant of PAO1 (white bar). (* $P<0.05$, ** $P<0.01$, Dunnett's test). The experiment was performed as four replicates. Data are representative of two independent experiments.

DNA microarray analysis of gene expression in the presence of AZM, CFT and CPR

In order to obtain knowledge of the mechanism by which virulence factor production was altered by presence of the three antibiotics we performed DNA microarray analyses on *P. aeruginosa* cultures treated with the three antibiotics AZM, CPR and CFT in concentrations not affecting growth. Our experience from screening large numbers of compound libraries is that toxic or growth inhibitory compounds may show QSI-like activity (unpublished observations). This is likely to be caused by pleiotropic effects rather than a "true" QSI activity (such as interaction with the QS receptor), since the effect on QS controlled gene expression disappears when the concentration of the compound is lowered to a level that allows the bacteria to attain the optimum growth rate in a given medium. Furthermore, since obstruction of QS (either by mutation or antagonistic drugs) will not inhibit growth, we consider it essential to investigate potential QSI activities of the three antibiotics under conditions not affecting growth.

The medium of exponentially growing *P. aeruginosa* cultures was either supplemented with AZM (2 and 8 µg/mL), CFT (0.25 µg/mL) or CPR (0.04 µg/mL) at OD_{600} 0.1 or left untreated as a control. These concentrations clearly did not affect growth. Growth curves for *P. aeruginosa* grown in

the presence or absence of AZM, CPR and CFT in different concentrations (including concentrations with slightly growth inhibiting effects) and the Δ*lasI*Δ*rhlI* mutant are presented in Fig. 7.

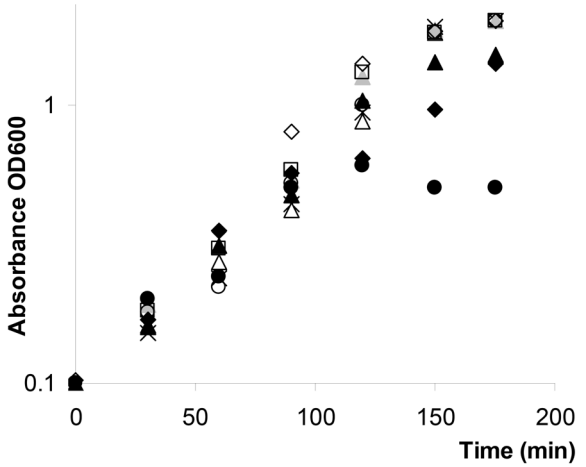

Figure 7. Growth curves for the Δ*lasI*-Δ*rhlI* mutant (□) and PAO1 grown without antibiotics (×) or PAO1 grown in the presence of 2 μg/mL AZM (Δ), 8 μg/mL AZM (▲), 16 μg/mL AZM (▲), 0.04 μg/mL CPR (○), 0.08 μg/mL CPR (●), 0.25 μg/mL CFT (◊) and 0.5 μg/mL CFT (♦).

Applying the growth conditions described in the Materials and methods section, OD_{600} 2.0 corresponds to the transition from exponential phase into stationary phase, and at this point a substantial fraction of QS regulated genes are maximally regulated (induced/repressed) (78). OD_{600} 2.0 was therefore chosen as sample point for the transcriptomic analysis. Total RNA from the samples were extracted and subjected to cDNA synthesis. The absolute expression values from cultures grown in the presence of antibiotics were compared with untreated cultures and the changes in expression reported as simple fold. According to (36, 88) expression changes below 5 fold are disregarded. Only "present genes" – as validated by Affymetrix software GeneChip Operating System (GCOS) version 1.4 – were included in the analyses. (Table I).

According to these criteria, non-growth inhibitory concentrations of AZM altered the expression of 488 (8 μg/mL) and 276 (2 μg/mL) genes, with most genes (427 and 227, respectively) being down-regulated. Non-growth inhibitory concentrations of CFT (0.25 μg/mL) and CPR (0.04 μg/mL) affected the expression of 146 (136 repressed) and 281 genes (223 repressed), respectively. (Table II).

AZM, CFT and CPR affect expression of QS regulated genes

The QS regulon has been defined by Rasmussen *et al*., 2005 (73). This particular study considers genes to be QS regulated if their expression are consistently altered >5 fold in both Δ*lasI*-Δ*rhlI* and Δ*lasR*-Δ*rhlR* mutants compared to their wild-type counterpart. The majority of all QS regulated genes defined this way are inducible with exogenous AHLs in a Δ*lasI*-Δ*rhlI* background. Only one gene (PA1556, a probable cytochrome c oxidase subunit) is consistently upregulated in both the Δ*lasI*-Δ*rhlI* and the Δ*lasR*-Δ*rhlR* mutant and thus QS repressible. Using this QS regulon we analyzed the fraction of QS regulated genes among the genes repressed by the three antibiotics. These analyses showed that AZM (8 μg/mL) repressed 70% of the 174 QS regulated genes in *P. aeruginosa* PAO1 and that 28% percent of the total number of genes being repressed by AZM are members of the QS regulon. Lowering the concentration of AZM to 2 μg/mL caused a relief in inhibition of QS regulated genes (47% of all QS genes repressed) and the total number of genes down-regulated fell to 227 however, the specificity toward the QS regulon increased to 36%. CPR accounts for the highest specificity towards the QS regulon (40%) and targeted 51% of all QS genes in *P. aeruginosa*, whereas the specificity for CFT was 36% and CFT only repressed 28% of all QS genes. For comparison, the specificity of C30 is 48% and it down-regulates 72% of all QS genes in *P. aeruginosa* (C30 data from (36), applied to the same data treatment and criteria).

As outlined above, members of the QS regulon are overrepresented among the genes repressed by subMIC levels of AZM, CFT and CPR. This suggests that these antibiotics exhibit some specificity for QS regulated gene expression through interaction with, or effect on QS regulatory components. Investigating the expression profile of LasR, RhlR or LasR-RhlR regulated genes, we further aimed to elucidate potential QS target(s) of the antibiotics. The QS regulated genes can be divided into 4 groups. Group A contains 44 LasR dependent genes, *i.e.* genes down-regulated in both a Δ*lasR* and a Δ*lasR*-Δ*rhlR* mutant. Group B consists of RhlR dependent genes (14 genes), genes down-regulated in both a Δ*rhlR* and a Δ*lasR*-Δ*rhlR* mutant. Group C comprises the main fraction of QS regulated genes (94 genes in total) and consists of genes which require both LasR and RhlR to be expressed

Table I. The effect of subMIC of AZM, CFT, and CPR on *P. aeruginosa* genes belonging to the QS regulon defined by (73). This regulon consist of genes down regulated in both a Δ*lasI*-Δ*rhlI* and a Δ*lasR*-Δ*rhlR* mutant of PAO1.

PaNumber	Gene	*pprB*	*	Reg.	AZM 2	AZM 8	CFT	CPR	*lasIrhlI*	*lasR-rhi*	*lasR*	*rhlR*	Description
PA0059	*osmC*			C	-28.1	-61.6	-10.9	-15.9	-22.0	-31.8	-44.0	-25.8	osmotically inducible protein OsmC
PA0105	*coxB*			C	-11.2	-30.3	-10.0	-11.5	-11.5	-13.5	-18.7	-7.9	cytochrome c oxidase, subunit II
PA0107				C	-14.2	-30.7	-10.3	-20.5	-17.4	-14.1	-18.6	-11.4	conserved hypothetical protein
PA0108	*coIII*			C	-10.9	-44.8	-9.9	-11.9	-27.8	-8.5	-15.8	-6.3	cytochrome c oxidase, subunit III
PA0122		-		C	-2.0	-15.3	-1.3	-2.7	-44.8	-26.1	-7.1	-6.0	conserved hypothetical protein
PA0188				C	-3.3	-61.2	-5.5	-5.2	-8.1	-21.3	-28.2	-8.8	hypothetical protein
PA0355	*pfpI*			C	-10.2	-15.6	-3.9	-7.5	-11.8	-21.5	-17.7	-11.6	protease PfpI
PA0567				C	-13.2	-21.8	-3.4	-8.8	-249.4	-25.2	-22.1	-9.5	conserved hypothetical protein
PA0572				A	-1.8	-7.1	-2.8	-2.9	-10.4	-13.7	-8.2	-1.1	hypothetical protein
PA0737				C	-2.5	-4.4	-3.4	-5.6	-9.4	-7.7	-94.4	-5.8	hypothetical protein
PA0843	*plcR*			A	-3.4	-11.1	-2.5	-4.4	-7.1	-6.2	-25.0	-4.6	phospholipase accessory protein PlcR precursor
PA0852	*cbpD*		-	C	-4.8	-36.5	-5.3	-10.7	-63.7	-74.4	-33.2	-8.3	chitin-binding protein CbpD precursor
PA0990				A	-15.5	-3.7	-2.6	-3.4	-5.7	-19.9	-6.6	-4.4	conserved hypothetical protein
PA0996	*pqsA*	-	+	A	1.3	1.5	1.2	-1.3	-7.0	-20.8	-34.5	3.3	probable coenzyme A ligase
PA0997	*pqsB*	-		A	1.2	-1.1	1.1	-1.1	-12.6	-15.4	-59.2	3.1	Homologous to beta-keto-acyl-acyl-carrier protein synthase
PA0998	*pqsC*	-		A	1.3	-1.2	1.0	-1.1	-8.2	-13.9	-29.9	2.8	Homologous to beta-keto-acyl-acyl-carrier protein synthase
PA0999	*pqsD*	-		A	1.4	-1.1	-1.0	-1.3	-8.2	-9.8	-20.2	2.6	3-oxoacyl-[acyl-carrier-protein] synthase III
PA1000	*pqsE*			A	1.7	1.3	1.4	1.3	-7.9	-12.0	-28.9	3.6	Quinolone signal response protein
PA1001	*phnA*	-		A	1.7	1.1	-1.0	-1.5	-6.6	-9.7	-20.9	3.2	anthranilate synthase component I
PA1002	*phnB*			A	1.5	-1.0	1.2	-1.3	-7.3	-5.2	-6.3	5.5	anthranilate synthase component II
PA1131				B	-2.4	-6.8	-1.4	-2.9	-5.9	-11.1	-2.9	-6.6	probable major facilitator superfamily (MFS) transporter
PA1168				A	-4.6	-3.7	-5.2	-1.5	-21.5	-19.1	-26.6	-3.9	hypothetical protein
PA1190				C	-71.4	-18.2	-6.7	-10.9	-14.6	-12.4	-12.5	-10.4	conserved hypothetical protein
PA1242				C	-29.8	-5.1	-5.0	-11.1	-19.6	-21.3	-6.7	-9.2	hypothetical protein
PA1249	*aprA*			D	-7.4	-52.8	-4.3	-5.7	-7.3	-8.4	-4.1	-2.7	alkaline metalloproteinase precursor
PA1250	*aprI*			D	-1.2	-1.5	1.3	-1.4	-9.7	-5.5	-3.0	-1.0	alkaline proteinase inhibitor AprI
PA1323				C	-23.1	-41.3	-4.0	-8.4	-25.3	-39.3	-56.5	-15.5	hypothetical protein
PA1324				C	-20.2	-25.6	-5.2	-7.9	-22.1	-26.7	-39.2	-17.0	hypothetical protein
PA1412				C	-13.1	-9.0	-30.5	-4.8	-5.7	-5.6	-8.8	-6.6	hypothetical protein
PA1431	*rsaL*	-		A	-1.9	-2.2	-1.5	-1.7	-3619.1	-3441.9	-3038.3	-1.9	regulatory protein RsaL
PA1432	*lasI*	-		A	-1.2	1.3	2.0	2.0	-97.0	-133.1	-39.0	-1.5	autoinducer synthesis protein LasI
PA1471				C	-4.9	-6.5	-1.8	-2.4	-7.8	-5.6	-10.3	-9.7	hypothetical protein
PA1556					9.4	18.8	9.2	10.1	7.9	6.0	7.8	5.3	probable cytochrome c oxidase subunit
PA1625				C	-5.4	-3.1	-3.2	-7.2	-6.6	-9.0	-6.5	-5.5	conserved hypothetical protein
PA1657				C	3.1	-1.5	1.5	3.3	-12.7	-15.4	-12.7	-5.2	conserved hypothetical protein
PA1662				D	-1.1	-4.0	-2.0	-1.4	-5.7	-9.0	-4.3	-4.4	probable ClpA/B-type protease
PA1664		-		C	1.9	-2.8	-1.3	1.1	-5.2	-11.0	-15.5	-8.7	hypothetical protein
PA1665				A	1.3	-3.0	-3.6	1.1	-5.6	-46.9	-15.1	-2.8	hypothetical protein
PA1667				A	1.3	-3.0	-1.2	-1.1	-5.5	-5.1	-5.0	-3.1	hypothetical protein
PA1669				B	1.2	-4.0	-1.9	-1.7	-28.6	-12.7	-3.8	-10.3	hypothetical protein
PA1784				A	-2.9	-18.5	-2.5	-4.7	-9.4	-11.2	-9.9	-1.4	hypothetical protein
PA1869		-		C	-1.0	-3.1	-1.0	-2.6	-137.1	-287.9	-23.6	-25.1	probable acyl carrier protein
PA1870				C	-21.6	-16.2	-10.3	-6.6	-22.0	-12.7	-8.8	-13.8	hypothetical protein
PA1871	*lasA*	-	-	C	-5.4	-35.6	-3.2	-8.5	-105.4	-147.8	-31.3	-7.1	LasA protease precursor
PA1874				A	-6.8	-13.6	-7.8	-9.2	-8.0	-6.4	-7.9	1.9	hypothetical protein
PA1875				A	-4.7	-7.0	-6.8	-12.9	-34.2	-9.0	-6.7	1.1	probable outer membrane protein precursor
PA1901	*phzC2*	-		C	1.0	-25.3	-3.4	-4.0	-21.8	-25.3	-19.1	-51.2	phenazine biosynthesis protein PhzC
PA1902	*phzD2*	-		C	-1.2	-20.5	-3.0	-4.1	-133.5	-132.0	-97.7	-91.0	phenazine biosynthesis protein PhzD
PA1903	*phzE2*	-		C	1.2	-18.4	-2.8	-4.7	-17.2	-24.4	-18.6	-19.7	phenazine biosynthesis protein PhzE
PA1904	*phzF2*	-		C	1.3	-18.5	-3.2	-4.0	-134.0	-61.6	-77.4	-44.7	probable phenazine biosynthesis protein
PA1905	*phzG2*	-		C	1.3	-18.6	-2.5	-4.0	-14.1	-32.7	-20.8	-30.0	probable pyridoxamine 5'-phosphate oxidase
PA1914				A	-29.6	-36.2	-6.9	-8.7	-92.8	-15.1	-100.0	-1.4	conserved hypothetical protein
PA2021				C	-22.1	-17.3	-5.6	-12.5	-8.6	-12.1	-12.2	-13.2	hypothetical protein
PA2046				C	-3.6	-35.7	-7.5	-8.2	-32.6	-22.1	-38.7	-45.5	hypothetical protein
PA2067				A	-1.8	-16.7	-2.0	-7.5	-6.0	-5.7	-5.3	-4.2	probable hydrolase
PA2068		-		C	-2.3	-14.0	-1.9	-6.7	-8.5	-19.4	-18.5	-25.0	probable major facilitator superfamily (MFS) transporter
PA2069		-		C	-2.1	-38.3	-2.0	-8.1	-29.3	-72.9	-26.1	-26.8	probable carbamoyl transferase
PA2137				C	-36.0	-160.1	-73.0	-22.6	-11.4	-15.9	-46.4	-71.3	hypothetical protein
PA2139				C	-74.8	-8.7	-15.2	-15.6	-9.5	-10.0	-7.7	-24.3	hypothetical protein
PA2141				C	-19.5	-42.7	-11.5	-56.0	-5.8	-45.3	-80.1	-69.0	hypothetical protein
PA2142				C	-45.6	-27.7	-12.6	-19.7	-13.4	-16.4	-18.2	-26.8	probable short-chain dehydrogenase
PA2143				C	-17.6	-23.8	-6.3	-23.3	-67.1	-60.1	-191.3	-83.7	hypothetical protein
PA2144	*glgP*			A	-5.0	-14.3	-7.6	-8.5	-7.3	-20.8	-11.4	-4.8	glycogen phosphorylase
PA2146			-	C	-7.1	-17.0	-11.0	-8.0	-6.9	-10.8	-15.5	-9.7	conserved hypothetical protein
PA2148				A	-17.0	-11.9	-4.5	-7.5	-8.1	-8.1	-6.4	-4.0	conserved hypothetical protein
PA2149				C	-7.8	-13.0	-5.2	-17.5	-6.3	-7.0	-39.5	-11.2	hypothetical protein
PA2151				C	-81.5	-135.8	-7.3	-10.8	-39.3	-124.4	-41.2	-30.6	conserved hypothetical protein
PA2153	*glgB*			C	-19.8	-51.5	-4.0	-5.9	-6.9	-11.3	-19.1	-9.2	1,4-alpha-glucan branching enzyme
PA2158				C	-8.7	-87.7	-10.9	-29.9	-65.0	-10.3	-35.9	-52.4	probable alcohol dehydrogenase (Zn-dependent)
PA2163				C	-21.0	-6.7	-3.9	-10.9	-8.2	-8.2	-6.9	-5.3	hypothetical protein
PA2165				C	-8.4	-26.9	-6.4	-13.9	-8.7	-5.4	-6.2	-7.5	probable glycogen synthase
PA2166				C	-28.2	-26.2	-6.2	-19.0	-15.4	-38.5	-24.4	-19.2	hypothetical protein
PA2167				C	-21.1	-147.4	-7.4	-14.8	-8.7	-8.1	-7.1	-15.4	hypothetical protein
PA2170				C	-12.4	-7.7	-2.9	-14.3	-8.2	-14.1	-5.4	-6.5	hypothetical protein
PA2171			-	C	-9.2	-15.0	-6.3	-38.5	-27.6	-109.9	-58.6	-22.5	hypothetical protein
PA2176				C	-109.1	-145.0	-8.6	-15.5	-19.2	-25.2	-30.0	-12.0	hypothetical protein
PA2178				D	-13.5	-4.3	-3.8	-37.5	-11.2	-6.3	-4.3	-4.9	hypothetical protein
PA2184				C	-6.8	-8.7	-5.5	-8.9	-15.5	-13.9	-17.5	-20.8	conserved hypothetical protein
PA2190			-	C	-16.4	-22.0	-5.5	-10.7	-17.0	-14.9	-9.9	-6.9	conserved hypothetical protein
PA2193	*hcnA*	-	+	B	2.1	-1.5	1.8	1.3	-262.2	-256.6	-3.0	-9.1	hydrogen cyanide synthase HcnA
PA2194	*hcnB*	-		B	1.6	-4.0	-1.1	-1.4	-123.1	-43.1	-2.3	-8.3	hydrogen cyanide synthase HcnB
PA2195	*hcnC*	-		B	1.5	-4.4	1.1	-1.3	-20.5	-38.9	-2.7	-6.9	hydrogen cyanide synthase HcnC
PA2300	*chiC*		-	C	-14.0	-58.3	-8.3	-25.1	-33.8	-66.7	-82.8	-34.0	chitinase
PA2302		-		A	-2.1	-10.0	-1.9	-3.5	-13.2	-17.5	-10.1	-1.2	probable non-ribosomal peptide synthetase
PA2303		-		A	-2.2	-8.6	-1.4	-2.4	-87.9	-212.8	-59.2	-1.3	hypothetical protein
PA2304		-		A	-1.3	-6.5	-1.8	-2.0	-18.1	-23.2	-9.2	1.1	hypothetical protein
PA2305				A	-1.2	-3.1	1.0	-1.3	-6.5	-16.7	-8.8	1.1	probable non-ribosomal peptide synthetase

