Pseudomonas aeruginosa quorum-sensing - A factor in biofilm development, and an antipathogenic drug target

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Pseudomonas aeruginosa quorum-sensing – A factor in biofilm development, and an antipathogenic drug target

Ph.D. Thesis

by

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Pseudomonas aeruginosa biofilm development and inhibition
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Cover illustration: Crystal structure of the LasR protein from *P. aeruginosa*

Preface

This dissertation is submitted as a partial fulfillment of the requirements to obtain the Ph.D. degree at the Technical University of Denmark (DTU). The work presented in this thesis was carried out at the Department of Systems Biology, under the supervision of Associate Professor Tim Tolker-Nielsen (Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen) and Professor Søren Molin (Infection Microbiology Group, Department of Systems Biology, DTU), in the time period from August 2006 to July 2009. The Ph.D. scholarship was granted by DTU.

Liang Yang
Virum, July 2009

Acknowledgements

First and formost, I would like to thank my two supervisors: Associated Professor Tim Tolker-Nielsen and Professor Søren Molin. I appreciate Tim for introducing me to this fascinating topic in science and his precise guidance for all my research project, which is a great help for my research life. I also appreciate Søren for accepting me into his group after Tim sought new challenges in University of Copenhagen. Thank you for creating a great scientific environment with an international atmosphere and plenty of visitors for new inspiration.

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I appreciate all the members from Professor Mingqiang Qiao and Professor Yanling Bai's group at Nankai University. I would not start my research life without you.

I want to express my gratitude to my parents, for their endless support and my girlfriend Yang Liu, for coming to Denmark to support me.

Virum, July 2009 Liang Yang

Summary

Bacteria have evolved chemical-mediated cell-to-cell communication systems (quorum sensing) to coordinate gene expression and group activities within communities. Quorum sensing systems exist in a wide range of different bacterial species and play major roles in determining virulence factor production, adaption and biofilm formation.

Biofilms are complex microbial communities consisting of microcolonies embedded in self-produced extracellular polymer substances. It has been estimated that 65% of microbial infections in the developed countries are associated with biofilms. Biofilm bacteria show much greater resistance to antimicrobial agents than their free-living counterparts, and therefore give rise to persistent infections.

Biofilm development is a dynamic and complicated process which involves many different components and group activities such as cell surface structures (e.g. type IV pili), motility, chemotaxis, subpopulation differentiation, and iron siderophore synthesis. Understanding the regulation mechanisms of bacterial biofilm development can provide knowledge for better control of biofilm related problems.

Pseudomonas aeruginosa is an important opportunistic pathogen, and a model organism for biofilm studies. The *P. aeruginosa* biofilm matrix contains primarily polysaccharides, proteins and extracellular DNA (eDNA). This Ph.D. project aimed at understanding roles of quorum sensing in *P. aeruginosa* biofilm structure development, and in addition an identification of potential antipathogenic drugs targeting *P. aeruginosa* quorum sensing was carried out. The *Pseudomonas* quinolone signal (PQS)-mediated quorum sensing system was identified to be necessary for release of eDNA which was shown to play a key role in *P. aeruginosa* biofilm structure development and maintenance. The spatiotemporal pattern of PQS synthesis gene expression in *P. aeruginosa* biofilms was monitored and found to be in accordance with the pattern of eDNA release. A high iron concentration was found to reduce *P. aeruginosa* PQS synthesis, eDNA release and biofilm formation. A transposon insertion mutant library was constructed and mutants deficient in eDNA release were identified. The majority of the eDNA release deficient mutants had the transposon inserted in genes directly involved in the PQS synthesis pathway. A *tatA* mutant, which is defective in the twin-arginine translocation (Tat) system, was DNA release deficient, and was found to synthesize the same amount of PQS as its parent strain, but failed to release PQS to the extracellular environment. These studies indicated that PQS synthesis and its release process are involved in eDNA release. In addition, the two different group activities, PQS mediated eDNA release, and pyoverdine mediated iron up take, were studied and found to mediate subpopulation interactions in *P. aeruginosa* biofilms. Finally, a high-throughput virtual screening of *P. aeruginosa* quorum sensing inhibitors was performed via computer based molecular docking methods. Several recognized drugs were identified and verified as quorum sensing inhibitors, which could significantly reduce virulence and biofilm formation by *P. aeruginosa*.

中文简介

细菌生物膜（biofilm）是指由细菌黏附于固体或有机腔道表面后，并分泌多糖蛋白聚合物（Extracellular Polymeric Substances）将自身包裹其中而形成的膜状物。细菌生物膜广泛存在于自然界，在医疗、食品、工业、军事等诸多领域给人类社会带来了严重的危害，造成巨大的经济损失。细菌生物膜的成分比较复杂，其主要生物大分子有蛋白质、多糖、DNA、肽聚糖、脂和磷脂等物质。这些物质对细菌的初期黏附和维持生物膜的结构都有重要的作用。这些大分子的合成和释放机制还不是非常的清楚。生物膜的形成是一个非常复杂的动态过程。细菌的表面附属结构（如鞭毛和纤毛），分泌系统（secretion），移动能力（motility），密度感应系统（quorum sensing），化学趋化（chemotaxis）系统等都在生物膜的形成中发挥作用。

目前，由于铜绿假单胞菌(*Pseudomonas aeruginosa*)形成生物膜而引发的感染已成为医院亟待解决的问题之一。铜绿假单胞菌是一种非发酵革兰阴性杆菌。对人类而言，铜绿假单胞菌属于条件致病菌。长期应用激素、免疫抑制剂，进行肿瘤化疗、放射治疗、大面积烧伤等导致病人免疫功能低下，以及手术后或某些治疗操作后（如气管切开、保留导尿管等）的病人易导致本菌感染，故认为该菌为医院内感染的重要病原菌之一。铜绿假单胞菌感染是高加索人群最常见遗传病囊肿性纤维化（Cystic Fibrosis，CF）患者的主要致死原因。

本研究以铜绿假单胞菌作为模式菌株，通过转座文库的构建，荧光标记，基因芯片，蛋白质组学等手段，以激光共聚焦显微镜为工具来研究生物膜形成的分子机制、生理生化特点及其调控机理。同时，本研究还通过分子对接及虚拟筛选来寻找抑制细菌密度感应系统及生物膜形成的新药物。本研究的主要结果如下：

（1）铜绿假单胞菌生物膜的组成。

通过建立转座文库，本研究发现了调控铜绿假单胞菌生物膜膜基质多糖蛋白聚合物中主要成分胞外核算（extracellular DNA）相关的一系列基因。研究显示 DNA 主要来自一部分细胞的死亡，而这种细胞死亡又是受环境中的营养因素（比如铁离子浓度）及密度感应系统调控的。同时该文库还表明表面附属结构，分泌系统，移动能力及信号传导系统相关的基因都能调控细胞死亡，由此推断细胞死亡是在生物膜形成的动态过程中发生的。

（2）铜绿假单胞菌生物膜的形成及其对耐药性的影响。

通过荧光标记和激光共聚焦显微镜研究，揭示了铜绿假单胞菌生物膜动态发展过程（粘附，菌落的形成、发展和成熟）中细胞死亡的发生及其对生物膜结构和生理生化的影响。并进一步阐明了其与抗生素耐药性与生物膜中不同亚群的分化有关。生物膜中不同的亚群有着不同的生长速率和对抗生素的耐药性。

（3）计算机辅助药物设计在铜绿假单胞菌感染中的应用。

通过分子对接（Molecular Docking）其对大量化合物数据库进行虚拟筛选（Virtual Screen），从已知的药物库中筛选出了 3 中能够抑制铜绿假单胞菌的密度感应系统，毒素释放及生物膜形成的药物，这些药物将在小鼠肺部感染模型中进行测试。

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3. **Yang L**, Nilsson M, Gjermansen M, Givskov M, Tolker-Nielsen T. Pyoverdine and PQS mediated subpopulation interactions involved in *Pseudomonas aeruginosa* biofilm development. Submitted to Mol Microbiol.
4. Gjermansen M, Nilsson M, **Yang L**, Tolker-Nielsen T. Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms: genetic elements and molecular mechanisms. Accepted by Mol Microbiol.
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14. Allesen-Holm M, Barken KB, **Yang L**, Klausen M, Webb JS, Kjelleberg S, Molin S, Givskov M, Tolker-Nielsen T. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Mol Microbiol. 2006, 59(4):1114-28.

Table of Contents

1 Introduction

This initial chapter aims at introducing bacterial cell-to-cell communication (quorum sensing), the biofilm mode of growth, and the problems caused by biofilms.

1.1 Quorum sensing - small molecules play a big role

Bacteria have evolved chemical-mediated cell-to-cell communication systems to coordinate gene expression and group activities within communities, which was defined as 'quorum sensing' by Dr. Peter Greenberg in 1994 (Fuqua *et al.*, 1994). Quorum sensing is widely spread in the bacterial kingdom, with many different species 'speaking' systems (Gray & Garey, 2001). Within a community, individual bacterial cells produce and respond to their specific secreted small molecules (often referred to as autoinducers). Despite the fact that there are a variety of different quorum sensing autoinducers, the principles of quorum sensing are conserved in a wild range of bacteria (Gray & Garey, 2001). As a bacterial population is growing, the level of the released autoinducer increases with the increasing cell number. When the autoinducer level reaches a crucial threshold level, the quorum sensing system will be activated and cause a population-wide alteration of gene expression. Autoinducers thus coordinate the gene expression of individual cells within communities to perform group activities and this phenomenon only happens when bacterial cells live together in communities.