PaNumber	Gene	*pprB*	*	Reg.	AZM 2	AZM 8	CFT	CPR	*lasIrhlI*	*lasR-rhi*	*lasR*	*rhlR*	Description
PA2414				C	-13.4	-87.3	-4.2	-15.2	-32.2	-17.6	-16.4	-7.7	L-sorbosone dehydrogenase
PA2415				C	-6.8	-9.7	-2.9	-6.7	-13.8	-9.3	-9.2	-5.2	hypothetical protein
PA2423			+	A	-1.6	-1.2	-1.2	-1.7	-5.8	-5.9	-7.6	1.6	hypothetical protein
PA2433				C	-28.5	-103.6	-6.0	-23.8	-25.9	-67.3	-70.0	-32.8	hypothetical protein
PA2485		+		C	-12.6	-7.3	-2.4	-6.6	-12.0	-18.0	-15.5	-12.3	hypothetical protein
PA2486				C	-6.9	-14.9	-2.4	-4.5	-8.9	-10.6	-8.5	-5.7	hypothetical protein
PA2566				A	-1.6	-6.9	1.1	-3.2	-9.6	-7.1	-6.3	-1.7	conserved hypothetical protein
PA2570	*lecA*			C	-9.0	-32.6	-3.7	-10.9	-45.4	-49.2	-27.2	-30.3	LecA
PA2587	*pqsH*	-		A	-1.3	-1.9	-1.5	-1.7	-46.1	-36.1	-20.4	1.3	probable FAD-dependent monooxygenase
PA2588				A	-2.9	-4.8	-2.5	-5.6	-6.7	-34.3	-87.8	-2.4	probable transcriptional regulator
PA2591				D	1.1	-1.3	1.1	-1.1	-7.1	-14.8	-2.2	1.1	probable transcriptional regulator
PA2592				D	1.3	-1.2	1.5	-1.1	-6.7	-17.9	-3.8	-1.6	probable periplasmic spermidine/putrescine-binding protein
PA2708				C	-6.3	-5.5	-3.9	-5.7	-5.8	-7.8	-8.7	-12.5	hypothetical protein
PA2747			+	C	-8.5	-7.1	-2.3	-7.0	-32.6	-37.3	-37.5	-10.3	hypothetical protein
PA2751				C	-2.5	-5.7	-2.2	-5.2	-8.5	-7.0	-10.3	-6.0	conserved hypothetical protein
PA2754				C	-7.1	-10.5	-2.3	-3.5	-11.5	-7.3	-9.1	-5.4	conserved hypothetical protein
PA2777				C	-13.3	-10.1	-4.2	-9.1	-8.4	-6.2	-10.5	-8.9	conserved hypothetical protein
PA2873				D	-1.7	-9.2	-1.4	-2.9	-13.2	-5.8	-1.6	-1.3	hypothetical protein
PA2937				C	-12.6	-57.1	-6.6	-12.5	-6.9	-8.6	-9.1	-5.4	hypothetical protein
PA2939		-		A	-4.7	-56.1	-3.5	-6.6	-39.8	-9.1	-12.7	1.8	probable aminopeptidase
PA3231				C	-23.3	-10.2	-4.8	-23.7	-33.7	-13.2	-27.7	-10.0	hypothetical protein
PA3273				C	-73.9	-12.0	-4.0	-7.9	-13.3	-13.2	-13.2	-27.1	hypothetical protein
PA3274				C	-20.2	-34.7	-6.2	-19.0	-45.1	-37.5	-112.5	-17.5	hypothetical protein
PA3326				D	1.1	-4.8	1.4	-1.1	-12.3	-11.0	-2.4	-4.1	probable Clp-family ATP-dependent protease
PA3327		-		C	1.2	-5.8	-1.0	1.3	-5.4	-12.7	-5.0	-9.8	probable non-ribosomal peptide synthetase
PA3328		-		B	-1.0	-4.4	-1.1	-1.2	-6.5	-26.0	-3.2	-12.4	probable FAD-dependent monooxygenase
PA3329		-		C	2.3	-3.2	1.8	1.4	-54.9	-173.5	-6.1	-69.3	hypothetical protein
PA3330		-		B	2.3	-3.3	1.8	1.3	-62.4	-179.6	-4.4	-19.5	probable short chain dehydrogenase
PA3331		-		B	1.7	-4.3	1.7	1.3	-19.8	-62.0	-3.8	-23.8	cytochrome P450
PA3332		-		B	1.5	-4.6	1.2	1.2	-10.2	-18.7	-5.0	-17.7	conserved hypothetical protein
PA3333	*fabH2*	-		B	-1.1	-6.0	1.0	-1.3	-17.0	-33.4	-4.1	-10.4	3-oxoacyl-[acyl-carrier-protein] synthase III
PA3334		-		C	1.4	-4.1	1.6	1.5	-42.8	-17.4	-5.1	-11.2	probable acyl carrier protein
PA3335		-		D	1.2	-4.4	1.9	1.3	-18.4	-9.4	-2.3	-3.9	hypothetical protein
PA3336				B	1.4	-3.7	1.7	-1.3	-8.2	-9.3	-4.3	-22.8	probable major facilitator superfamily (MFS) transporter
PA3361	*lecB*			C	-1.3	-13.9	-1.4	-2.2	-22.4	-28.9	-13.2	-15.3	fucose-binding lectin PA-IIL
PA3369			+	C	-10.8	-11.0	-4.3	-12.9	-19.9	-42.2	-36.5	-14.2	hypothetical protein
PA3370			+	C	-41.9	-25.2	-8.2	-30.1	-39.0	-66.4	-87.1	-31.2	hypothetical protein
PA3371			+	C	-21.0	-11.4	-8.0	-19.4	-23.8	-51.7	-69.2	-25.7	hypothetical protein
PA3460				A	-10.4	-18.9	-1.8	-3.4	-5.0	-6.0	-7.0	-4.7	probable acetyltransferase
PA3476	*rhlI*	-		D	-1.2	-2.0	-1.0	-1.5	-19.0	-41.4	-2.4	-1.8	autoinducer synthesis protein RhlI
PA3478	*rhlB*		-	C	-4.8	-32.3	-2.7	-6.8	-120.2	-122.7	-19.5	-53.0	rhamnosyltransferase chain B
PA3479	*rhlA*	-		C	-3.5	-30.5	-1.9	-5.1	-320.2	-196.0	-14.5	-55.0	rhamnosyltransferase chain A
PA3520				C	-2.6	-11.1	-1.8	-4.7	-28.3	-18.3	-13.9	-7.4	hypothetical protein
PA3581	*glpF*			C	-42.3	-18.4	-10.5	-45.8	-61.0	-88.9	-19.1	-35.1	glycerol uptake facilitator protein
PA3584	*glpD*			C	-8.9	-16.4	-8.5	-7.6	-5.7	-20.4	-12.0	-8.4	glycerol-3-phosphate dehydrogenase
PA3691				C	-8.5	-10.3	-1.8	-2.8	-11.6	-19.2	-28.8	-12.3	hypothetical protein
PA3692				C	-17.9	-26.1	-3.3	-3.6	-30.7	-16.8	-37.5	-12.3	probable outer membrane protein precursor
PA3724	*lasB*	-		C	-2.6	-20.9	-2.3	-6.3	-167.2	-224.3	-15.1	-5.2	elastase LasB
PA3734				C	-4.0	-10.8	-3.9	-5.3	-7.2	-21.3	-6.6	-8.5	hypothetical protein
PA3788				A	-8.1	-6.6	-3.0	-4.9	-9.2	-5.8	-6.1	-4.0	hypothetical protein
PA3819				C	-5.6	-4.3	-3.4	-2.7	-6.3	-7.0	-9.7	-7.1	conserved hypothetical protein
PA3888				A	-5.7	-3.8	-2.4	-3.8	-5.8	-10.2	-10.5	-4.6	probable permease of ABC transporter
PA3890				C	-4.4	-5.2	-2.6	-7.1	-17.8	-87.2	-64.7	-31.8	probable permease of ABC transporter
PA3904		-		A	1.5	1.7	1.5	1.7	-68.3	-40.7	-172.1	-1.0	hypothetical protein
PA3906		-		A	-1.0	-1.1	-1.3	-1.1	-7.5	-176.5	-9.5	-1.4	hypothetical protein
PA3907				A	1.3	-1.2	1.2	1.9	-5.8	-13.4	-5.2	1.0	hypothetical protein
PA3908		-		D	1.3	-3.7	-1.6	1.2	-6.2	-49.8	-4.5	1.1	hypothetical protein
PA4078			-	A	-4.4	-15.3	-3.8	-7.4	-5.6	-11.0	-7.4	-4.8	probable nonribosomal peptide synthetase
PA4130		-		D	1.9	-1.9	-1.2	1.1	-9.6	-6.2	-3.3	1.5	probable sulfite or nitrite reductase
PA4133		-		A	2.4	-1.8	-2.3	1.1	-57.4	-13.0	-6.1	1.6	cytochrome c oxidase subunit (cbb3-type)
PA4134		-		A	1.3	-3.2	-3.8	-1.5	-12.0	-5.6	-18.0	-1.2	hypothetical protein
PA4139				A	-6.4	-12.6	-4.2	-3.6	-6.1	-10.2	-7.0	-4.2	hypothetical protein
PA4141		-		C	-1.6	-10.3	-1.0	-1.4	-73.1	-105.0	-27.6	-57.6	hypothetical protein
PA4142				C	-2.0	-11.6	-2.2	-4.2	-27.2	-71.7	-25.6	-26.4	probable secretion protein
PA4143				C	-1.8	-16.7	-2.4	-3.9	-8.1	-15.3	-7.3	-9.0	probable toxin transporter
PA4171				C	-93.8	-10.9	-6.1	-6.8	-5.4	-15.5	-13.6	-9.5	probable protease
PA4175	*prpL*			A	-4.2	-21.5	-6.5	-17.1	-28.4	-47.1	-30.4	-1.6	Pvds-regulated endoprotease, lysyl class
PA4209	*phzM*			C	1.5	-15.4	-1.8	-2.1	-9.8	-18.8	-11.9	-7.5	probable phenazine-specific methyltransferase
PA4210	*phzA1*			C	1.0	-10.2	-2.1	-5.5	-65.1	-123.9	-79.7	-20.2	probable phenazine biosynthesis protein
PA4211	*phzB1*	-		C	1.3	-19.8	-2.1	-2.3	-51.0	-61.2	-59.7	-46.1	probable phenazine biosynthesis protein
PA4217	*phzS*	-		C	1.6	-11.4	-2.5	-2.3	-11.4	-12.9	-8.9	-10.9	flavin-containing monooxygenase
PA4290				C	-33.4	-22.9	-31.2	-31.5	-7.2	-6.0	-14.2	-15.9	probable chemotaxis transducer
PA4345				C	-12.2	-7.2	-4.0	-6.8	-10.6	-9.1	-7.7	-6.6	hypothetical protein
PA4377			-	A	-11.1	-17.2	-3.7	-8.0	-10.1	-7.0	-7.8	-3.2	hypothetical protein
PA4677				A	-3.4	-4.4	-3.8	-5.8	-17.0	-40.0	-22.5	-1.1	hypothetical protein
PA4738				C	-18.1	-19.4	-5.5	-10.8	-39.6	-54.4	-58.1	-16.6	conserved hypothetical protein
PA4739		-		C	-11.9	-14.4	-3.3	-5.2	-34.3	-34.3	-43.8	-16.6	conserved hypothetical protein
PA4876	*osmE*			C	-9.8	-11.0	-3.4	-4.4	-18.2	-9.1	-10.8	-6.7	osmotically inducible lipoprotein OsmE
PA4880				C	-7.2	-18.2	-4.7	-5.1	-6.0	-29.6	-12.9	-12.9	probable bacterioferritin
PA5097				D	-1.9	-32.9	-21.1	-5.7	-9.5	-5.2	-3.4	-4.8	probable amino acid permease
PA5099				C	-237.8	-531.6	-61.1	-753.1	-16.2	-14.7	-40.5	-39.9	probable transporter
PA5212				C	-6.6	-5.6	-2.0	-2.8	-8.8	-8.7	-12.1	-5.7	hypothetical protein
PA5220				B	-2.3	-6.3	-1.9	-2.6	-7.7	-8.9	-4.2	-6.9	hypothetical protein
PA5235	*glpT*			B	-3.4	-3.2	-2.1	-5.6	-15.2	-32.4	-2.8	-13.9	glycerol-3-phosphate transporter
PA5297	*poxB*			D	-22.7	-8.6	-2.3	-4.1	-17.8	-6.0	-3.8	-2.8	pyruvate dehydrogenase (cytochrome)
PA5480				B	-1.2	-3.1	1.0	-3.6	-14.2	-19.9	-2.5	-6.1	hypothetical protein
PA5481			+	C	-25.8	-26.4	-5.4	-14.1	-45.6	-50.7	-96.6	-22.6	hypothetical protein
PA5482			+	C	-13.6	-13.4	-3.9	-8.8	-59.6	-43.0	-81.1	-12.2	hypothetical protein

Text to table I. The effect of subMIC levels of AZM, CFT and CPR on genes belonging to the QS regulon defined by (73). This regulon consists of genes down-regulated in both a Δ*lasI*-Δ*rhlI* and a Δ*lasR*-Δ*rhlR* mutant of *P. aeruginosa* PAO1. Genes more than 5 times down regulated are marked grey and genes more than 5 times upregulated are marked black.

The column *pprB* reports genes being more than 5 times upregulated (+) or 5 times down regulated (-) in a Δ*pprB* mutant of PAO1 according to (24).

The column designated * reports genes previously reported to be upregulated (+) or down regulated (-) by AZM according to (59).

The column Reg. assigns the genes into one of the four QS regulation groups. Group A; Las dependent genes, group B; Rhl dependent genes, group C; genes requiring both a functional Las and Rhl system and group D are Las-Rhl regulated genes. See further explanation in the text.

Table II

Description	AZM 8 µg/mL (11 µM)	AZM 2 µg/mL (2.7 µM)	CFT 0.25 µg/mL (0.4 µM)	CPR 0.04 µg/mL (0.1 µM)	C30[c] 2.5 µg/mL (10 µM)
No. of genes > 5 fold down-regulated	427	227	136	223	260
No. of genes > 5 fold up regulated	61	49	10	58	0
No. of QS genes[a] affected (> 5 fold)	121	81	49	89	125
Percent of QS genes[a] affected (> 5 fold)	70%	47%	28%	51%	72%
Specificity [b]	28%	35%	36%	40%	48%

[a] Genes belonging to the QS regulon as defined by (73)

[b] Specificity; QS genes[a] repressed/total number of genes repressed

[c] Data for the QSI furanone C-30 at OD_{600} 2.0 included for comparison (Data presented in (36))

at normal levels, that is genes which are down-regulated in all three types of mutants; either Δ*lasR* or Δ*rhlR* or Δ*lasR*-Δ*rhlR*. Finally, group D consists of 14 LasR-RhlR regulated genes, which are only down-regulated if both LasR and RhlR are knocked out as in the Δ*lasR*-Δ*rhlR* mutant. Interestingly it seems that AZM, CFT and CPR have a low preference for the group B genes (the RhlR specific genes), whereas they have a high specificity for the group C genes. (Fig. 8).

Among the genes affected by AZM, CFT and CPR many encode previously identified QS regulated genes such as chitinase (*chiC,* PA2300) and chitin-binding protein (*cbpD,* PA0852) (30, 78, 88). Other known QS induced genes such as the genes encoding the osmotically inducible protein (*osmC*, PA0059) and cytochrome c oxidase (coxA-B and coIII, PA0105-PA0108) are down-regulated by the presence of the three antibiotics (36, 78). The endoprotease gene *piv* (also known as *prpL*, PA4175) is also down-regulated by AZM, CFT and CPR. This protease is capable of degrading elastin, decorin and the transferrin family of proteins, including lactoferrin (91). The only QS repressed genes from the regulon; PA1556 (cytochrome c oxidase) was upregulated by all three antibiotics together with PA1557.

Many genes from the PA2134 – PA2190 region of the genome were down-regulated by AZM, CFT and CPR. This region has been found to be

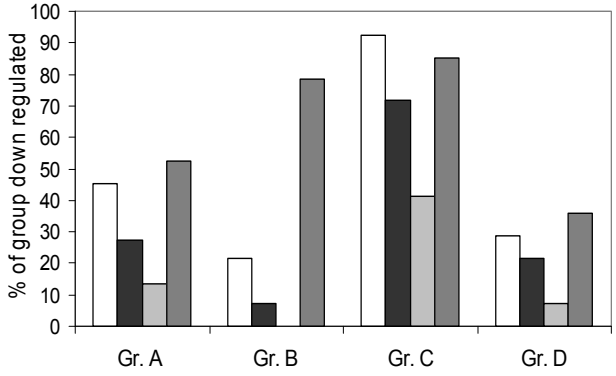

Figure 8. LasR, RhlR and LasR-RhlR specificity of AZM (white bars), CFT (black bars) and CPR (light grey bars) (and C30 (dark grey bars)). See definitions for group A, B, C and D in the text.

associated with stationary growth and to be under QS/*rpoS* control (77, 78, 88, 89). Out of the 57 genes in this 60.69 Kbp region, 46 were evaluated as present in the all arrays. Out of the 46 genes, 39 genes were more than 5 fold repressed by AZM, CFT and CPR. The general stress protein PA2190 was down-regulated in the presence of all three antibiotics along with genes involved in nutrient stress (*glgA* PA2165, *glgB* PA2153 and *glgP* PA2144). A *glgA* transposon insertion in *Escherichia coli* has been shown to cause the mutant to become severely defective in biofilm formation (43).

AZM and CPR seem to exhibit higher target specificity for QS genes than CFT which is in accordance with the virulence factor assays described earlier in this section. Many QS regulated virulence factors such as the alkaline metalloproteinase (*aprA*, PA1249), protease PfpI (*pfpI*, PA0355) LasA protease (*lasA,* PA1871) elastase B (*lasB*, PA3724) the cytotoxic galactophilic lectin (PA-IL, *lecA*, PA2570) and rhamnolipid (*rhlA, rhlB* PA3478-3479) were more than 5 fold repressed by AZM and CPR but not by CFT. Investigations have shown that biofilms of *P. aeruginosa* QS mutants have reduced tolerance to antibiotics compared with their wild-type parent. In line with that, inhibition of QS have been shown to promote eradication by antimicrobial treatments and make the biofilm more susceptible to phagocytosis by neutrophils (19, 36, 72). Since rhamnolipid and the adhesive lectin LecA are both QS regulated and involved in biofilm maturation (18, 22, 51, 64) repression of these genes may contribute to the development of a less structured and more antibiotic sensitive biofilm seen in QS mutants. Agents targeting expression of rhamnolipid, lectins and other biofilm factors either via QS or by other pathways are desirable in clinical settings as biofilm formation complicates the eradication of bacterial infections. Moreover, rhamnolipid and lectin LecA have been reported to cause cytotoxic effects on mammalian cells, including neutrophils and macrophages, and are also important in the infectious process of *P. aeruginosa* (4, 44, 56, 57, 79, 80, 94).

AZM, CFT and CPR alter gene expression of a variety of genes

A few genes were consistently upregulated by AZM, CFT and CPR. The QS repressed gene PA1556 (cytochrome c oxidase subunit) and PA1557 were more than 5 times induced. PA1673 (unknown function, 44% similar to hemerythrin, used for oxygen storage/transfer by a marine, sipunculid worm) was >5 fold induced by AZM and CPR but only 4.1 fold by CFT treatment. PA2260 (hypothetical protein, identified by (88) to be QS repressed) was induced by AZM and CFT but not CPR.

Several genes are solely induced by AZM. A high proportion of these genes have also been reported as AZM inducible by (59), and many encodes for ribosomal subunits such as PA3742 (*rplS,* 50S ribosomal protein L19) and PA4671 (probable ribosomal protein L25). The overexpression of ribosomal genes may be a mechanism to compensate for the effect of AZM which targets the 50S ribosomal subunit. The initiation factor *infA* (PA2619) was also induced in the presence of AZM in accordance with the findings by (59).

In agreement with two recent studies (11, 52) we found that CPR induces the expression of the pyocin S genes (PA0985, pyocin S5, PA1150 pyocin S2 and PA3866 pyocin S3 like). The S-type pyocins are protease sensitive bacteriocins and have colicin like structures and mode of actions; they contain an effector component possessing DNase and lipase activity as well as an immunity component.

The ribosome modulation factor (*rmf,* PA4296) was repressed by AZM but not affected by treatment with CFT and CPR. The transcriptome analyses by (59) also shows that *rmf* is repressed by AZM. *rmf* of *P. aeruginosa* shows a high homology to *rmf* of *E. coli* (similarity 66%). *rmf* mutants of *E. coli* have been shown to gradually lose viability in stationary phase cultures (60) however, a recent study concluded that *rmf* (in *E. coli*) may only be required under competitive growth conditions (12).

AZM induces expression of genes related to Type III secretion

In accordance with the findings by (59) we also found that AZM induced expression of genes involved in Type III secretion (T3S). *P. aeruginosa* uses its T3S system to secrete and translocate several cytotoxic products into the cytosol of eukaryotic cells. Expression of T3S increases pathogenicity of *P. aeruginosa* and is associated with more severe infections and higher mortality rate in animal models (1, 74, 76). QS has been shown to negatively modulate expression of the T3S system in *P. aeruginosa* (9), which means that the T3S system is down-regulated under high cell density conditions and thus presumably primarily plays a role in the early stages of the infection. Genes in the T3S system such as PA1700-1701 (conserved hypothetical proteins in T3S), *pcrGV* (PA1705 regulator in T3S and PA1706), *exsB* (PA1712, exoenzyme S synthesis protein B), *exsD* (PA1714, part of the *pscB-L* operon) and *pscBCEO* (PA1715, T3S export apparatus protein, PA1716, T3S outer membrane protein, PA1718, T3S export protein, PA1696 translocation protein in T3S) were upregulated by AZM. Neither CFT nor CPR however, induced genes in the T3S system gene cluster PA1690-

PA1725. Interestingly, a study by (49) showed that treatment of *P. aeruginosa* with subMIC macrolides (AZM, clarithromycin, erythromycin) prior to intranasal challenge of mice significantly enhanced the mortality rate (from 0% to 80-100%) In the same study however, macrolide antibiotics were also administered to mice *after* inoculation of *P. aeruginosa* PAO1, and in this case the mortality of mice did not increase (49). Thus it seems that activation of genes by macrolides only causes enhanced virulence if the macrolides are applied prophylactically and not as a part of the treatment. Still the potential risk of inducing virulence and cytotoxicity through the T3S system should be considered along with the potential benefits when applying AZM (and possibly other macrolides) to patients infected by *P. aeruginosa.*

AZM, CPR, CFT and the two component regulator system pprA-B

The QS induced chemotaxis transducer PA4290 was down-regulated by AZM, CFT and CPR. AZM and CPR also repressed the main part of the genes in the region PA4290-PA4306. Many of these genes have been shown to be QS regulated in previous studies where QS genes were identified by mapping genes down-regulated in Δ*lasI*-Δ*rhlI* mutants and restored to wild-type levels by addition of signal molecules (36, 78, 88). The two component regulator system *pprA-B* (PA4293, PA4296) was down-regulated by AZM and CPR but not by CFT. *pprA* was down-regulated 30 and 7.9 times by AZM and CPR, respectively, with *pprB* down-regulated 12.9 and 5.7 times by AZM and CPR, respectively. Expression of *pprA-B* has been shown to be QS regulated (78, 88), but the gene products have also been identified having QS modulatory activity, as yet another auto-feedback system of QS in *P. aeruginosa* (24). Knock out of *pprB* leads to reduced influx of the *P. aeruginosa* QS signal OdDHL thereby globally influencing the expression of QS regulated genes (24), but PprB is also involved in regulation of sensitivity to antibiotics. Overexpression of *pprB* results in an increased sensitivity to antibiotics, especially aminoglycosides, probably due to a decrease in membrane permeability (90). *In vivo* and *in vitro* studies have shown that *P. aeruginosa* develops an increasing tolerance to aminoglycosides following pre-treatment with these antimicrobials (17, 46, 92). It is possible that a similar mechanism operates in the presence of other antibiotics such as AZM and CPR and perhaps also CFT thereby inducing an adaptive down-regulation of *pprB* to decrease membrane permeability and further causing decreased OdDHL influx, which results in a change in expression of QS regulated genes. Support for this hypothesis can found in the observation that the three antibiotics have higher specificities for *las* regulated genes than *rhl* regulated genes (group B). The *rhl* signal molecule BHL is capable of freely diffusing over the membrane and is – unlike OdDHL - not dependent on active transport.