There are generally three major classes of bacterial quorum sensing systems based on the type of autoinducer signals and the receptors used for its detection. First, gram negative bacteria typically use LuxI/R quorum-sensing systems (Fuqua *et al.*, 1994) (Figure 1, left column). LuxI is an enzyme that synthesizes an acylated homoserine lactone (AHL) based autoinducer which interacts with the transcriptional regulator LuxR and then controls transcription of quorum-sensing target genes. LuxI/R systems are found existing in more than 70 species of gram negative bacteria (Henke & Bassler, 2004; Miller & Bassler, 2001). The second class of quorum sensing systems exists in gram positive bacteria, where modified oligopeptides are used as autoinducers (Figure 1, center column). The extracellular peptide autoinducer is recognized by a two-component signal transduction protein called sensor histidine kinase (Hakenbeck & Stock, 1996). The sensor histidine kinase then transmits sensory information via phosphorylation of a two-component response regulator protein and modifies its DNA binding activity and thus enables it to control transcription of quorum-sensing target genes (Hakenbeck & Stock, 1996). The third class of quorum sensing systems is a combination of the gram negative and gram positive systems (Figure 1, right column). Small molecule autoinducer (e.g. AHL) is synthesized and released into the environment like the first quorum sensing systems among gram negative bacteria. However, the sensing of the autoinducer and control of transcription of quorum-sensing target genes is through a two-component signal transduction system like the second class of quorum sensing system among gram positive bacteria (Bassler *et al.*, 1993; Bassler *et al.*, 1994; Chen *et al.*, 2002). Besides the above three quorum sensing systems, there are many minor quorum sensing systems which only exist in certain organisms.

One thing that needs to be taken into consideration is that some autoinducer signals can be used for interspecies interactions. For example, *P. aeruginosa*, which does not possess the *luxS* gene and does not produce autoinducer II was shown be able to respond to autoinducer II produced by indigenous (nonpathogenic) populations in cystic fibrosis (CF) sputum samples from host microflora (Duan *et al.*, 2003). In mixed biofilms formed by *P. aeruginosa* and *Burkholderia*

cepacia, *B. cepacia* was capable of sensing the AHL signals produced by *P. aeruginosa* (Riedel et al., 2001).

Quorum sensing systems are used to regulate important behaviors that are most effective when coordinated within a large population, including production of virulence factors, motilities, production of iron chelators, exoenzymes, antibiotics, biofilm formation and so on (Williams et al., 2000). Many of these quorum sensing regulated behaviors share another important feature – energy costing. Thus quorum sensing provides a way where bacteria can coordinate cooperative group activities. Quorum sensing determined virulence factors play an important role in infection diseases caused by pathogenic bacteria and quorum sensing systems are potential drug targets for infection diseases treatments (Hentzer *et al.*, 2003).

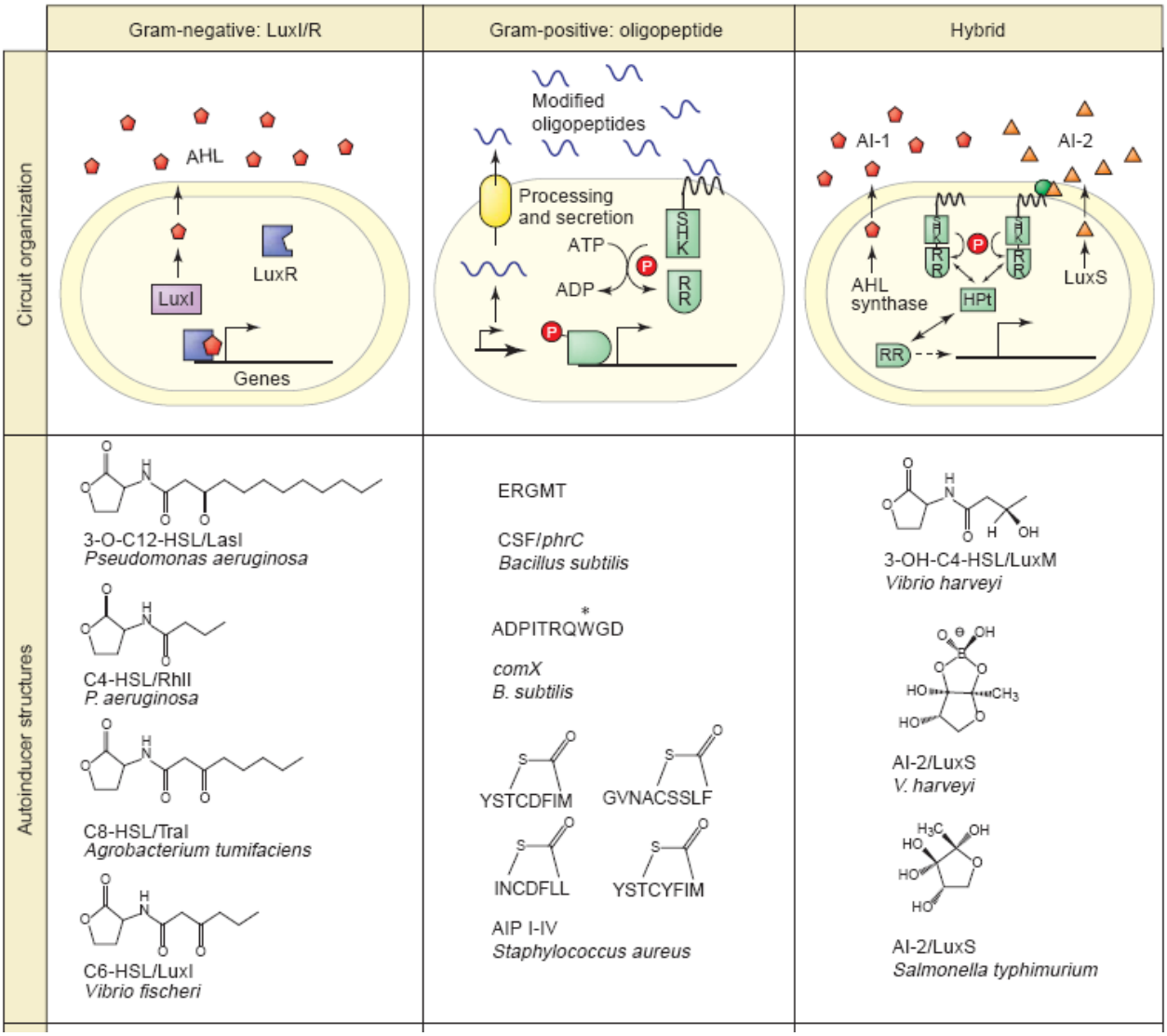

Fig. 1. The three general classes of quorum-sensing systems in bacteria (Henke & Bassler, 2004).

In *P. aeruginosa*, there are three interconnected quorum sensing systems *las*, *rhl* and *pqs* and together they control expression of a wide range of virulence factors (Latifi *et al.*, 1995; Passador *et al.*, 1993; Pesci *et al.*, 1999). The major signal molecules involved are 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL) in the case of the *las* system, C4-HSL in the case of the *rhl* system, and 2-heptyl-3-hydroxy-4-quinolone, designated the *Pseudomonas* quinolone signal (PQS), in the case of the *pqs* system (Latifi *et al.*, 1996; Pesci *et al.*, 1999). Fig. 2 summarized the synthesis and interactions of the three different quorum sensing systems in *P. aeruginosa* (Dubern & Diggle, 2008). In *P. aeruginosa*, the *las* system is required for full expression of *pqsH*, and *pqsR* is positively regulated by LasR/3-oxo-C12-HSL. In contrast, both *pqsA* and *pqsR* are repressed by the *rhl* system. PQS can induce the expression of *pqsA* in a *PqsR* dependant manner (Dubern & Diggle, 2008).