The study by (24) identified 53 genes more than five fold down-regulated by a Tn5 insertion in *pprB*. In comparison we found, as presented in this current study, that expression of 40 of those genes more than 5 fold reduced were down-regulated to half the level or less in the presence of AZM. This include 21 genes which were more than 5 fold down-regulated. We found that CPR down-regulated 20 out of the 53 genes more than 2 fold, including 6 genes more than 5 fold down-regulated. CFT caused a down-regulation of 14 of the 53 genes down-regulated by the *pprB* mutation with no genes being more than 4 times down-regulated. Genes reported to be PprB regulated include virulence factor genes such as *lasB, rhlAB, lecA, prpL* (24) which are also down-regulated by AZM and CPR. The expression of the QS transcriptional regulator RhlR and the AHL synthetase genes *lasI* and *rhlI* are also decreased by the *prpB* knock out mutation (24). This is not surprising as *rhl* is subordinate to the *las* system and the AHL synthetase genes are under QS control, which may provide negative feedback that enhances the QS repressing effect of the *pprB* mutation. Only AZM however, causes a down-regulation of *rhlR*, and none of the three antibiotics down-regulates the AHL synthetase genes. Additionally AZM decreased the expression of *lasR* 5 fold, which may also contribute to the QS inhibitory effect of AZM.

Docking of AZM, CFT and CPR against the Ligand Binding Domain of the LasR protein.

In order to explore the possibility that AZM, CFT and CPR directly interacts with the LasR protein, we performed an *in silico* docking of the three antibiotics against the recently published Ligand Binding Domain (LBD) of the LasR protein (10). None of the three antibiotics gave a high affinity score in the docking. In contrast to this, previously reported QSI compounds (*e.g.* C-30, patulin, furanone and 4-nitropyridine-*N*-oxide) exhibited

high affinity scores (Table III). Further the *in silico* analysis suggested that AZM, CFT and CPR do not fit inside the binding site cavity of LasR because they exhibit of steric clashes with the protein target. This suggests that AZM, CFT and CPR effects on QS may be pleiotrophic in nature.

Final comments

In the present paper we present the screening results of 12 antibiotics for QSI activity using the QSI screening system QSIS1. Three of the 12 antibiotics; AZM, CFT and CPR showed strong positive results in the screening system and were further investigated for their effect on production of virulence factors in *P. aeruginosa*. Administered in concentrations not affecting growth, AZM, CPR and CFT, inhibited production of the important virulence factors chitinase, protease, elastase and rhamnolipid production. Our recent data suggest that rhamnolipid functions as shield to protect biofilms against the predatory behavior of neutrophils, and rhamnolipid may thus be a pivotal virulence factor (44). DNA microarray analyses showed that QS genes were over-represented among the genes down-regulated by AZM, CPR and CFT, suggesting that the three antibiotics possess QS inhibitory activity. Furthermore, the three antibiotics exhibited a higher specificity for LasR regulated genes than RhlR dependent genes.

We speculate that the effect of AZM and CPR and perhaps also CFT is pleiotropic and channeled through the two component regulator system encoded by *PprAB*. Expression of this two component regulator system was down-regulated by AZM and CPR. PprB has been reported to control membrane influx of OdDHL and thereby affect QS in *P. aeruginosa. pprB* was first identified to be involved in sensitivity to antimicrobials by regulating membrane permeability in response to the presence of antibiotics. This suggests that the mechanism by which AZM and CPR affects QS is to lower influx of OdDHL over the *P. aeruginosa* membrane by down-regulating expression of PprB in response to the presence of antibiotics.

QSIs constructed using AHL scaffolds are designed to bind to the LuxR homologue without activating it thereby blocking the binding and activation by AHLs. Thus, these QS antagonists have a high structural similarity to AHLs. Other QSIs all sharing the 2(5*H*)-furanone moiety such as the natural occurring halogenated furanones, and the mycotoxins patulin and penicillic acid seem to exert their effect by destabilizing LuxR and thereby inducing turnover of QS regulator proteins (55, 73). Structures of BHL and OdDHL and previously published QSI compounds are shown in Fig. 9.

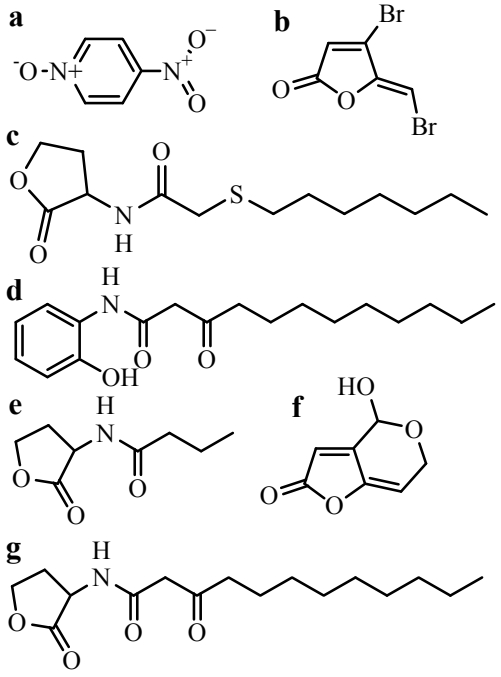

Figure 9. Inhibitors and signal molecules of *P. aeruginosa* QS. **a** 4-nitropyridine-N-oxide (72), **b** C30 (36), **c** 2-heptylthioacetyl homoserine lactone (69), **d** 3-oxo-C_{12}D10 (82), **e** BHL, **f** patulin (73), **g** OdDHL.

The three antibiotics AZM, CFT and CPR are structurally very different from all until now published QSIs, moreover, as many other antibiotics they are very bulky molecules (Fig. 10). *In silico* docking of AZM CFT and CPR against LBD of the LasR protein showed a low affinity of the antibiotics to the LasR receptor site mainly due to spatial penalties. This supports the idea that these antibiotics may exert their QS regulatory effect through mechanisms different to the other QSI's thereby opening new interesting perspectives for combination therapy with QSI's having different modes of action. The usefulness of the combination of QSI's and traditional bactericidal compounds has been proven by several *in vitro* experiments (19, 36, 73). The understanding of some antibiotics' dual course of action may have the potential to further add to anti-pathogenic therapies of which the key element is attenuating rather than killing bacteria directly. This strategy allows the host defense system eliminate the attenuated bacteria.

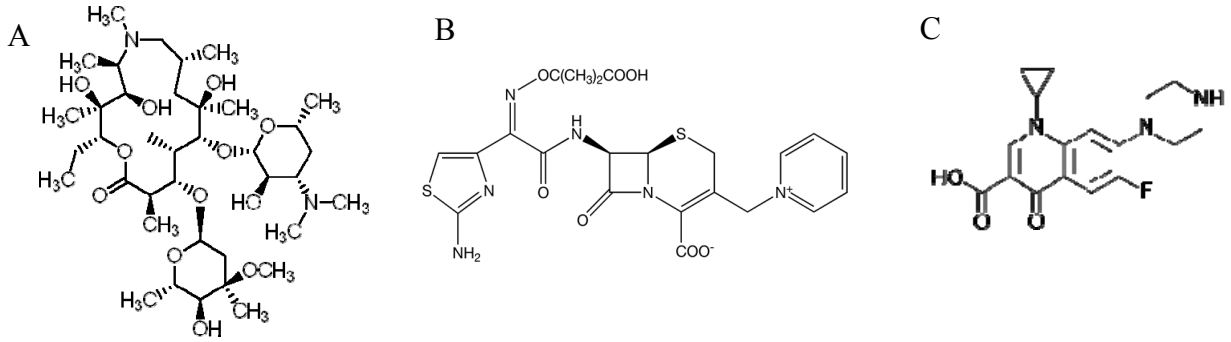

Figure 10. Structures of the three antibiotics investigated for potential quorum sensing inhibitory activities. A. Azithromycin, B. Ceftazidime and C. Ciprofloxacin

Most antibiotics originate from natural sources such as soil microorganisms, or are derivatives of compounds originating from nature. A general point of view has been that the ecological purpose of antimicrobials in the environment is to fight competitors. However, in soil the concentration of free antibiotics are likely to be well below MIC.

Table III. *In silico* docking

Name of ligand	Rerank Score
OdDHL (template)	-107.3
3-oxo-C_{12}D10	-94.5
2-heptylthioacetyl homoserine lactone	-88.8
BHL	-82.2
Patulin	-69.8
4-nitropyridine-*N*-oxide	-62.9
C30	-50.4
Ciprofloxacin	317.3
Ceftazidime	373.5
Azithromycin	3316.2

As described in this paper and by others (52, 93) subMIC antibiotics may have various effects on bacteria such as enhanced biofilm formation, altered motility patterns, increased cytotoxicity and changes in expression of virulence factors, effects which are totally different from the impact of high doses (37, 52, 59). The authors of (52) suggest that the function of antibiotics in nature may be intermicrobial signaling rather than bullets aimed to kill enemies in order to defend niches or food. This hypothesis offers an evolutionary explanation of why some antibiotics may be capable of interfering with QS controlled gene expression.

In line with the present report, it has recently been shown that OdDHL (and other 3-oxo AHLs) as well as its tetramic acid degradation product exhibit antibacterial activity against Gram positive bacteria (47) suggesting that the signal molecules, like some antibiotics, posses dual activity. Furthermore, our data suggest that antibiotics which function to down-regulate QS in *P. aeruginosa* may be administered in subMIC concentrations (where there is a reduced selection pressure for the development of resistance) to block QS and thereby attenuate the pathogen. Such interesting properties highlight the huge unexplored potential of using compounds derived from natural scaffolds in the search for new pharmaceuticals with a variety of targets (*i.e.* multifunctional antimicrobials). It seems that we have not fully explored and exploited the potential of the drugs we know today.

The dual activity of some antibiotics (e.g. AZM, CFT and CPR investigated here) may help explaining that CFT has been shown to be superior to comparative anti-pseudomonal antibiotic regimes of CF patients in the Danish CF center (68). Caution must be taken however. The subsequent shift to CFT as the predominant anti-pseudomonas antibiotic in the Danish CF center lead to an epidemic spread of a multi-resistant non-mucoid strain (67). The study by (33) shows that subMIC concentrations of antibiotics may still modulate transcriptional patterns in members of the beneficial human flora causing a variety of unwanted effects. Likewise, it has been shown, that the long-term use of AZM in CF patients with chronic *P. aeruginosa* infection may lead to macrolide resistance in *Staphylococcus aureus* in these patients (86). This underlines the requirement for further development of QSIs without classical bactericidal or bacteriostatic activity.

Materials and Methods

Bacterial strains applied in this study:
The sequenced *P. aeruginosa* PAO1 wild-type where obtained from the Pseudomonas Genetic Stock Center (www.pseudomonas.med.ecu.edu, strain PAO0001). The *lasI-rhlI* mutant was constructed using previously reported knock out system (6). The QSI selector strain QSIS1 were described by (72).

Bacterial screen for QS inhibition
The Quorum Sensing Inhibitor Selector 1 (QSIS1) has been described in detail elsewhere (72). QSIS1 harbors the plasmid pUC18Not-*luxR*-P_{luxI}-RBSII-*phlA* T_0-T_1, Ap^r maintained in CSH37 (58), an *E. coli* strain constitutively expressing β-galactosidase.

Briefly, preparation of QSIS1 screens were performed as follows: BT medium (B medium (15) plus 2.5 mg thiamine pr. liter) containing 2% agar (wt/vol) was melted, added 10% A10 (15) and cooled to 45°C. *N*-3-oxo-hexanoyl-L-homoserine lactone (Sigma-Aldrich, Germany), ampicillin (Ap, VepiDan, Denmark), X-Gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside, Apollo Scientific, UK) and 1-methyl-2-pyrrelidon (MERCK, Germany) were added in final concentrations of 100 nM, 100 µg/mL, 40 µg/mL and 0.2% (vol/vol) respectively and the medium was supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) casamino acid. QSIS1 over night culture (grown in ABT media supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) casamino acid and 100 µg/mL ampicillin) was added to final concentrations of 0.4%. Plates for the screening assay were subsequently made by pouring the mixture into a box giving an agar thickness of 5 mm. Wells with a diameter of 5 mm were made in the agar plates. Upon solidification, 50 µL of a 1-10 µg/mL solution of antibiotics to be tested for QSI activity was added to the wells. The plate was then incubated at 30°C for 16 hours. Appearance of a circular zone of growth (appearing blue due to the presence of hydrolyzed X-gal), indicates the presence of QSI active compounds in the sample tested.