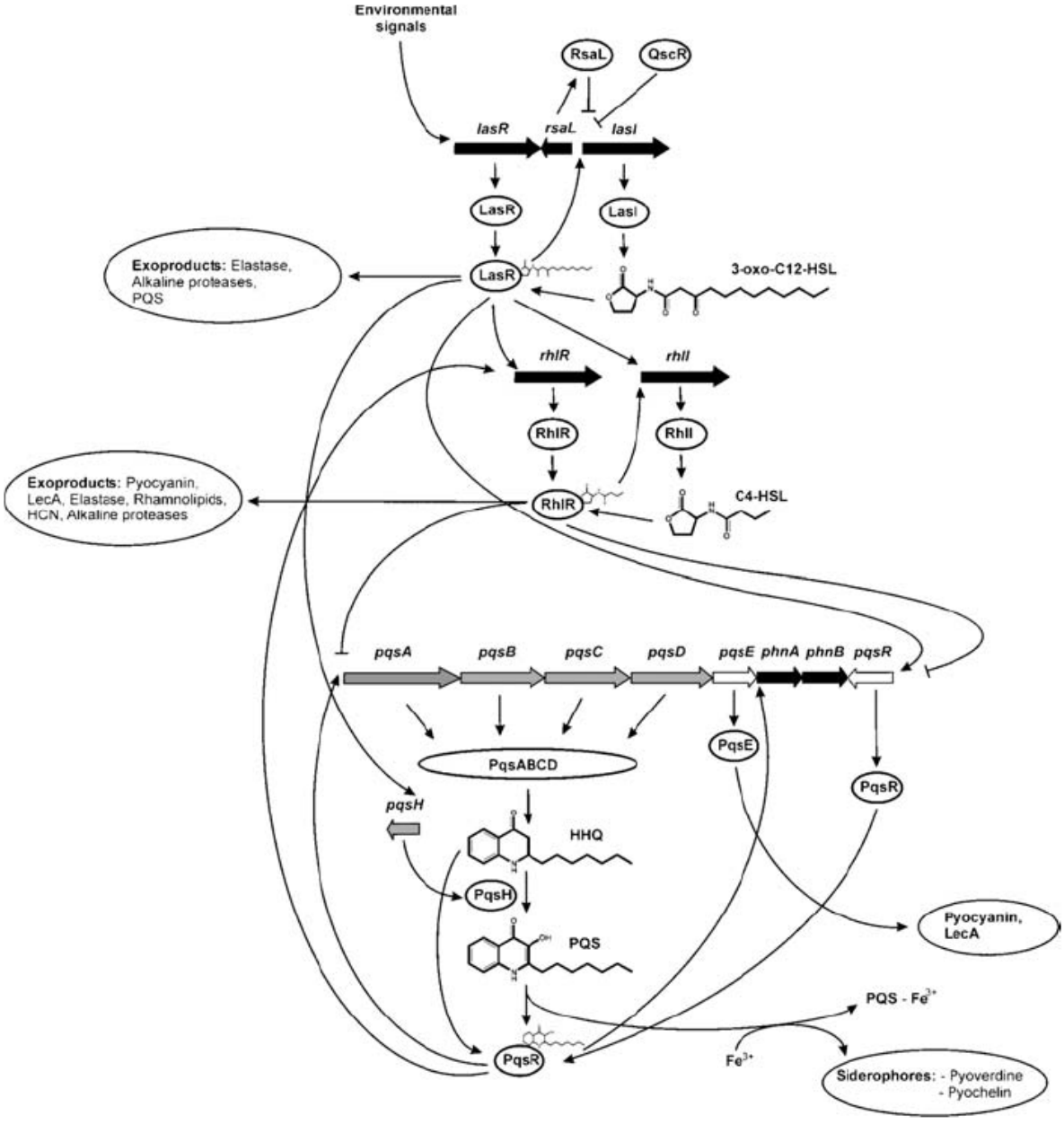

Fig. 2. *P. aeruginosa* quorum sensing systems. → represents positive regulation; ⊣ represents negative regulation.

1.2 Biofilms - together we stand

1.2.1 Biofilm definition and problems

In the past centuries, it was well known by human beings that bacteria live as unicellular lives. The traditional way of culturing bacteria in liquid medium has been adopted in studying of microbial pathogenesis and revealed some of the essential facets of microbial physiology. However, pure culture planktonic cells are rarely how bacteria exist in nature. Recent development in microscopy and molecular technologies has enabled us to observe the complex microbial communities *in situ* in great detail. Direct observation of a wide range of natural microorganisms has revealed the fact that the majority of microbes persist as surface attached communities surrounded by self-produced matrix materials, also called biofilms (Costerton *et al.*, 1995). Fig. 3 shows a staphylococcal biofilm on the inner surface of an indwelling medical device, where bacterial cells are entrapped by an extensive amount of slime matrix material.

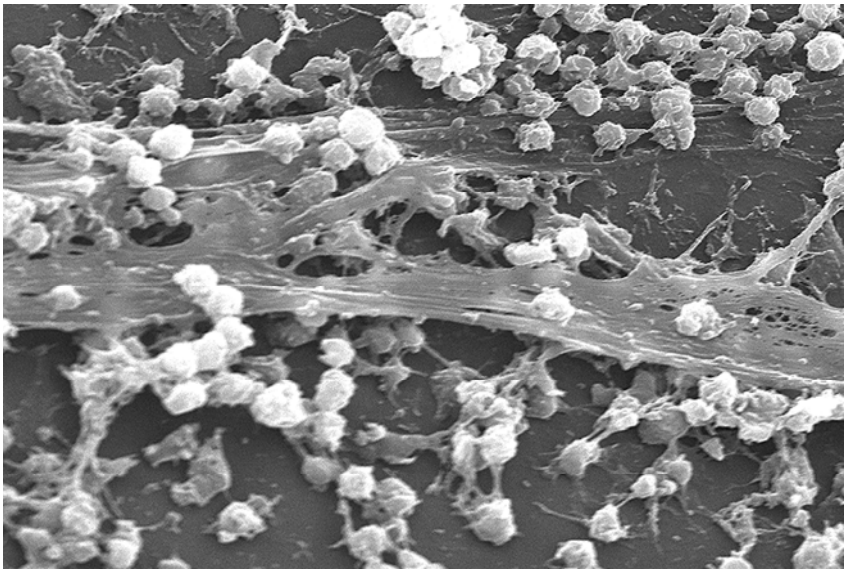

Fig. 3. Scanning electron micrograph of a staphylococcal biofilm on the inner surface of an indwelling medical device. Photograph by Janice Carr, CDC (http://www.medscape.com).

What drive bacteria to produce biofilms? It was proposed by Jefferson in his review that there could be at least four explanations: (1) protection from harmful conditions in the host (defense), (2) sequestration to a nutrient-rich area (colonization), (3) utilization of cooperative benefits (community), (4) bacteria normally grow as biofilms and planktonic cultures are an *in vitro* artifact (biofilms as the default mode of growth) (Jefferson, 2004). There will be more details about the biofilm formation mechanisms discussed in the next chapter.

Biofilm formation has brought huge amounts of problems to our everyday life (Fig. 4 and Fig. 5). Microbial biofilms cost billions of dollars every year world wide in equipment damage, product contamination, energy losses and medical infections. Conventional methods for eradicating bacteria such as antibiotics, and disinfection are often ineffective to biofilm populations due to their special physiology and physical matrix barrier (Stewart, 2002). So novel strategies based on a better understanding of the biofilm formation mechanisms are urgently needed by many industries as well as hospitals.

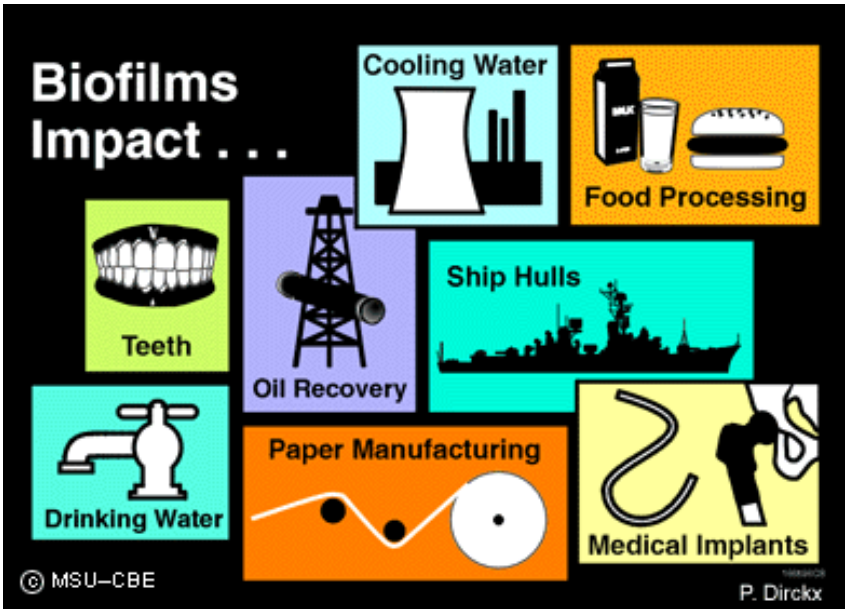

Fig.4. Problems duo to bacterial biofilms (http://www.erc.montana.edu/)