Sample preparation for P. aeruginosa GeneChip® Analysis
ABT medium supplemented with 0.5% cas amino acids were inoculated with exponentially growing *P. aeruginosa* PAO1 cells to an OD_{600} of 0.05 and grown at 37°C, 200 rpm in 5 x 500 mL flasks containing 100 mL each. At OD_{600} 0.3 AZM (Zithromax®, Pfizer, 8 and 2 µg/mL Azithromycin), CPR (Fluka, 0.04 µg/mL Ciprofloxacin) and CFT (Fortum®, GlaxoSmithKline, 0.25 µg/mL ceftazidime) were added. These concentrations of antibiotics did not affect growth of *P. aeruginosa.* Samples were retrieved at OD_{600} 2.0, and immediately transferred to two volumes RNAlater (Ambion) and stored at -80°C. RNA was isolated using the "RNeasy Mini Purification Kit" (QIAGEN) according to the provided protocol including the on-column DNase treatment. Synthesis of cDNA was performed with 12 µg RNA, 300 ng/µL random primers (Invitrogen™), 1500 U SuperScript III Reverse Transcriptase (Invitrogen™) and 30 U SUPERase·In™ Rnase Inhibitor (Ambion) according to Affymetrix's Expression Analysis Protocol. Synthesized cDNA was purified using the QIAquick PCR Purification Kit (QIAGEN) and 3-4 µg cDNA was fragmented using 0.2 U DNase I, FPLC pure™ (Amersham Bioscience) pr. µg cDNA. Fragmented cDNA was terminal labelled with biotin-ddUTP (Enzo Bioarray™ terminal labeling kit) and hybridised for 18 h at 55°C to *P. aeruginosa* genome microarray GeneChip® (Affymetrix). The chips were washed and stained according to the Affymetrix protocol. The microarray hybridization signal intensity was scaled to an overall signal average of 2500, and present/marginal/absent (based on evaluation of PerfectMatch (PM) and MisMatch (MM) intensities) was evaluated by the use of the Affymetrix GCOS v1.4 software. Only expression value genes estimated being "Present" were included in the further analyses.
DNA microarray data (CHP, CEL, EXP files) have been submitted to Gene Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/geo/. Experiment series number GSE8953.

Virulence factor assays

Supernatents for virulence factor assays
P. aeruginosa PAO1 cultures were grown for either 16-20 hours (for hemolysis assay) or to OD_{600} 2.0 (for protease and elastase assays) as described above with 8 or 2 µg/mL AZM, 0.04 µg/mL CPR or 0,25µg/mL CFT and a control with no antibiotics added. The *P. aeruginosa* PAO1 QS mutant Δ*lasI*-Δ*rhlI* was also grown along with the

treated and untreated *P. aeruginosa* PAO1 cultures. Cells were harvested by centrifugation and the supernatants were sterile filtered (TPP syringe filter 0.22 µm, Switzerland). Supernatants were either used immediately or stored at -20°C.

Hemolysis assay

Sterile filtered supernatants were autoclaved and mixed with venous blood from a healthy individual in volumetric ratio of 30:1 (supernatant:blood), observed for hemolysis after 20 minutes and left at room temperature for four hours to allow sedimentation of intact erythrocytes. Hemolytic activity was observed as a clearing and a non-precipitating red coloring of the blood solution, while lack of hemolytic activity was characterized by the precipitation of intact erythrocytes. The experiment was performed four times with supernatants from four individual growth experiments.

Protease assay

Sterile filtered supernatants (300 µL each) were added to wells in ABT agar plates (ABT medium supplemented with 2% agar) containing 5% skimmed milk and incubated overnight at 37°C. Clearing zones were measured on photos of the plates by the use of a ruler. The experiment was performed twice as four replicates with supernatants from two individual growth experiments.

Elastase assay

Sterile filtered supernatants were mixed with phosphate buffer (0.1 M, pH 6.3) 2:1 and 2 mg/mL Elastin Congo Red (Sigma) was added. The mixture was incubated at 37°C with shaking (200 rpm) for 1 week. After centrifugation, the absorbance of the supernatant at 495 nm was measured with a spectrophotometer zeroed on an elastin Congo-red sample incubated with media alone. Procedure modified from (62). The experiment was performed twice in triplicate with supernatants from three individual growth experiments.

Chitinase assay

Chitinase was measured using a modified chitin azure assay (30, 34). Sterile filtered supernatants were mixed with Na-citrate buffer (0.1 M, pH 4.8) 2:1 and 0.5 mg/mL Chitin azure (Sigma) was added. The supernatant-chitin azure mixtures were incubated at 37°C with shaking (200 rpm) for 1 week. The samples were then centrifuged at 15,000 × *g* for 10 min, and the absorbance at 570 nm was determined. Samples were compared to blanks incubated with media only. The experiment was performed twice as four replicates with supernatants from two individual growth experiments.

Quantification of rhamnolipid

A standard curve for rhamnolipid B (concentration vs. total ionisation current (TIC)) was calculated from LC-ESI-MS data. To minimize potential differences in ionization levels of rhamnolipid between the samples the rhamnolipid standards used for calculating the concentration curve were analyzed immediately prior to, as well as after the samples undergoing analysis. The TIC level was determined on the $[M+NH_4]^+$ ion at 668.4, over 7 seconds at which rhamnolipid B was eluted. HPLC-MS analysis was performed with an agilent 1100 series HPLC connected to a micromass LCT TOF MS.

Docking of AZM, CFT and CPR against the LBD of the LasR protein

The x-ray crystallographic structure of the LBD of the LasR protein (PDB ID code 2UV0) was used for docking of AZM, CFT and CPR to the LasR receptor site using the Template Docking model with default settings, (Molegro Virtual Docker (MVD) (85)). OdDHL was set as the template ligand in the LBD of the LasR protein. Ligands were ranked using the MVD re-rank scoring function.

Data analysis and statistics

Statistical analyses; one-way analysis of variance (ANOVA) followed by Dunnett's test were used to evaluate the effect of AZM, CFT and CPR treatment and the effect of the *lasI-rhlI* mutation on the expression of elastases, chitinase and protease compared to the untreated *P. aeruginosa* PAO1 wild-type. Tests were performed using InStat 3 from GraphPad (GraphPad Software, San Diego, CA). Differences were considered significant if $P<0.05$ or lower.

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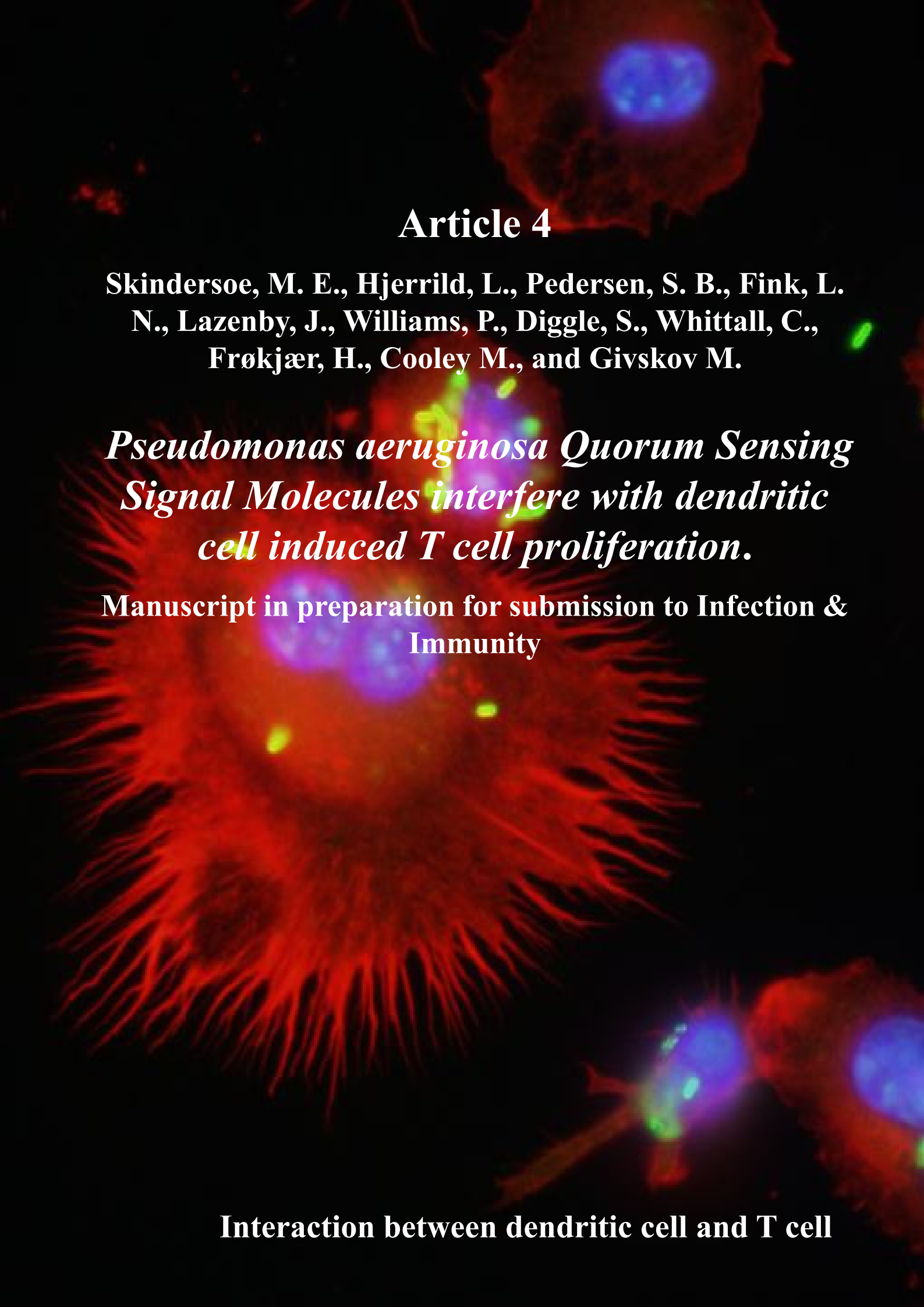
Article 4

Skindersoe, M. E., Hjerrild, L., Pedersen, S. B., Fink, L. N., Lazenby, J., Williams, P., Diggle, S., Whittall, C., Frøkjær, H., Cooley M., and Givskov M.

Pseudomonas aeruginosa Quorum Sensing Signal Molecules interfere with dendritic cell induced T cell proliferation.

Manuscript in preparation for submission to Infection & Immunity



Interaction between dendritic cell and T cell

Further experiments to be carried out for the manuscript in progress (article 4):

PSEUDOMONAS AERUGINOSA QUORUM SENSING SIGNAL MOLECULES INTERFERE WITH DENDRITIC CELL INDUCED T CELL PROLIFERATION

Besides the analyses of the effect of quorum sensing signal molecules (QSSMs) on production of interleukin- (IL)-10 and IL-12 by bone marrow derived dendritic cells (BM-DCs), it would be relevant to investigate the influence of QSSMs on the production of the proinflammatory cytokines IL-6 and TNF α.

IL-6 is secreted in response to specific microbial molecules and is an important mediator of the acute phase response. Even though IL-6 is a proinflammatory cytokine it has been shown to polarize naive $CD4^+$ T cells to effector T_H2 cells by inducing the initial production of IL-4 in $CD4^+$ T cells and to impede T_H1 induced response by pulmonary DCs (2, 3) Thus IL-6 may play an important role in the T_H1/T_H2 balance and for this reason it would be relevant to investigate the effect of QSSMs on DC IL-6 production.

TNF α is another very important acute phase cytokine. TNF α production by peritoneal macrophages has been shown by (6) to be inhibited by OdDHL. As TNF α is a T_H1 associated cytokine it would be relevant to investigate whether DCs' TNF α production is affected by QSSMs.

It would be interesting to investigate the effect of AHLs with varying acyl chain length and AHL like structures to elucidate which structural features are critical for the ability of these molecules to function as immunomodulators and induce cytokine profile changes in the model described in the manuscript.

As C57BL/6 mice have been suggested to be T_H1 biased (1, 5) it would be relevant to repeat the investigations of the effect of QSSM on the cytokine profile with BALB/c mice which has been reported to be T_H2 biased (4, 5).

The fate of PQS in cell culture media in the presence and absence of cells should also be investigated. This can be done by a recently constructed P*pqsA::gfp*(ASV) fusion.

The investigation of surface marker expression (MHC II, CD40, CD80 and CD86) will be repeated with a lower concentration of *Escherichia coli* LPS. Hopefully this will give clarify the effect of BHL and PQS on the surface phenotypes of BM-DCs.

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PSEUDOMONAS AERUGINOSA QUORUM SENSING SIGNAL MOLECULES INTERFERE WITH DENDRITIC CELL INDUCED T CELL PROLIFERATION

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ABSTRACT
Pseudomonas aeruginosa secretes a wide array of toxins and tissue degrading enzymes. The release of these malicious virulence factors is controlled by interbacterial communication in a process known as quorum sensing. An expanding body of evidence indicates that the quorum sensing mediator *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) has immune-modulating capacity. Recently was yet another quorum sensing signal molecule; the *Pseudomonas* quinolone signal (PQS) shown to reduce the release of interleukin-2 (IL-2) by mitogen stimulated human T cells (D. Hooi *et al.,* Infect. Immun. 72:6463-6470, 2004). In this article we demonstrate that OdDHL and PQS decrease the production of IL-12 by *E. coli* lipopolysaccharide-matured bone-marrow-derived dendritic cells (BM-DCs) without altering the IL-10 release. Moreover, BM-DCs exposed to PQS and OdDHL during antigen stimulation exhibit a decreased ability to induce T cell proliferation in a syngeneic mixed leukocyte reaction. Collectively, this suggests that OdDHL and PQS change the maturation pattern of stimulated DCs away from a proinflammatory T_H1 directing response, thereby decreasing the antibacterial activities crucial for the eradication of an infection. OdDHL and PQS thus seem to possess dual activities in the infection process; as inducers of virulence factors as well as immune-modulators facilitating the infective properties of this pathogen.

KEY WORDS:
Dendritic cells, immunomodulation, OdDHL, PQS, *Pseudomonas aeruginosa,* quorum sensing

ABBREVIATIONS:
15d-PGJ$_2$;15deoxy$\Delta^{12\text{-}14}$prostaglandin J$_2$, APC; antigen presenting cells, BHL; *N*-butanoyl-L-homoserine lactone, BM-DC; Bone Marrow-derived Dendritic Cells, INF; interferon, IL; interleukin, LPS; lipopolysaccharide, MLR; Mixed Leukocyte Reaction OdDHL; *N*-(3-oxododecanoyl)-L-homoserine lactone, PBMC; Peripheral Blood Mononuclear Cell, PQS; Pseudomonas quinolone signal (heptyl-3-hydroxy-4-quinolone); QS(SM); quorum sensing (signal molecules), TNF; Tumor Necrosis Factor

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen, which often infects decubitus ulcers and burn wounds and thereby retards healing (10). Moreover, this versatile bacterium is a major cause of pneumonia in immunocompromised persons and in cystic fibrosis (CF) patients where chronic *P. aeruginosa* infections are the main cause of morbidity and mortality (2, 34). The persistent infection is dominated by an exaggerated host response causing tissue destruction. During the infectious process, *P. aeruginosa* expresses virulence factors and tissue damaging products in a cell number-dependent process termed quorum sensing (QS) (13, 28). Several *in vivo* surveys have demonstrated that QS is important for virulence and pathogenicity of *P. aeruginosa* (16, 20, 29, 48, 49). The QS system of *P. aeruginosa* is mainly mediated by three signal molecules; the two *N*-acyl L-homoserine lactones; *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL)

and *N*-butanoyl-L-homoserine lactone (BHL) and the *Pseudomonas* quinolone signal, PQS (heptyl-3-hydroxy-4-quinolone).

The major *P. aeruginosa* QS signal molecule (QSSM) OdDHL has been shown to affect functions and activities of a range of mammalian cell types. OdDHL inhibits contraction of arterial smooth muscle (19) and modulates expression of an array of cytokines in various cells of the immune system. OdDHL induces interleukin (IL)-8 production in respiratory epithelial and lung fibroblasts cells (11, 42) and inhibits tumour necrosis factor α (TNF-α)-production by lipo-polysaccharide (LPS)-stimulated macrophages (45). Both OdDHL and PQS have been shown to inhibit cell proliferation of human peripheral blood mononuclear cells (PBMCs), PQS being far more potent than OdDHL (17). This study also showed differential immunomodulatory effects of PQS and OdDHL, as OdDHL but not PQS inhibits IL-2 release by T cells activated via anti-CD3/anti-CD28 antibodies (17).

Dendritic cells (DCs) are professional antigen presenting cells (APCs) and the principal stimulators of naïve T cells, and are thus important in the initiation and modulation of immune responses. The T_H1 response, which promotes cell mediated immunity, is characterized by high interferon γ (IFN-γ)-production by $CD4^+$ T cells and thus an elevated IFN-γ/IL-4 ratio. The T_H1 response is induced by APCs - in particular DCs - producing IL-12, but is also promoted by IFN-γ (22, 46). The T_H2 type response is dominated by IL-4 production and low IFN-γ and promotes humoral immunity. These two T_H responses are sustained by their representative cytokines, while they antagonize each other; for example, the cytokine IFN-γ is produced by T_H1 cells and while this cytokine further enhances the T_H1 responses it inhibits proliferation of the T_H2 subset (26, 27). Another example of cross-regulation is the T_H2 cytokine IL-10 which down-regulates IL-12 (50).

OdDHL suppresses the production of the T_H1 cytokines IL-12 and TNF-α by macrophages, and hinders lymphocyte proliferation while promoting IgG_1 production, thereby supporting the hypothesis that OdDHL pushes the T_H1/T_H2 balance away from a T_H1 dominated response (45). Clinical surveys have shown that the T_H response in CF patients is predominantly of the T_H2 type, and that patients with a T_H1-dominated immune response have improved lung function and a better prognosis suggesting that a change from a T_H2 to T_H1 dominated response might improve the prognosis in these patients (24). Thus OdDHL may play a dual role in the pathogenesis of *P. aeruginosa*; 1) as mediator of bacterial communication by which it controls virulence and 2) as immunomodulator changing the milieu from the host-protecting proinflammatory T_H1 response towards the T_H2 direction, thus favouring the establishment of bacteria within the host.

Here we investigate the effect of the three *P. aeruginosa* QSSMs; OdDHL, BHL and PQS on DC functions and maturation pattern, in particular the effect on cytokine profile and activation of T cell proliferation.

MATERIALS AND METHODS

Mice

Animal experiments were approved by the Danish Council of Animal Ethics (mice used for experiments with cytokine production and cell surface markers) and University of New South Wales (UNSW) Animal Care and Ethics Committee (ACEC 06/16A) (mice used for syngeneic Mixed Leukocyte Reaction, MLR). C57BL/6 mice were used for investigations on cytokine production and cell surface markers. Transgenic mice on a B10.BR genetic background bred in house at UNSW from homozygous breeding pairs (kindly provided by B. Fazekas de St. Groth) were used for syngeneic MLR. These mice overexpress a T cell receptor (TCR) specific for the COOH-terminal epitope of pigeon moth cytochrome *c* (MCC) on 50-80% of peripheral $CD4^+$ T cells (3). All mice used were 8–12 wk old, kept under SPF conditions and given sterile mouse chow and water *ad libitum*.

Quorum sensing signal molecules (QSSMs)

The QSSMs applied were *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), Pseudomonas quinolone signal (PQS, heptyl-3-hydroxy-4-quinolone) and *N*-butanoyl-L-homoserine lactone (BHL). OdDHL was synthesized, purified and validated as described by (6), PQS was synthesized, purified and validated as described by (17, 31), and BHL was purchased from Sigma-Aldrich.

Bone marrow cell culture

Bone marrow cells were isolated and cultured as described by (21), with minor modifications.

Briefly, femora and tibiae from C57BL/6 and MCC TCR transgenic B10.BR mice were removed and stripped of muscles and tendons. After soaking the bones in 70% ethanol for 2 min. and rinsing in PBS, both ends were cut and the marrow was flushed with PBS using a 27-gauge needle. Cell clusters were dispersed by repeated pipetting. The resulting cell suspension (containing 5–7.5×10^7 leukocytes per mouse) was centrifuged for 10 min at 300 × *g* and washed once in PBS. Cells were resuspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 4 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM β-mercapto ethanol and 10% (v/v) heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA). For cytokine production and cell surface markers, 15 ng/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was added as culture supernatant (containing 550 ng/mL GM-CSF) harvested from a GM-CSF-producing cell line (GM-CSF transfected Ag8.653 myeloma cell line) (54). The GM-CSF-producing cell line was kindly provided by Dr. R. Tisch (University of North Carolina, Chapel Hill, NC). For generation of DCs for syngeneic MLR, 15 ng/mL GM-CSF purchased from Peprotech, Rocky Hill, NJ was used.

BM-DC for cytokine production

For cytokine production, 10 mL of cell suspension containing 2×10^6 leukocytes/mL was seeded per 100-mm bacteriological petri dish and incubated for 8 days at 37 °C in 5% CO_2. An additional 10 mL of freshly prepared media was added to each plate on day 3. On day 6, 9 mL from each plate was withdrawn without removing cells and replaced with 10 mL fresh media. On day 8, nonadherent cells were gently harvested and used for investigations of cytokine release in the absence or presence of QSSM.

BM-DC for induction of surface markers and MLR

For Mixed Leukocyte Reaction (MLR) and investigation of surface markers, 0.7 mL of cell suspension containing 2×10^6 leukocytes/mL was seeded into wells in a 12 well plate (Nunc, Germany) at day 0. An additional 0.7 mL fresh media was added on day 3, and on day 6, 0.6 mL used media was carefully withdrawn and replaced with 0.7 mL fresh media. On day 8 cells were stimulated with LPS (*Escherichia coli* O26:B6 LPS; Sigma-Aldrich) in the presence or absence of QSSM.

Induction of cytokine release

Cells from bone marrow-derived cell cultures were centrifuged for 5 min at 260 × *g* and resuspended in media supplemented with 10 ng/mL GM-CSF. Cells were seeded in 48-well tissue culture plates at 1.2 × 10^6 pr. well in 500 µl. 50 µl/well PBS containing QSSMs (1-30 µM final concentration, stock solution 50 mM in DMSO) were then added. Controls were PBS containing DMSO. All wells, except wells used for control of the effect of DMSO, were adjusted to contain 0.1% DMSO. 50 µl/well LPS (*E. coli* O26:B6; Sigma-Aldrich) at 1 µg/mL final concentration in PBS was added to induce maturation. After a stimulation period of 15 h at 37°C in 5% CO_2, culture supernatant was collected and stored at -80 °C until cytokine analysis. Viability of the cells was investigated by propidium iodide exclusion using a BD FACS array flow cytometer (BD Biosciences, San Jose, CA) and indicated that QSSM treated as well as untreated cultures contained ≥90% viable cells. The entire experiment (including analysis of cell viability and ELISA) was repeated 4 times.

Cytokine quantification in culture supernatants

Culture supernatants were aliquoted and stored at -80 °C until they were analyzed for the presence of cytokines. Quantitative assays for IL-12, IL-10 and TNF-α in the culture supernatants were performed using enzyme-linked immunosorbant assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, polystyrene microtitre plates (Nunc, Germany) were coated with the first antibody diluted in coating buffer overnight at 4°C. Various concentrations of recombinant cytokines were added as external standards. Standards and supernatants were incubated for 2 h in dilutions of 1:2 (for IL-10 and IL-12) and 1:40 (for TNF α). Bound cytokine was detected with biotinylated second antibody, streptavidin-peroxidase conjugate and 3,3′,5,5′-Tetramethylbenzidine (TMB). The reaction was stopped after 20 min. incubation with 2 M H_3PO_4 and absorbance was measured using a microplate reader (PowerWaveX Microplate Spectrophotometer, BioTek Instruments, Winooski, VT) wavelength 450 nm and 630 nm

as reference. Cytokine concentrations were calculated using standard curves that were performed for each ELISA plate in order to minimize interassay variability. Results were expressed as the mean concentrations in duplicate culture supernatants.

Induction of surface marker expression

0.7 mL of spent medium was carefully removed and discarded from 8-day old DC-enriched cultures in 12 well plates (described in the section *BM-DC for induction of surface markers and MLR*), each containing 1.4 mL. Then, 0.6 mL of media without GM-CSF was added to each dish. 50 µl/well PBS containing QSSMs (5-30 µM final concentration, stock solution 50 mM in DMSO) were then added. Controls were PBS containing DMSO. All wells, except wells used for internal control of the effect of DMSO were adjusted to contain 0.1% DMSO. 50 µl/well LPS (*E. coli* O26:B6; Sigma-Aldrich) at 1 µg/mL final concentration in PBS was added to induce maturation. Cultures were incubated for 14 h at 37°C in 5% CO_2. At the end of the incubation period, 3 mL of PBS containing 1% (v/v) FBS and 0.15% (w/v) sodium azide (PBS-Az) was added to each well to prevent internalization of surface markers during subsequent handling of cells. Nonadherent cells were collected by gentle pipetting, centrifuged for 5 min at 300 × *g*, and subjected to surface marker staining for flow cytometric analysis as described below. Trypan blue and propidium iodide exclusion indicated that the QSSM-treated as well as untreated cultures contained ≥90% viable cells. Cell number pr. well was not affected by treatment with QSSMs.

Immunocytostaining and flow cytometry

Centrifuged cells were resuspended in cold PBS-Az and transferred to a round-bottom 96-well plate (4×10^5 cells/well). During all work, cells were kept at or below 4°C and at low light exposure. Cells were centrifuged and incubated 10 min. in 50 µl/well PBS-Az containing anti-mouse FcγRII/III (3 µg/mL; BD Biosciences, San Jose, CA) to block nonspecific binding of Ab reagents. After incubation, an additional 50 µl/well of PBS-Az containing fluorochrome-conjugated Abs in pre-optimized concentrations was added and incubated for 45 min. Cells were then washed twice in 200 µl/well PBS-Az and finally resuspended in 300 µl/well PBS-Az for flow cytometric analysis in a BD FACS Array flow cytometer (BD Biosciences, San Jose, CA). The analysis was based on counting of 30,000 cells. The following Abs used for staining were purchased from eBioscience, San Jose, CA (eB) or Southern Biotech, Birmingham (SB): APC-conjugated anti-mouse CD11c (SB), PE-conjugated anti-mouse MHCII (SB), APC-conjugated CD86 (SB), PE-conjugated anti-mouse CD80 (eB) and PE-conjugated anti-mouse CD40 (eB). Non-viable cells were gated out by forward and side light scatter. The results were expressed as median fluorescence of cells.

Mixed lymphocyte reaction (MLR)

Mixed lymphocyte reaction was performed in triplicate wells. 100 µl $CD4^+$ T cells (1×10^6 cells/mL) isolated from mouse splenocytes by positive selection with anti-CD4 microbeads (Miltenyi Biotec) were co-cultured with 100 µl sodium azide harvested syngeneic DCs (1.5×10^5, 7.5×10^4 and 3.8×10^4 cells/mL) treated with QSSMs as described in *Induction of surface markers* in 96-well round-bottom microtitre plates. As negative controls, T cells (1×10^6 cells/mL) in triplicate wells or DC (1.5×10^5 cells/mL) in triplicate wells were cultured in media alone. The plates were incubated in a 5% CO_2 incubator at 37 °C. On day 5 each well was loaded with 20 µl alamarBlue™ (BioSource) and incubated at 37 °C, 5% CO_2 for 5 h. Plates were read fluorometrically (excitation wavelength 544 nm and emission wavelength 590 nm) at a Polar Star microplate reader (BMG Lab Technologies Pty Ltd).

Measurement of BHL and OdDHL in cell cultures

AHLs in the supernatant were quantified by the use of the two QS monitor strains; MH205 (*ahyR-ahyI*::*gfp(ASV)*) (44) and MHLAS *lasB*::*gfp*(ASV) (15) for BHL and OdDHL, respectively. The QS monitor strains were applied as follows: 150 µl ABT media (9) supplemented with 0.5% cas-amino acids were added to wells in a microtiter dish (Black Isoplate®, Perkin Elmer). A 2-fold serial dilution series of supernatants containing AHL was made, leaving the last well for reference (concentration of AHL = zero). Finally, 150 µl 50× diluted overnight culture of either MH205 or MHLAS (OD_{450} of diluted culture = 0.1) was added to the wells. The Gfp(ASV)-expression (measured as

fluorescence; excitation and emission wave length 485 and 535 nm, respectively) was measured every 15 min. during the following 18 hours by the use of a multilabel plate reader Wallac 1420 VICTOR^{2}™ (Perkin Elmer). OD_{450} was also determined as a measure for growth. During the assay the temperature was held constant at 34 ˚C. Expression of Gfp(ASV) by the monitor bacteria was used as a relative measure of the concentration of OdDHL and BHL in the supernatants.

Data analysis and statistics

Statistical analyses; one-way analysis of variance (ANOVA) followed by Dunnett's test comparing the effect of QSSM with the control stimulus, were used for all analyses. Tests were performed using InStat 3 from GraphPad (GraphPad Software, San Diego, CA). Differences were considered significant if $P<0.05$ or lower. Values for 50% inhibitory concentration (IC_{50}) were calculated from the regression equation using Microsoft Office® Excel 2003 SP2.