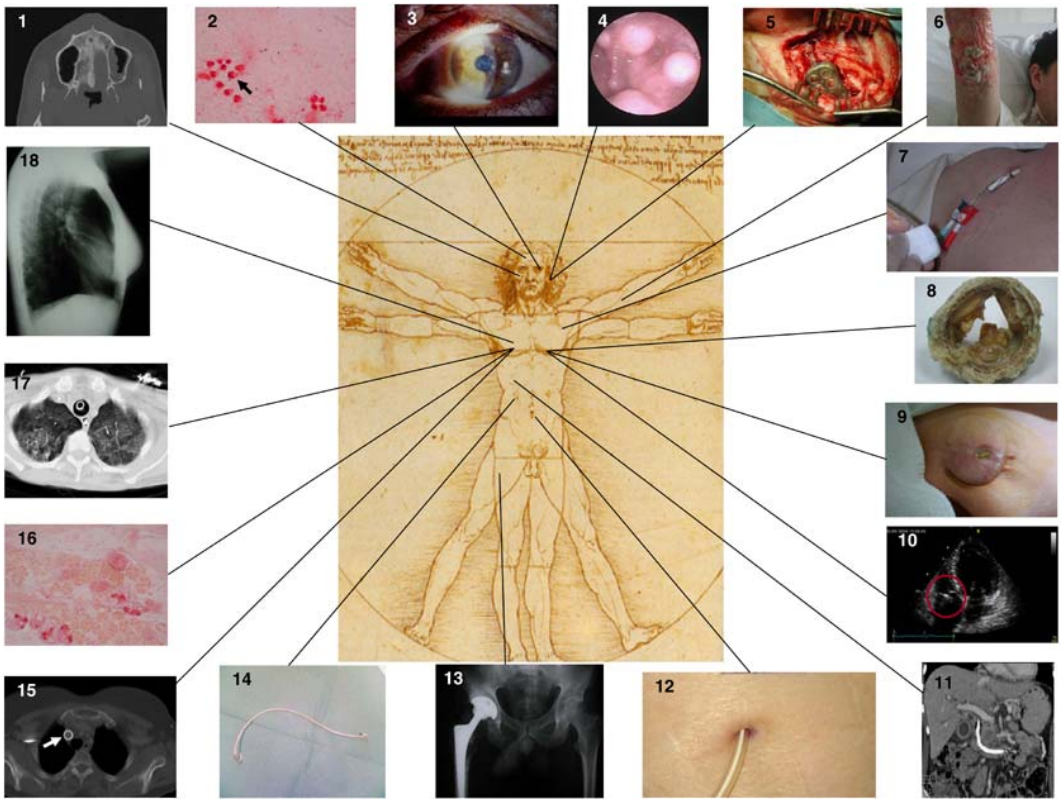

Fig. 5. Human infections diseases caused by bacterial biofilms (del Pozo & Patel, 2007). (1) Chronic sinusitis. (2) Central nervous system shunt infection. (3) Contact lens-associated keratitis. (4) Chronic otitis media. (5) Cochlear implant infection. (6) Burn-related infection. (7) Intravascular catheter infection. (8) Prosthetic valve endocarditis. (9) Pacemaker infection. (10) Electrophysiological wire endocarditis. (11) Biliary stent infection. (12) Peritoneal dialysis catheter infection. (13) Prosthetic joint infection. (14) Urinary stent infection. (15) Intravascular stent infection. (16) Pulmonary infection in cystic fibrosis patient. (17) Ventilator associated pneumonia. (18) Breast implant infection.

The formation of biofilms is a complicated process which involves many physiological activities and is highly dependent on the environmental factors (e.g. oxygen level, shear force, nutrition). Scientists often term this process 'biofilm development'. Biofilm development is a dynamic process which can be shown as Fig. 6. In a recent review by Monds and O'Toole, a criteria for microbial biofilm development was proposed as following (Monds & O'Toole, 2009):

"

- Changes in form and function that are part of the normal life cycle of the cell.
- Genetic pathways regulate stage-specific transitions in form and function in response to environmental cues.
- Genetic pathways directly couple changes in form with changes in function.
- Genetic pathways are hierarchically ordered, with hierarchical divisions serving as checkpoints enforcing coordination and directionality of development.
- Developmental pathways have evolved as dedicated systems for regulation of biofilm formation.
- The developmental model describes biofilm formation in terms of a multicellular process, wherein genetic pathways are presumed to have evolved to facilitate cooperation among members of the biofilm.

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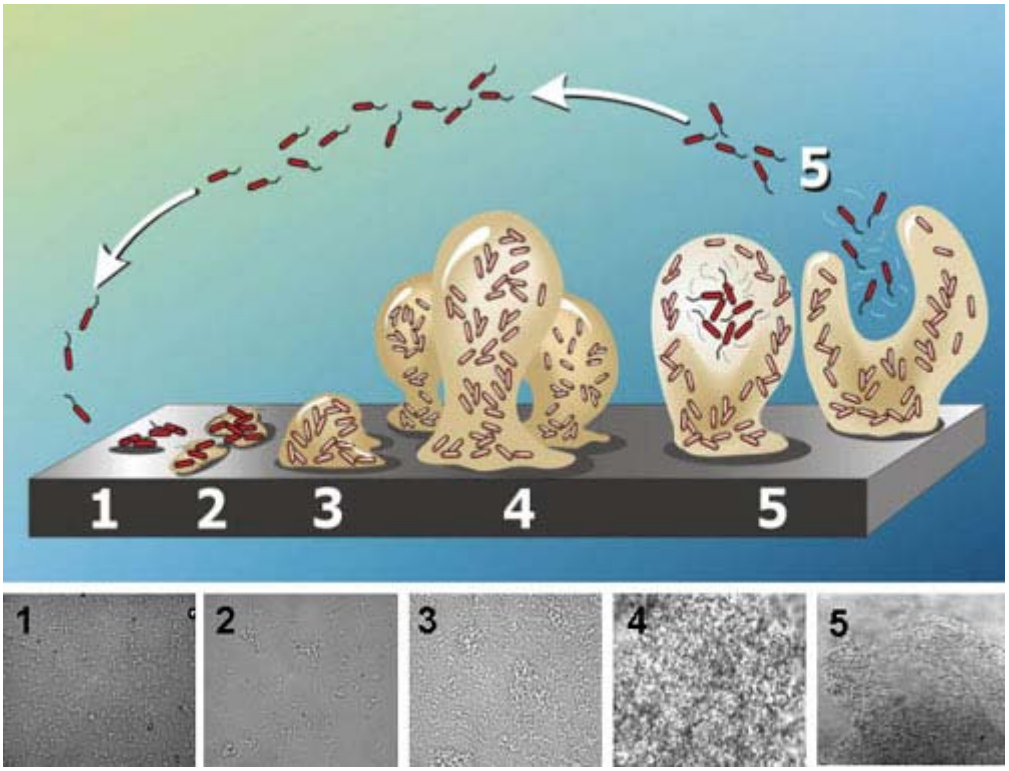

Fig. 6 An example showing the development of a biofilm as a five-stage process under continuous-flow conditions. Stage 1: initial attachment of cells to the surface. Stage 2: production of EPS resulting in more firmly adhered cells. Stage 3: early development of biofilm architecture. Stage 4: maturation of biofilm architecture. Stage 5: dispersion of single cells from the biofilm. The bottom panels (1-5) show each of the five stages of development represented by a photomicrograph of *P. aeruginosa* when grown under continuous-flow conditions on a glass substratum (Stoodley *et al.*, 2002).

No matter which models are used to describe biofilm development, the two things microbiologists interested in are always the biofilm cells and extracellular polymeric substances (EPS). The cell physiology has been extensively studied by using reporter genes, mutation analysis, transcriptomics and proteomics (Stewart & Franklin, 2008). These studies showed that cells with diverse genotypes, phenotypes, distinct metabolic pathways, stress responses and other specific biological activities coexist in biofilms (Stewart & Franklin, 2008). At the same time, the biofilm EPS matrix is also

well characterized with the help of chemical methods such as HPLC and specific staining. The EPS varies a lot in different biofilms and consists of a wide variety of proteins, glycoproteins, glycolipids, as well as extracellular DNA (e-DNA) (Flemming *et al.*, 2007). The mechanisms that contribute to the heterogeneity both in cells and EPS include microscale chemical gradients, interspecies interactions, stochastic gene expression and the genotypic variation that occurs through mutation and selection (Parsek & Tolker-Nielsen, 2008; Stewart & Franklin, 2008).

Matrix components may serve as a "wall" to hinder penetration of bactericidal agents into biofilms. The heterogeneity in biofilms can greatly facilitate the adaptation of bacterial cells under harmful conditions, which brings many problems (such as persister cells and antibiotic tolerant subpopulation development) for treatment of biofilm related infections diseases (see Fig. 7a & 7b).

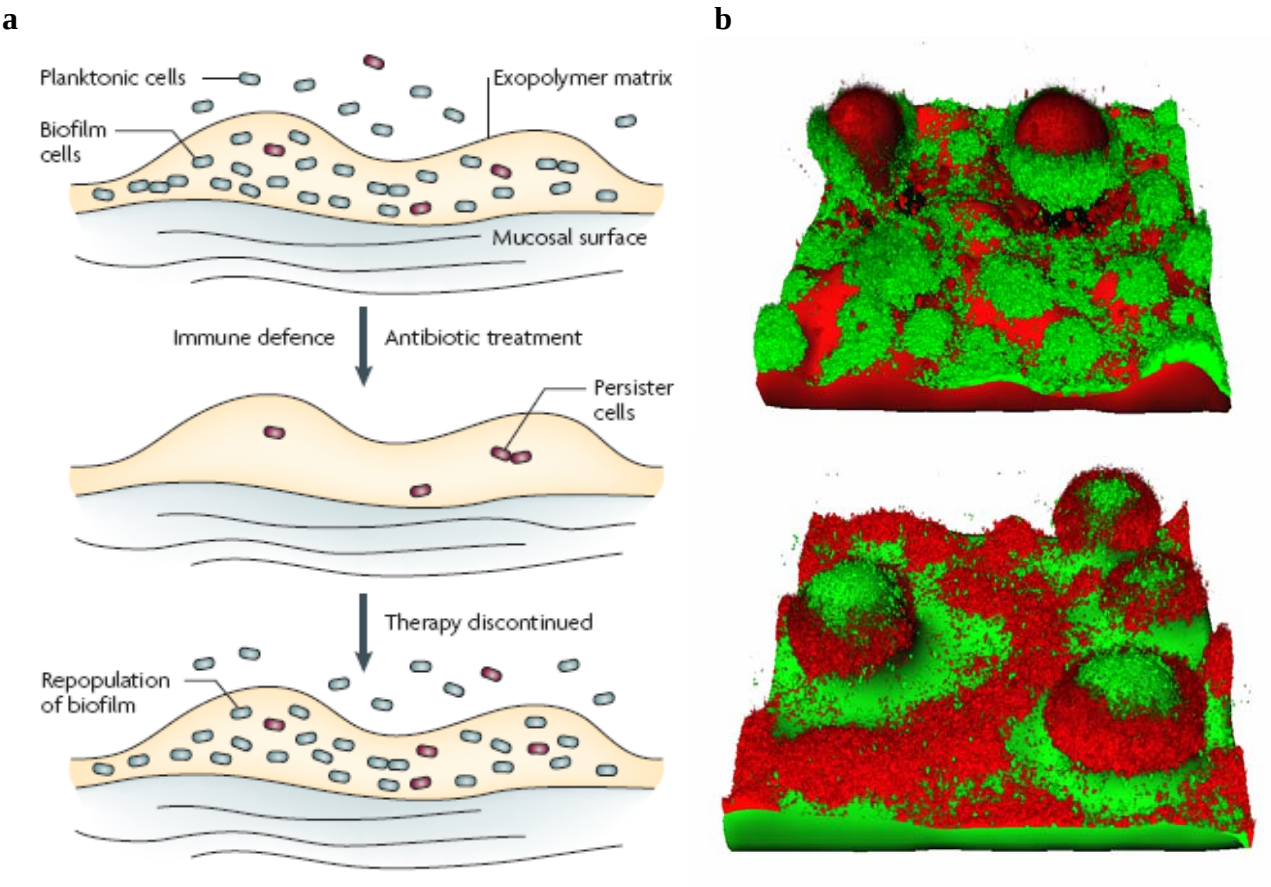

Fig. 7. Biofilm drug resistance. Figure 7a shows a model of biofilm resistance to killing based on persister survival. Initial treatment with antibiotic kills normal cells (coloured green) in both planktonic and biofilm populations. The immune system kills planktonic persisters (coloured pink), but the biofilm persister cells (coloured pink) are protected from the host defences by the exopolymer matrix. After the antibiotic concentration is reduced, persisters resuscitate and repopulate the biofilm and the infection relapses (Lewis, 2007). Figure 7b shows distribution of live (green) and dead (red) cells in EDTA (upper) and ciprofloxacin (down) treated *P. aeruginosa* biofilms (Liang Yang, unpublished).

1.2.2 Social activities in bacterial biofilms

It is well know now that bacteria and other microorganisms exhibit a wide range of social behaviors involving complex systems of cooperation, competition, adaptation, communication and synchronization just as mammals, birds and insects (Crespi, 2001). Microorganisms are ideal for social behavior studies since they have short generation times and are easy for genetic manipulations. The social lives of microbes have a significant impact on our everyday lives: cooperative behaviors play key roles in the microbial infections to humans, animals and agriculture plants (Kolter & Greenberg, 2006; Williams *et al.*, 2000). Biofilm formation involve many different group activities such as quorum sensing, iron siderophore production and rhamonolipid production (Banin *et al.*, 2005; Davey *et al.*, 2003; Davies *et al.*, 1998). These activities can determine the structure and antibiotic resistance of biofilms.

One of the most common forms of social behaviors in microbes is the production of public goods. Public goods are products manufactured by an individual cell that can be utilized by itself or its neighbors (West et al., 2006). However, the production of public goods will usually lead to the problem of cheating due to the fact that it is metabolically costly for the individuals who produce public goods. Cheaters who do not produce but could use public goods will evolve. According to the different consequences of both the actor and the recipient caused by the public goods, the social behaviors could be classified into four categories (Table 1).

		Effect on recipient	
		Positive	Negative
Effect on actor	Positive	Mutual benefit	Selfishness
	Negative	Altruism	Spite

Table 1. Social behaviors can be classified according to the fitness consequences they encounter for the actor and recipient. A behavior increasing the direct fitness of the actor is mutually beneficial if the recipient also benefits, while selfish if the recipient suffers a loss. A behavior reducing the fitness of the actor is altruistic if the recipient benefits, while spiteful if the recipient suffers a loss (West *et al.*, 2006).

Bacteria produce numerous extracellular public goods that are released into the environment (Table 2) (West *et al.*, 2007). The following figure has illustrated the problem of cooperation (Fig. 8). There are many unknown mechanisms regarding bacterial cooperative activities. For example, how a single bacterial cell can sense its relatedness with the neighbor cells around it? How the genes in different bacterial cells are coordinated to express at the same time to perform the cooperative activities?

Public good	Role
Siderophores	Iron-scavenging molecules (West & Buckling 2003).
Invertase	An enzyme for digesting sucrose (Greig & Travisano 2004).
β-lactamase	Inactivates and therefore gives resistance to antibiotics (Ciofu et al. 2000)
Biosurfactants	Extracellular matrices for facilitating movement over surfaces, e.g., Rhamnolipid (*P. aeruginosa*) and Serrawettin (*Serratia marcescens*) (Daniels et al. 2004, Velicer & Yu 2003)
Exopolysaccharides such as alginate or adhesive polymers	Providing structure for growth, and the ability to colonize different habitats (Davies & Geesey 1995, Rainey & Rainey 2003)
Host-manipulation factors	Increasing host susceptibility to predation, immune suppression, host castration (Brown 1999)
Shiga toxins	Breaking down host tissue (O'Loughlin & Robins-Browne 2001)
Protein synthesis	Growth (Turner & Chao 1999)
Toxic and lytic secondary metabolites	To kill and degrade prey organisms
Adhesive polymer	Colonization of the air-liquid interface (Rainey & Rainey 2003)
Quorum-sensing molecules	Cell-cell signals (Williams et al. 2007), iron chelation (Diggle et al. 2007, Kaufmann et al. 2005), immune modulators (Pritchard et al. 2003), biosurfactants (Daniels et al. 2006), plant systemic resistance (Schuhegger et al. 2006)
Proteases	Extracellular protein digestion (Hase & Finkelstein 1993)
Extracellular DNA	Structural component of biofilms (Spoering & Gilmore 2006)
Antibiotics	To kill competitors (although better to conceptualize as spiteful rather than public good
Membrane vesicles	Common biofilm component (Schooling & Beveridge 2006), transport of cell-to-cell signals (Mashburn & Whiteley 2005)
Rhamnolipids	Antiprotozoan defense mechanism (Cosson et al. 2002), mediate detachment from biofilms (Boles et al. 2005)
Microbial repellents	Repels competitors (Burgess et al. 2003)
Resources supplied by symbionts to their hosts, such as nitrogen fixation by rhizobia	Aids host growth and, in some cases, avoid enforcement by the host (Kiers et al. 2003, Kiers & van der Heijden 2006, West et al. 2002)

Table 2. Potential public goods (West *et al.*, 2007).

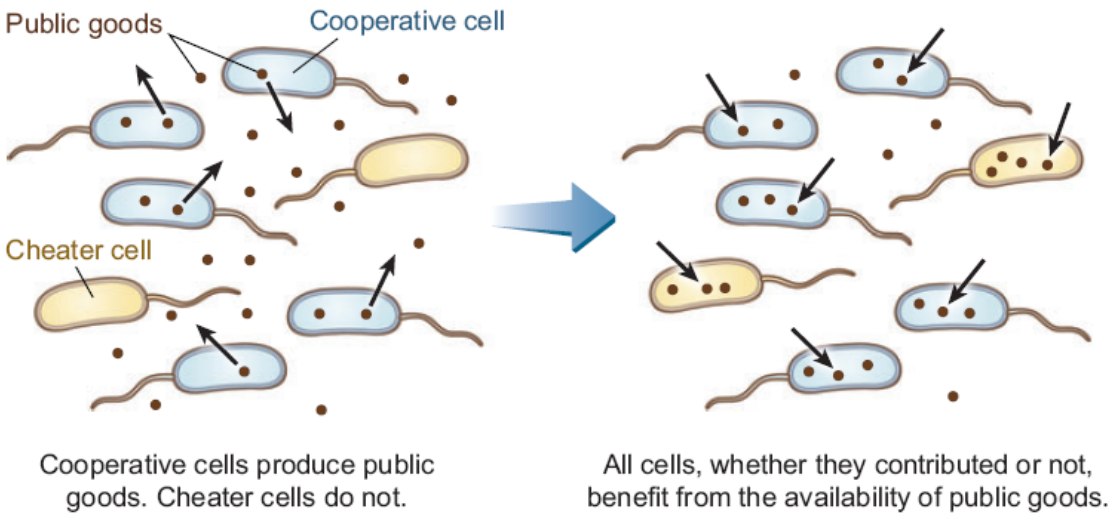

Fig. 8. The problem of cooperation (West *et al.*, 2007).