RESULTS

Cytotoxicity of OdDHL, PQS and BHL

First we tested the cytotoxicity of QSSMs on murine Bone Marrow-Derived DCs (BM-DC). Viability of cells after incubation with QSSMs were investigated by propidium iodide staining and showed that the highest non-cytotoxic concentration of both OdDHL and PQS was 50 μM. BHL was not toxic even at the highest concentration tested (100 μM). Previous studies have shown an anti-proliferative effect of PQS and OdDHL on ConA-stimulated human PBMCs (17). Therefore we also performed simple viable cell counts of DCs exposed to QSSM for 16 h in the presence of *E. coli* LPS, which showed no change in cell numbers even at the highest concentration of QSSM tested in LPS-stimulated cells (30 μM).

Fate of AHLs in cell culture media in the presence or absence of DCs

The concentration of AHLs may change during the duration of an experiment e.g. due to degradation. Therefore, we decided to investigate the fate of AHLs in cell culture medium in the presence or absence of cells. For this purpose, we applied QS biosensors activated by exogenous OdDHL or BHL to measure the relative concentration of biologically active AHLs during the first 2 hours and at the end point of the experiment (16 h). BHL retained its bioactivity during the 16 hours incubation both in the absence and presence of cells. By contrast, the bioactivity of OdDHL in the supernatant was almost completely lost after 16 hours incubation in the presence of cells; but if incubated in cell culture media without cells, OdDHL preserved 100% of its bioactivity after 2 h incubation and 90% after 16 h. (See figure 1).

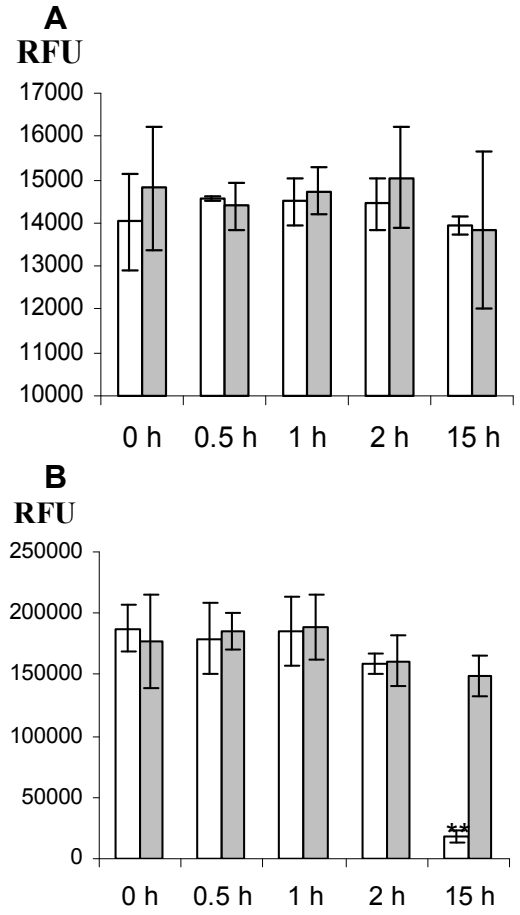

Figure 1. Fate of AHLs in cell culture media. A) BHL in cell culture media measured by the use of a BHL specific *ahyR-ahyI*::*gfp*(ASV) biomonitor.
B) OdDHL in cell culture media measured by the use of a OdDHL specific *lasR-lasB*::*gfp(ASV)* biomonitor. Background corrected maximal RFU are shown for samples taken at the time points 0, 0.5, 1, 2 and 15 hours. White bars represent AHL incubated in the presence of DCs, and grey bars represent AHL incubated in the absence of DCs (** $P<0.01$ for Tukey-Kramer Multiple Comparison test). The experiment was performed in duplicate. Data are representative of two independent experiments.

Cytokine profile in the presence of QSSM

In order to assess the effect of *P. aeruginosa* QSSMs on murine BM-DC cytokine production, either OdDHL, BHL or PQS or vehicle (DMSO) was added to cell cultures in final concentrations of 0, 5, 10 , 20 and 30 μM along with *E. coli* LPS. None of the QSSMs affected IL-10 production significantly (Figure 2 A). However, PQS and especially OdDHL but not BHL caused a dose-dependent decrease in IL-12 production (Figure 2 B). OdDHL showed a more pronounced effect on IL-12 production than PQS as the IC_{50} (the concentration required to lower the cytokine release to 50%) for OdDHL was 7.0 μM, whereas it was 17.2 μM for PQS.

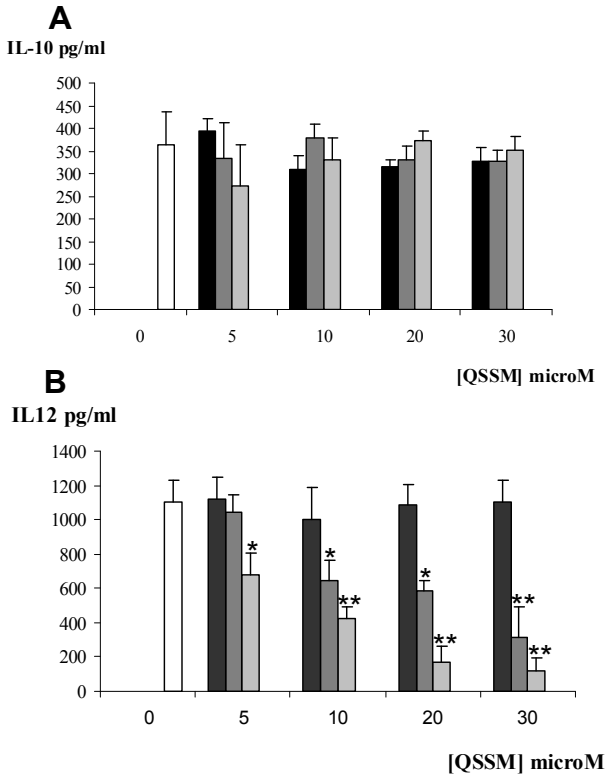

Figure 2. Effect of *P. aeruginosa* QSSM on cytokine production in murine BM-DCs stimulated with 1 μg/mL LPS for 16 h. A) IL-10 and B) IL-12. Control 0.1% DMSO (white bar), BHL (black bars), PQS (dark gray bars) and OdDHL (light grey bars).
(* $P<0.05$, ** $P<0.01$, Dunnett's test comparing the effect of QSSM with DMSO vehicle (control)). The experiment was performed in duplicate. Data are representative of four independent experiments.

Surface marker expression in the presence of QSSM

Upon culturing murine bone marrow cells with GM-CSF (Granulocyte-macrophage colony-stimulating factor), cells other than DC, such as myeloid progenitors, granulocytes, macrophages and lymphocytes may develop. Therefore, DC purity was verified by flow cytometry using the DC marker CD11c. In day 9 cultures, the proportion of $CD11c^+$ cells was in the range 90-95%, and microscopic inspection showed that the vast majority of cells appeared as irregularly shaped cells with clearly visible protrusions, which are characteristic morphological features for DC (not shown). The level of DC purity obtained in the cultures is consistent with results from previous studies (7, 21). DCs express many accessory molecules that interact with receptors on T cells to enhance adhesion and signaling. These molecules are regulated by exposure to various microbial stimuli collectively termed pathogen-associated molecular patterns (PAMPs). The maturation level of DCs can thus be followed by detection of several typical surface markers. In the current study we have investigated surface expression of MHC class II, CD80, CD86 and CD40 after LPS stimulation and in the presence or absence of QSSMs. The expression of CD80, CD86, CD40 and MHC II by unstimulated BM-DC or in LPS-stimulated BM-DC in the absence or presence of QSSM (BHL, PQS or OdDHL) is shown in figure 3. The results expressed as median of fluorescence intensity of cells either treated with QSSM or left untreated are summarized in table 1. OdDHL caused a decreased expression of the two T cell co-stimulatory molecules CD80 and CD86. While the expression of MHC II did not notably change by the presence of OdDHL, OdDHL slightly lowered the CD40 expression. The surface marker data showed no clear trend as response to the challenge by BHL and PQS.

Mixed Lymphocyte Reaction in the presence of QSSM

Since we noticed that OdDHL and PQS caused a decreased production of IL-12 and that OdDHL reduced the expression of surface markers related to maturation of LPS-stimulated DCs we decided to test whether OdDHL and PQS also influenced the ability of DCs to activate proliferation of T cells.

Immature BM-DCs derived from transgenic mice over-expressing a TCR specific for the COOH-terminal epitope of pigeon moth cytochrome *c* (MCC) (3) were generated as for cytokine production and investigation of surface markers.

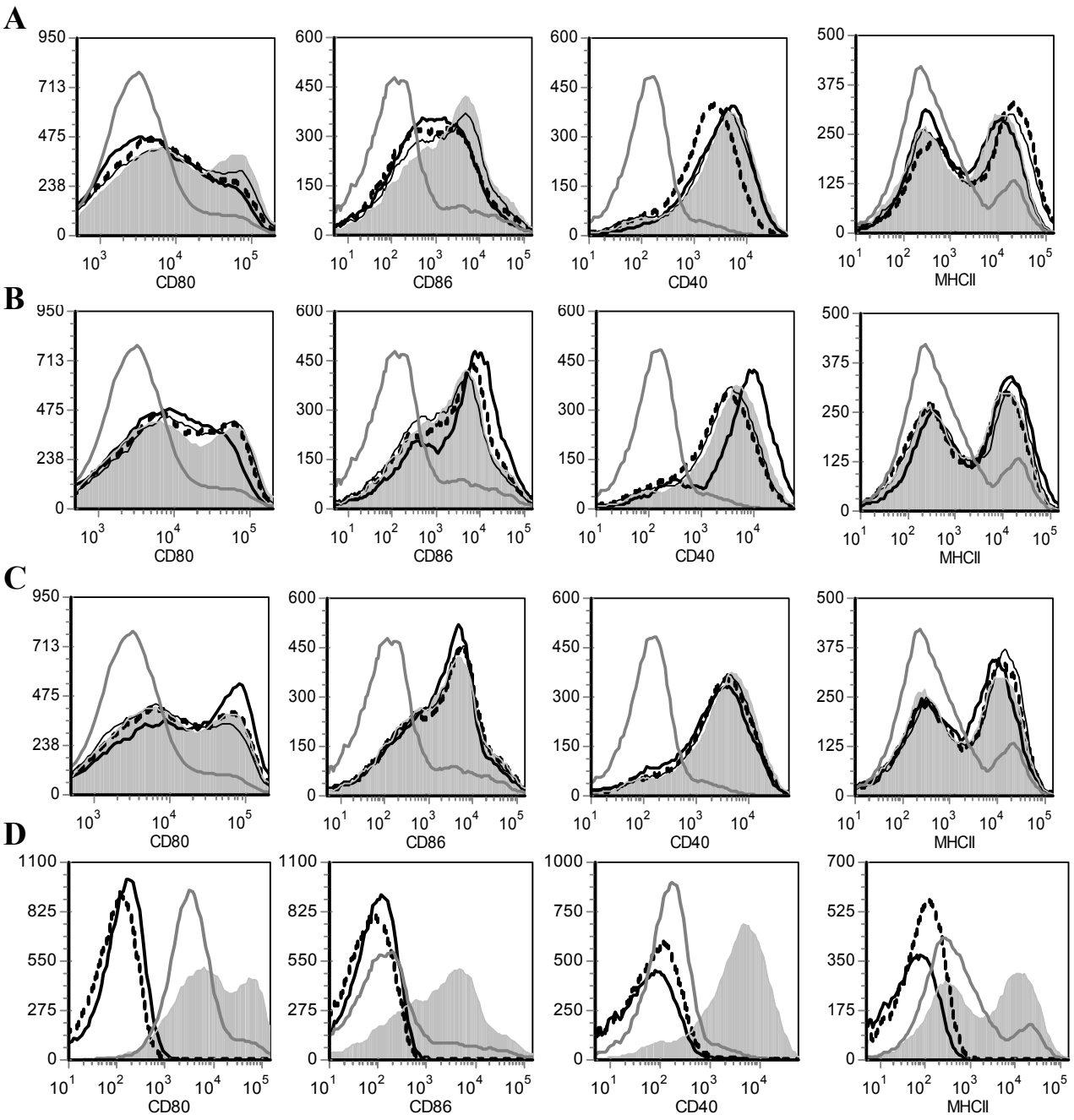

Figure 3. Effect of QSSM on expression of surface phenotypes A) OdDHL B) PQS, C) BHL, and D) isotype controls. Legend description for A), B) and C) Black solid line; cells stimulated with 1 µg/mL LPS along with 30 µM QSSM. Black dashed line; cells stimulated with 1 µg/mL LPS along with 10 µM QSSM. Black thin line; cells stimulated with 1 µg/mL LPS along with 5 µM QSSM. Filled light grey histogram; LPS stimulated cells and not added QSSM. Dark grey solid line; unstimulated cells. Legend description for D) Black solid line; binding of isotype-matched control antibody to LPS stimulated cells. Black dashed line; binding of matched isotype-control to unstimulated cells. Filled light grey graph; LPS stimulated cells with fluorochrome-conjugated Abs instead of isotype control (inserted for comparison). Dark grey solid line; unstimulated cells. with fluorochrome-conjugated Abs instead of isotype control (inserted for comparison). X-axis: Fluorescence intensity. Y-axis: Cell number.
Data shown are representative of three experiments performed.