1.2.3 Biofilm investigation approaches

Microtiter plates are used very often to quantify biofilm formation by crystal violate assay (O'Toole & Kolter, 1998). Colony morphology in agar plates containing Congo red dye is also used as a indication of production of biofilm EPS materials (Friedman & Kolter, 2004a). To visualize the dynamic biofilm development, scientists often use flow-cell technology in combination with confocal laser scanning microscopy (CLSM) as a gold standard. With the help of fluorescent reporter proteins and staining, researchers can observe the biofilm development such as spatial organization, gene expression and antibiotics resistance in real time under different conditions. A standard flow-chamber biofilm cultivation system is illustrated in Fig. 9. A detail protocol of flow-chamber biofilm system set up was described by Sternberg & Toker-Nielsen (Sternberg & Tolker-Nielsen, 2006).

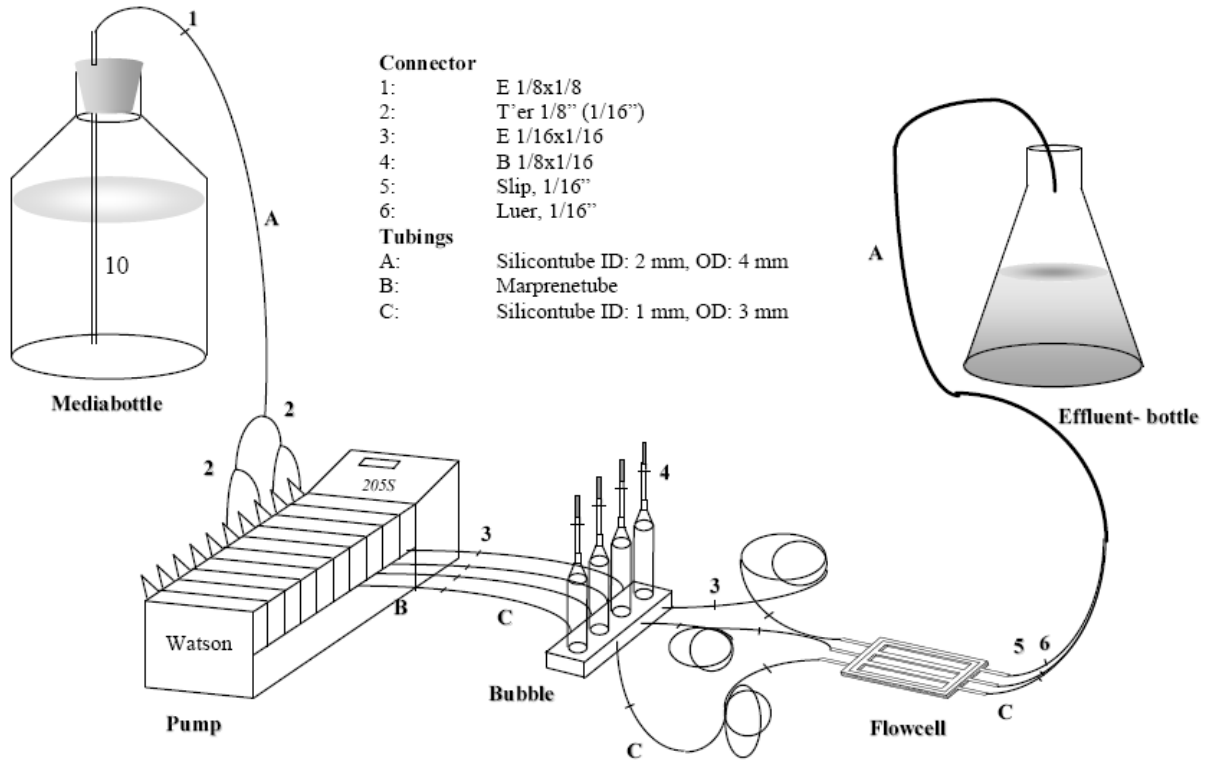

Fig. 9. Schematic representation of the flow-chamber biofilm cultivation system. A peristaltic pump is used to maintain a continuous flow of media through the flow-chambers and bubble traps are interconnected to catch air bubbles arising during cultivation (Barken & Haagensen, unpublished).

Other biofilm investigation technology includes scanning electron microscopy (SEM) and atomic force microscopy (AFM). These two technologies can observe biofilm structure and topography in really high resolution. However, the biofilm samples will usually be destroyed after observation of SEM or AFM.

2 *P. aeruginosa* biofilm development

A large part of the mechanisms of subpopulation differentiation and interactions during microbial biofilm development still remains unknown. Understanding these mechanisms can further help us to build novel strategies to eradicate bacterial biofilms. *P. aeruginosa* is used as a model organism to study biofilm development in this PhD thesis.

2.1 Extracellular polymeric substances (EPS) in *P. aeruginosa* biofilms

P. aeruginosa is a gram negative bacterium which exists in a varity of environmental conditions. *P. aeruginosa* is an important opportunistic pathogen which can cause a wide range of human infections such as pulmonary infections, medical-device-related infections, urinary tract infections, wound infections and bacteremia (Bodey *et al.*, 1983). *P. aeruginosa* is notorious for its tolerance to antimicrobial agents and this is to a large extent due to its ability to form biofilms (Costerton *et al.*, 1995; Costerton *et al.*, 1999).

The EPS materials of *P. aeruginosa* biofilms vary a lot in composition. Extracellular DNA, exopolysaccharide and proteinaceous compounds can all function as matrix components in *P. aeruginosa* biofilms, and that their relative importance as structural component may depend on the environmental conditions, the age of the biofilm and the particular *P. aeruginosa* strain forming the biofilm (Flemming *et al.*, 2007).

There are three well studied exopolysaccharides, Pel, Psl and alginate that contribute to biofilm formation in *P. aeruginosa* (Friedman & Kolter, 2004b; Ryder *et al.*, 2007; Stapper *et al.*, 2004). The Pel polysaccharide is synthesis through the *pel* gene cluster (Friedman & Kolter, 2004a). Friedman & Kolter showed that *P. aeruginosa* PA14 *pel* mutant in could not form a pellicle at the air-liquid interface of bacterial cultures grown in static conditions. They have further showed that the *pel* mutants are unable to form robust biofilms on a solid surface of plastic or glass. By using Congo red staining and carbohydrate linkage analysis, Friedman & Kolter provided evidence that the *pel* genes encode a glucose-rich matrix polysaccharide polymer, which does not appear to be cellulose (Friedman & Kolter, 2004a; Friedman & Kolter, 2004b). In a later study, Vasseur and colleges showed that Pel polysaccharide play a compensate role of type IV pili in the initial phase adherence of bacterial cells (Vasseur *et al.*, 2005). The Pel polysaccharide synthesis genes, pelA–G, are highly conserved in other *P. aeruginosa* strains (Ryder *et al.*, 2007). CF clinical isolates which autoaggregate in liquid culture and hyperadhere to solid surfaces were shown increased their expression of the *psl* and *pel* loci (Kirisits *et al.*, 2005). However, there is no immunological or lectin reagents available for probing *pel* expression or localization in developing *P. aeruginosa* biofilms until now (Ryder *et al.*, 2007).

The Psl polysaccharide is encoded from the polysaccharide synthesis locus (PA2231-2245), which is another crucial EPS component for bacterial adherence and biofilm development (Jackson *et al.*, 2004; Matsukawa & Greenberg, 2004). It was suggested that the Psl polysaccharide was mainly composed of mannose, galactose, rhamnose, glucose, and trace amounts of xylose (Ma *et al.*, 2007). Until recently, Ma and colleagues used fluorescently labeled lectins MOA (from *Marasmium oreades agglutinin*) and HHA (from *Hippeastrum hybrid*) to stain and visualize Psl polysaccharide in *P. aeruginosa* biofilms (Ma *et al.*, 2009). They have found that Psl polysaccharide forms a matrix to hold bacterial cells in the biofilm and on the surface at the early stage of biofilm development

and accumulates on the periphery of 3D-structured macrocolonies during the biofilm maturation (Ma *et al.*, 2009). The Psl polysaccharide synthesis genes and there putative functional annotations are listed in Fig. 10 as well as the Pel polysaccharide synthesis genes.

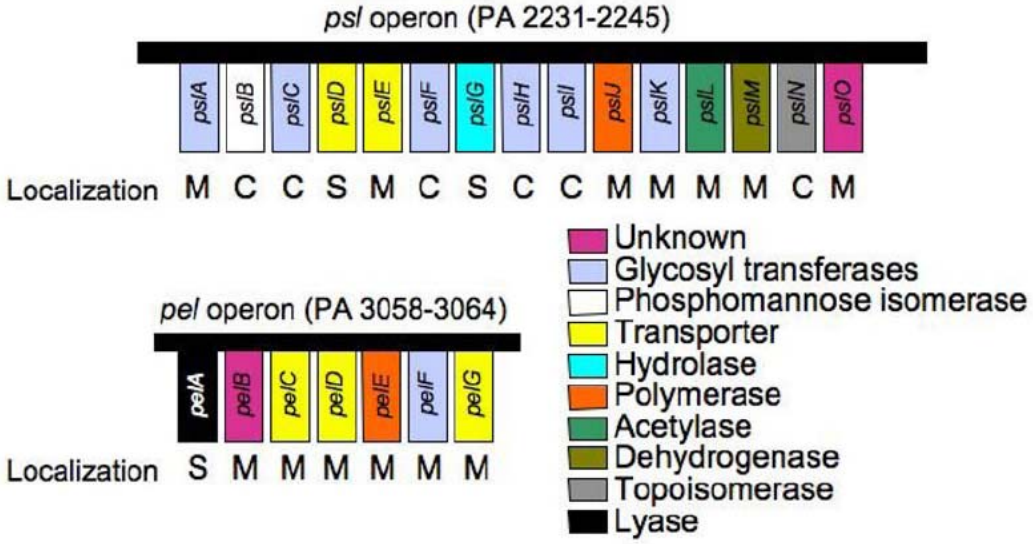

Fig. 10. Structure of *pel* and *psl* operons. Putative functions and localization of Pel and Psl polysaccharide enzymes are shown (M-membrane, C-cytoplasm, S-secreted) (Ryder *et al.*, 2007).

Unlike the above polysaccharides, alginate is a kind of special polysaccharide mainly found from *P. aeruginosa* clinical isolates from patients with life threating Cystic Fibrosis (CF) (Govan & Deretic, 1996; Hoiby, 1974). In CF patients, especially chronic pulmonary infected CF patients, *P. aeruginosa* mucoid phenotype can be isolated quite often (Fig. 11). The mucoid *P. aeruginosa* strains over produce copious quantities of alginate polysaccharide, which can help *P. aeruginosa* strains to adapt to the CF lung conditions (Govan & Deretic, 1996; Hoiby, 1974). Alginate is a high molecular weight, acetylated polymer composed of non-repetitive monomers of β-1,4 linked L guluronic and D-mannuronic acids (Govan & Deretic, 1996). Its special physical and chemical properties have multiple roles in protecting *P. aeruginosa* cells (Govan & Deretic, 1996). Nevertheless, alginate was shown not to be a necessary component for *in vitro P. aeruginosa* biofilm development (Wozniak *et al.*, 2003).

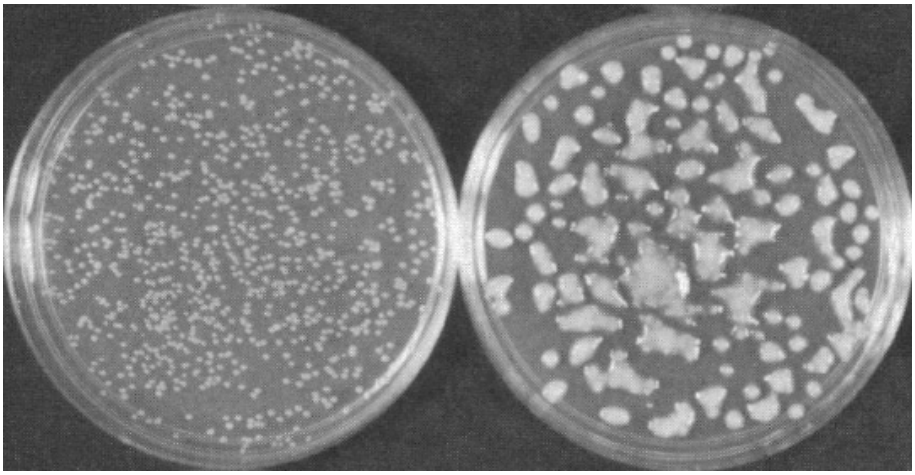

Fig. 11. *P. aeruginosa* nonmucoid strain (left) and mucoid strain (right) on plates (Darzins & Chakrabarty, 1984).

Recently, extracellular DNA (eDNA) was recognized as one of the major EPS matrix components of bacterial biofilms (Qin *et al.*, 2007; Rice *et al.*, 2007; Whitchurch *et al.*, 2002). It was shown that DNase treatment lead to dispersal of young *P. aeruginosa* biofilms (Fig. 12) (Whitchurch *et al.*, 2002). However, DNase will not disperse *P. aeruginosa* mature biofilms formed in this flow chamber system probably due to the increasing amount of other EPS materials.

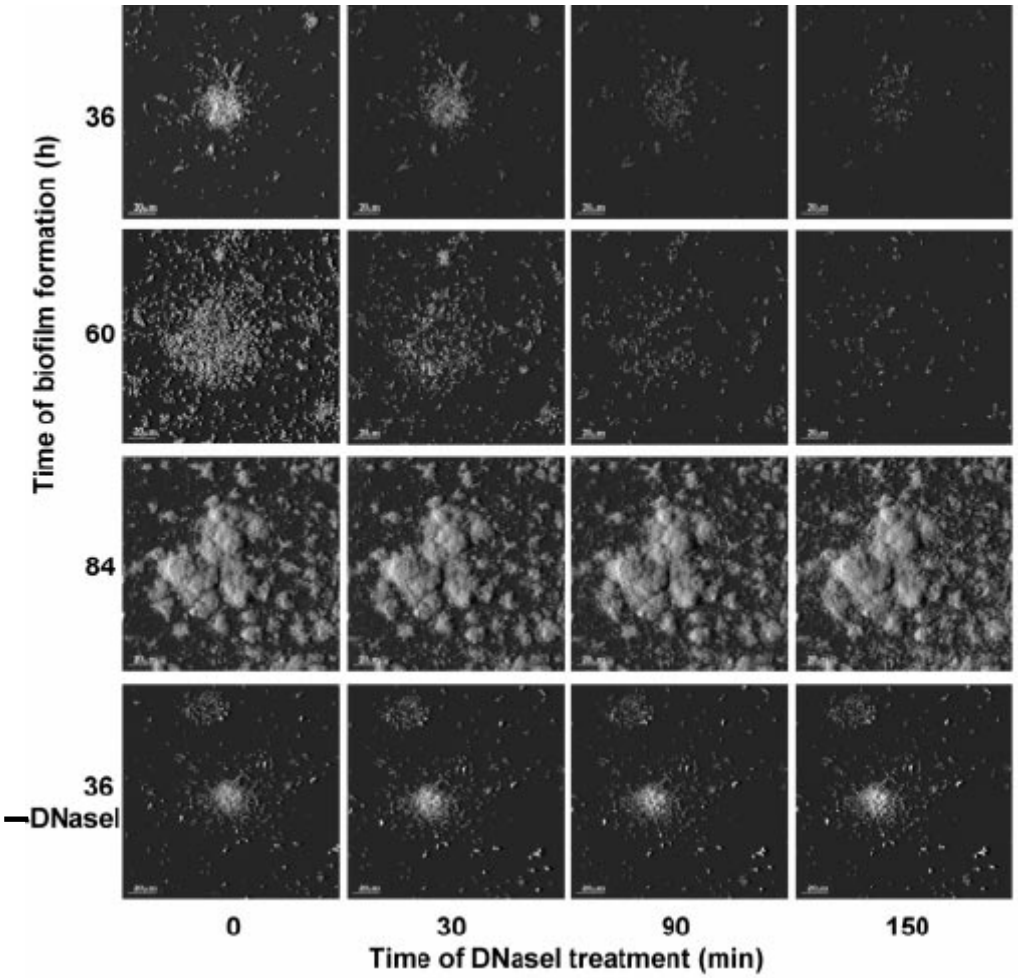

Fig. 12. DNase treatment of *P. aeruginosa* biofilms at different development stages (Whitchurch *et al.*, 2002).

In a recent study, our group showed that eDNA was mainly localized in the out layer of the stalk subpopulation of the 3D mushroom like structures of *P. aeruginosa* biofilm, which can be visualized by cell non-permeable fluorescent DNA specific staining propidium iodide (PI) and ethidium bromide (EB) (Fig. 13) (Allesen-Holm *et al.*, 2006). This localization was in accordance with the interface between motile subpopulations and non-motile subpopulations (Haagensen *et al.*, 2007; Klausen *et al.*, 2003), and which suggests us that eDNA release in *P. aeruginosa* biofilms might come from some group activities involved in subpopulation interactions. I will discuss about this in the next chapter.

Unlike the production of Pel, Psl and alginate polysaccharides as EPS materials, it is hard for us to understand why bacteria will release DNA as EPS material. Polysaccharides are relatively simple molecules and easy to synthesize while DNA is a complicated molecule containing genetic information of an organism. By chemical analysis, our group as well as other groups showed that

eDNA in the biofilm matrix was chromosome DNA (Allesen-Holm *et al.*, 2006; Qin *et al.*, 2007). eDNA was shown by our group to serve as an interconnector between the motile and non-motile subpopulations and it could significant reduce the dispersion effects of detergent sodium dodecyl sulfate (SDS) on *P. aeruginosa* biofilms (Allesen-Holm *et al.*, 2006; Barken *et al.*, 2008).