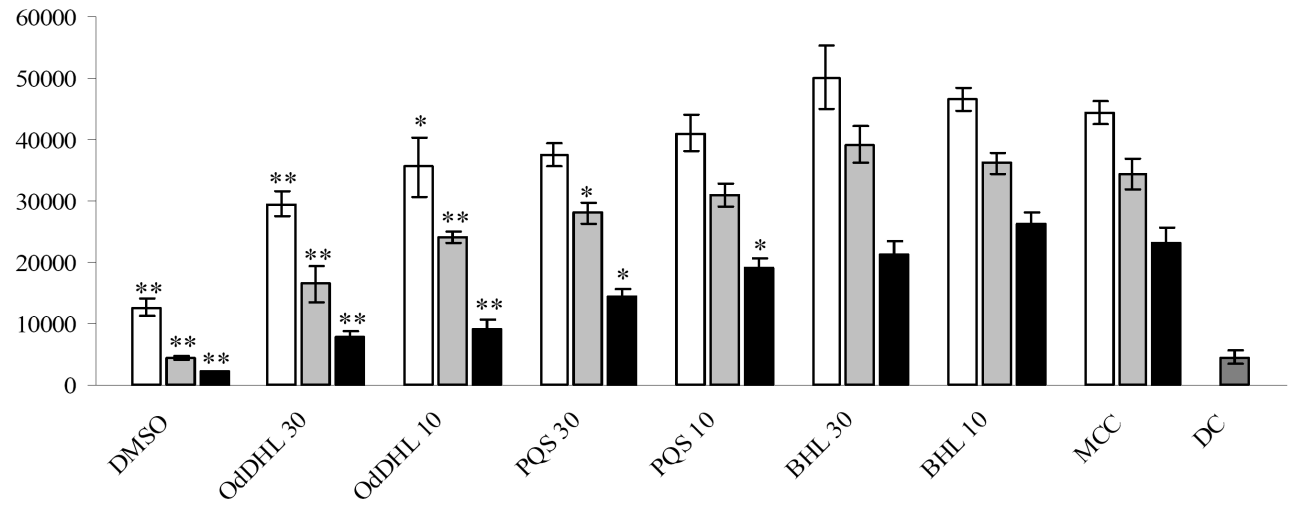

Figure 4. Effect of *P. aeruginosa* QSSMs on T cell proliferation in syngeneic MLR. Ratio of DC to $CD4^+$ cells: 1:7, (white bar), 1:13 (light grey bars) and 1:26 (black bars). DC cells alone (dark grey bar). No detectable proliferation of T cells alone. (* $P<0.05$, ** $P<0.01$, Dunnett's test comparing the effect of QSSMs+MCC vs. MCC, and DMSO (vehicle) vs. MCC). The experiment was performed in triplicate. Data are representative of four independent experiments.

Table 1. Table corresponds to figure 3 and summarizes the median of stained cells stimulated with LPS and challenged with QSSM compared to stained cell only stimulated with LPS (control) and unstimulated cells. In the table the median of QSSM challenged cells/median of control is shown as percent.

	Median of stained cells QSSM challenged vs. control (%)			
	CD80	CD86	CD40	MHCII
BHL 30 µM	175	107	92	87
BHL 10 µM	108	111	50	236
BHL 5 µM	89	104	85	136
PQS 30 µM	89	227	181	215
PQS 10 µM	105	111	64	77
PQS 5 µM	118	75	78	88
OdDHL 30 µM	55	38	63	87
OdDHL 10 µM	81	63	79	137
OdDHL 5 µM	66	38	84	126
Unstimulated	35	6	4	21
Control (LPS)	100	100	100	100

On day 8 the DCs were exposed to the MCC_{T102S} peptide (an altered version of the wild type MCC peptide (35)) in the absence or presence of QSSM and incubated for 20 h, after which they were mixed with syngeneic T cells and co-cultured for 5 days. On day five, proliferation of T cells was measured by a fluorometric Alamar Blue microassay. DCs stimulated with MCC peptide in the presence of OdDHL – and to a lower extent PQS – had a lowered ability to induce proliferation of T cells compared to cells only exposed to MCC or to MCC and BHL (Figure 4).

DISCUSSION

An expanding body of data shows that *P. aeruginosa* possesses the ability to interfere with the immune system via its signal molecule OdDHL. In the current study we have for the first time demonstrated that OdDHL and PQS alter the maturation pattern in LPS-stimulated DCs away from a T_H1-skewing phenotype. We have shown that OdDHL and PQS change the cytokine profile of LPS-stimulated DCs by reducing IL-12 release. IL-12 is an important factor in polarizing naïve T cells to develop into T_H1 cells, therefore a reduction of IL-12 is likely to modulate the T_H1/T_H2 balance. We also provide evidence that OdDHL inhibits LPS-stimulated DC expression of T cell stimulatory and maturation related surface markers (CD86, CD80 and CD40). Finally, we found that murine BM-DCs challenged with PQS and OdDHL during antigen stimulation exhibit a decreased capacity to induce T cell proliferation. Collectively, these results

suggest that the *P. aeruginosa* QSSMs OdDHL and PQS restrain the DCs in exerting their T cell stimulatory effects. These observations indicate that *P. aeruginosa* QSSMs impede the development of a host-protective pro-inflammatory T_H1 response. This is consistent with earlier findings showing an OdDHL-induced T_H2 skewing of the cytokine response by LPS-stimulated macrophages (45). A previous study has shown that OdDHL affects T cell differentiation and cytokine production in an early step of T cell maturation (35) and our observations suggest that this early step could involve the activation of T cell differentiation by DCs or perhaps other APCs.

In order to ensure that changes in cytokine patterns imposed by QSSMs were not caused by toxic or antiproliferating effects we examined the cytotoxicity and effect on DC proliferation. In the concentrations used, QSSMs did not effect neither cell proliferation nor viability measuring exclusion of Trypan blue and propidium iodide. Telford *et al*. assessed the cytotoxicity of OdDHL, OHHL and of DMSO alone by exposing Jurkat E6.1 cells to the compounds and found that none of the compounds were cytotoxic in the concentrations investigated (concentrations range: 0.1 to 100 μM) (45)

We also investigated the fate of AHL during the incubation with DCs. Elevated pH (pH above 8) is known to promote non-enzymatic lactonolysis of AHLs (53), and partial hydrolysis may take place under the conditions used for mammalian cell cultures, i.e. pH 6-7 and 37 °C, thereby reducing the concentration of bioactive AHL in the assays. The hydrophobic properties of long chain AHLs such as OdDHL may also cause it to bind to the plastic of the cell culture plates or sequestered by serum in the medium or partitioned into tissue cell membranes. We found that while BHL retained its bioactivity during the 16 hours incubation both in the absence and presence of cells, bioactivity of OdHDL in the supernatant was reduced to 10 % after 16 hours incubation in the presence of DCs. However, if incubated in cell culture media without cells, OdDHL preserved 90% of its bioactivity after 16 h incubation. Short chain AHLs such as BHL are more susceptible to non-enzymatic lactonolysis than long chain AHLs (53), and considering that OdDHL retained its bioactivity in the absence of cells, the result suggests that OdDHL may be taken up by the cells or degraded e.g. by enzymes released by the cells (e.g. paraoxonases) during incubation (8, 51). Recently Ritchie *et al*. (36) presented *in vitro* results indicating that OdDHL passively enters the cytoplasm of T cells and cultured DCs. The effect of OdDHL on cytokine release by spleen cells stimulated with anti-CD3 antibody or antigen pulsed APCs has been studied and it seems that OdDHL must be present within the first 2 hours after stimulation in order to significantly suppress cytokine production (35). Even though it remains to be elucidated whether a similar time-dependent effect of QSSMs is exerted on DCs, it is however likely that the "programming" of the DCs take place early after stimulation.

However, results indicating that OdDHL supports a T_H2 response are in contrast to the observations by (43). In this study OdDHL was found to activate splenic T cells activated via antigen and irradiated APCs to produce IFN-γ and hence potentially promote the T_H1 response. Another study by the same authors showed that expression of the T_H1 associated neutrophil attracting chemokine IL-8 were induced by OdDHL (42). Conflicting data and conclusions about the immune modulating T_H1/T_H2 bias mediated by OdDHL could be explained as suggested by Ritchie *et al.,* 2003; who expounded the theory that OdDHL accentuates the underlying T_H1/T_H2 bias of the T_H cell population used, rather than targeting either Th1 or Th2 (37). Concentration issues could also explain the apparent contradictions between observations on the ability of OdDHL to induce a skew in either the T_H1 or T_H2 direction, as some investigators tend to use high doses of OdDHL (50-100 μM) (43) while other studies have used low doses of OdDHL (≤10 μM) (45). Although a great number of studies deal with QS in *P. aeruginosa,* there are very few studies which aim to quantify the concentration of AHLs in *P. aeruginosa* liquid cultures and biofilms. The concentration of OdDHL in PAO1 cultures has been reported to be in the order of 5 μM (30). HPLC-(UV) have been used to measure the production of OdDHL by *P. aeruginosa* PAO1 grown in liquid culture (OD = 0.5-1.5) and the concentration of OdDHL was found to be in the range of 0.45 μM to 1.16 μM (33). In biofilms the concentration of OdDHL is much higher and has been measured to be as high as 251 μM to 1031 μM (5). Further complicating the picture is the possibility that host modulators of OdDHL, such as the OdDHL degrading

enzyme paraoxonase, are present in *in vivo* settings and may decrease the amount of bioactive OdDHL (8, 51). A small number of studies have aimed to measure the concentration of OdDHL in the sputum or lung tissue of CF patients and have detected OdDHLs concentrations in the pico to nanomolar range (12, 41), though OdDHL is likely to be unevenly distributed in the lungs, and may locally be higher e.g. in biofilm microcolonies. Moreover, the concentration may be underestimated due to enzymatic degradation of OdDHL during harvesting. However, the concentration of OdDHL *in vivo* is probably more likely to be in the low micromolar range (where OdDHL seems to be anti-inflammatory) rather than in the 50-100 μM range (which seems to promote a proinflammatory outcome).

PQS has been shown to inhibit proliferation of ConA stimulated human PBMCs (17), but the same study found that PQS in contrast to OdDHL does not decrease IL-2 secretion in a anti-CD3/anti-CD28 antibody driven T cell stimulation assay. Considering the high structural diversity between PQS and OdDHL, these results suggest that PQS and OdDHL may have differential targets as immunomodulators. However, in this study we found that PQS and OdDHL seems to have almost similar effects on DCs with respect to changes in the cytokine profile of LPS-stimulated DCs and effect in a MLR, where the major difference between the two QSSMs is that OdDHL seems to have a more pronounced immunosuppressive effect than PQS.

Both PQS and OdDHL are hydrophobic molecules (predicted log*P* values are 3.6 and 1.9 for OdDHL and PQS, respectively (23)) and as such they share their lipophilic properties with signaling compounds among members of *Fungi*, *Plantae* and *Animalia* (see review by (39)). This may facilitate binding to eukaryotic membranes and perhaps also uptake for PQS and OdDHL as well as for steroid hormones and other eukaryotic signal mediators. Uptake or sequestering of OdDHL could explain the observed decrease of OdDHL during incubation with murine BM-DCs and offer an alternative explanation to enzymatic degradation as the cause of the reduced OdDHL bioactivity during the experiment.

The immune suppressive effect of OdDHL-related compounds has been shown to be dependent on an 11-13 carbon side chain containing either a 3-oxo or a 3-hydroxy group and a high lipophilicity of the molecule (6). The possibility that the immuno-suppressive effect of OdDHL could involve insertion of the signal molecule within the lipid bilayer causing disruption of the association of molecules, including the TCR, within the plasma membrane, has recently been investigated, however, it seems more likely that the target of OdDHL is an (yet unknown) intracellular site (35). It appears that no matter the acyl chain length, AHLs (e.g. BHL, OdDHL and *N*-(3-oxooctanoyl)-L-homoserine lactone, OOHL) are able to cross the cell membrane of various cell lines and to function in mammalian cells (25, 36, 40, 47). However, whether PQS is also capable of crossing the eukaryotic cell membrane remains to be elucidated.

A highly relevant issue is how OdDHL and PQS exert their immunomodulatory effects. The effect of PQS on eukaryotic cells is not nearly as well investigated as for OdDHL. Compounds sharing structural properties with PQS have been found to have immunosuppressive effects. For example, quercetin (4*H*-1-Benzopyran-4-one) has been shown to decrease superoxide production and Platelet Activating Factor (PAF) released by neutrophils via inhibition of phosphatidylinositol 3-kinase (PI3K) and to decrease expression of the transcription factor nuclear factor κB (NF-κB) (4, 38). The antibacterial fluoroquinolones also have been shown to possess immunomodulatory effects, e.g. ciprofloxacin has been shown to inhibit production of TNF-α and IL-12 after LPS challenge in an *in vivo* mouse model (32). Nevertheless, whether PQS works in the same manner as some of these structurally related compounds will require further investigations.

NF-κB and Activator Protein-2 are both important factors for the maximal IL-8 transcription induced by OdDHL (42). The authors also found that OdDHL by activation of NF-κB induces the expression of inducible cyclooxygenase (COX-2) (43). Interestingly, it has been suggested that COX-2 plays a dual role in the inflammatory processes; as mediator of a proinflammatory response in the early phase of an inflammation via conversion of arachidonic acid to precursors of the 2-series prostanoids e.g. PGE_2 , and being involved in the resolution of the inflammation later by generating an alternative set of anti-inflammatory prostaglandins such as the anti-inflammatory cyclopentenone prostaglandins (14). These prostacyclins, such as

15deoxy$\Delta^{12\text{-}14}$prostaglandin J_2 (15d-PGJ_2), bind to and activate the peroxisome proliferator-activated receptors which mediate anti-inflammatory effects on several immune cells, including inhibition of the IL-2 production by T cells and down-regulation of DC maturation and IL-12 production by DCs (18, 52). The prostacyclin 15d-PGJ_2 has also been shown to inhibit toll like receptor-mediated activation of DCs via the MAP kinase and NF-κB pathways (1). Thus, consistent with the above mentioned findings, one hypothesis for the immunomodulatory mechanism put forth by OdDHL could be that OdDHL induces COX-2 expression which further generates either pro-inflammatory prostaglandins which promote a T_H1 dominated response as observed by (42, 43) or anti-inflammatory prostacyclins which merely gives a T_H2 or anergy-like effect which would lead to impaired maturation of DCs, decreased IL-12 production and inhibition of IL-2 release from stimulated T cells.

CONCLUSIONS

We have investigated the effect of PQS, OdDHL and BHL on LPS-matured murine BM-DCs and found that OdDHL and PQS, but not BHL decrease the production of IL-12 by murine BM-DCs without altering the IL-10 release. OdDHL also inhibited LPS stimulated DCs' expression of T cell stimulatory and maturation related surface markers. Murine BM-DCs exposed to PQS and OdDHL during antigen stimulation exhibit a decreased ability to induce specific T cell proliferation. These results suggest that the *P. aeruginosa* QSSMs OdDHL and PQS impede DCs in exerting their T cell stimulatory effects. As DCs are essential APCs and play an imperative role in the immune response, the immunosuppressive effect of these two signal molecules may facilitate establishment of *P. aeruginosa* infections. OdDHL and PQS may therefore be considered as modulators of the immune system along with their ability to mediate release of QS-controlled toxins and tissue degrading enzymes. The immunosuppressive effects of OdDHL and PQS may however also help to protect the host against excessive tissue destruction due to prolonged T_H1 response during a chronic lung infection e.g. in CF patients.

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