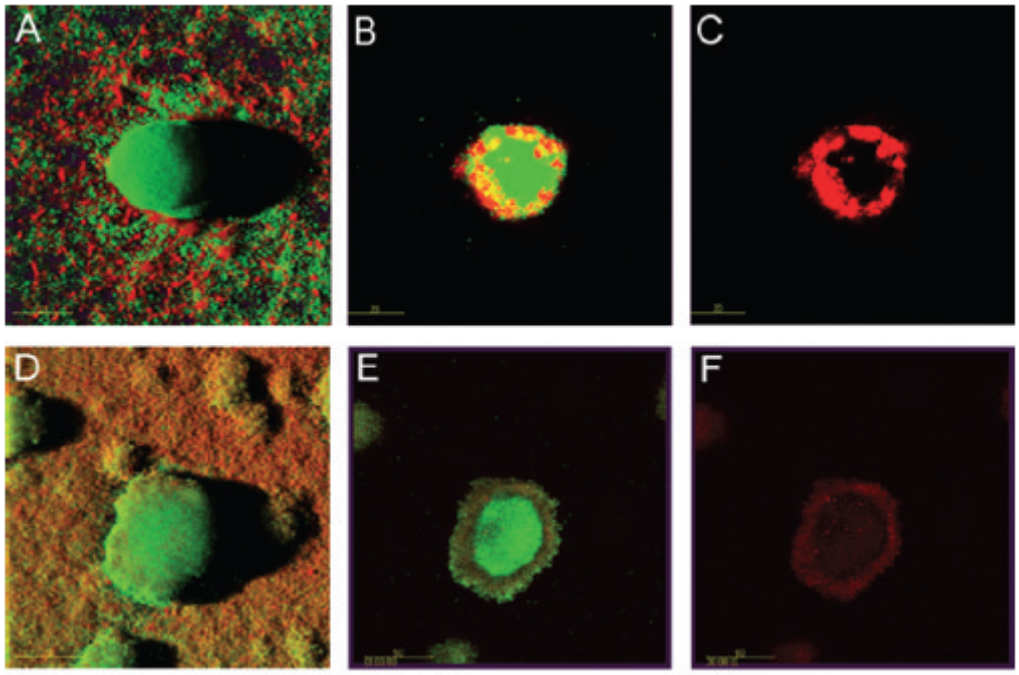

Fig. 13. eDNA visualized by cell non-permeable fluorescent DNA specific staining propidium iodide (PI) (A, B, C) and ethidium bromide (EB) (D, E, F). 4-day-old gfp tagged PAO1 biofilms were showed by a top-down views (A, D) and horizontal optical sections (B, C, E, F) (Allesen-Holm *et al.*, 2006). The bars represent 50 μM.

To investigate the mechanisms of eDNA release in *P. aeruginsoa* biofilms, Allesen-Holm and colleagues screened a couple of *P. aeruginosa* lab strains and mutants (Allesen-Holm *et al.*, 2006). They found that release of eDNA is regulated through the N-Acyl Homoserine Lactone (AHL) mediated quorum sensing systems and *Pseudomonas* quinolone signal (PQS) mediated quorum sensing system (Allesen-Holm *et al.*, 2006). AHL quorum sensing system was reported to be able to control PQS quorum sensing system and this means that studying the PQS quorum sensing system could help to identify genes that directly control eDNA release of *P. aeruginosa* (Diggle *et al.*, 2003; McGrath *et al.*, 2004). The detail mechanism of PQS mediated cell lysis is still unclear. Membrane vesicles containing DNA and other virulence factors were shown to be released from the outer membrane of *P. aeruginosa* cells under PQS regulation (Mashburn & Whiteley, 2005). Membrane vesicles were found to be a common particulate feature of the matrix of *Pseudomonas aeruginosa* biofilms (Schooling & Beveridge, 2006). Other studies showed that PQS was an iron chelator (Bredenbruch *et al.*, 2006) and it was wrapped together with iron and other molecules as inclusion bodies in periplasmic vacuoles (Royt *et al.*, 2007). Recently, PQS was reported acting as a pro-oxidant and sensitizes the bacteria towards oxidative stress and other stresses (Haussler & Becker, 2008) and this may provide another explanation of cell lysis. To better understand *P. aeruginosa* biofilm development, more studies are needed to be carried out about PQS mediated DNA release.

It is mentioned above that localization of eDNA is in accordance with the interface between motile subpopulation and non-motile subpopulation of *P. aeruginosa* biofilms (Allesen-Holm *et al.*, 2006).

It seems that eDNA release might be due to interactions between motile and non-motile subpopulations during *P. aeruginosa* biofilm development.

2.2 Group activities in *P. aeruginosa* biofilm development

Several models describing *P. aeruginosa* biofilm development were summarized by Kirisits and Parsek in a recent review (Kirisits & Parsek, 2006). Many group activities are involved in biofilm structure development and maintenance. eDNA release described above is just an example of group activity in *P. aeruginosa* biofilm development.

2.2.1 Motilities

Our group has used a two strain model to study different group activities in *P. aeruginosa* biofilm development. In this model, a mixture of yellow fluorescent protein (Yfp) tagged *P. aeruginosa* wild type and cyan fluorescent protein (Cfp) tagged *pilA* mutant (deficient in biogenesis of type IV pili) was grown in minimal medium supplemented with glucose as carbon source and developed mushroom-like biofilm structures. As shown in Fig. 14, the *pilA* mutants could only form stalks whereas the wild-type bacteria formed all the caps, suggesting that type IV pili are necessary for cap formation (Klausen *et al.*, 2003). Because cap formation was shown to occur via bacterial migration and require type IV pili, the process was suggested to involve type IV pili mediated migration (Klausen *et al.*, 2003).

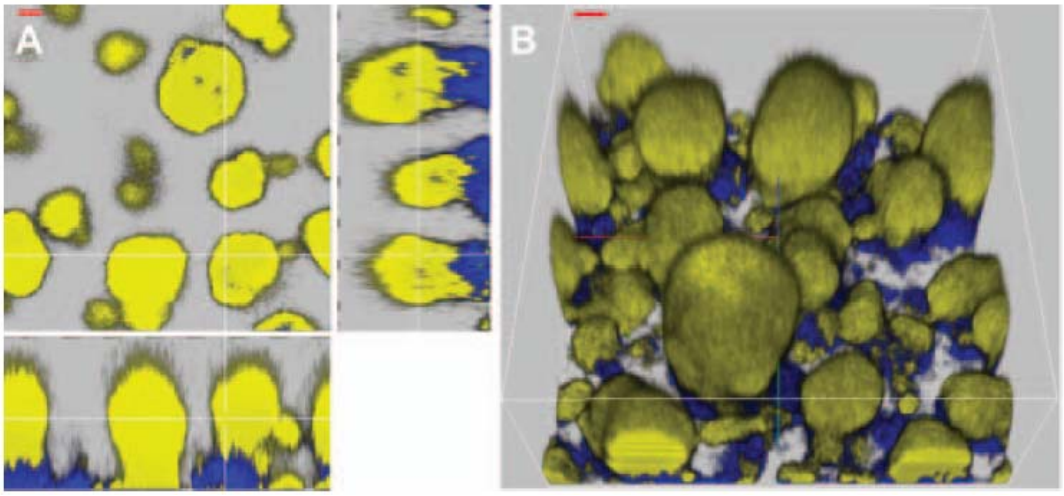

Fig. 14. Mushroom-shaped multicellular biofilm structures with yellow caps and cyan or yellow stalks. CLSM images were acquired in a 4-day-old biofilm which was initiated with a 1:1 mixture of Yfp tagged *P. aeruginosa* PAO1 wild type and Cfp tagged *pilA* derivative and grew on glucose minimal medium. The bars represent 20 μM. Biofilms were showed by a horizontal optical section view (A) and 3D view (B) (Klausen *et al.*, 2003).

Since we also found that eDNA was mainly localized within the interface between motile subpopulation and non-motile subpopulation (Allesen-Holm *et al.*, 2006), it would be interesting to study whether eDNA play a role in the migration of *P. aeruginosa* PAO1 wild type onto the *pilA* stalk. Based on this concept, a *pilAlasRrhlR* mutant was constructed and tagged with Cfp by Barken and collegues (Barken *et al.*, 2008). This strain was defective at both type IV pili and AHL mediated quorum sensing as well as eDNA release. When this mutant was mixed with a Yfp tagged PAO1 wild type in biofilms, PAO1 wild type tend to form independent mushroom like biofilm

structures instead of associating with the *pilAlasRrhlR* mutant (Fig. 15) (Barken *et al.*, 2008). In addition, feeding the *pilA* Cfp/PAO1 Yfp mixed biofilm medium with DNaseI after 2 days of cultivation could significant repress the association of the two strains and mushroom like biofilm structure development (Barken *et al.*, 2008). However, quorum sensing controls production of a wide range of other factors such as rhamnolipid, which is also necessary for *P. aeruginosa* biofilm development (Pamp & Tolker-Nielsen, 2007). Thus the structures of *pilAlasRrhlR* Cfp/PAO1 Yfp mixed biofilms and *pilA* Cfp/PAO1 Yfp mixed biofilms fed with DNaseI in the medium were different (Barken *et al.*, 2008).

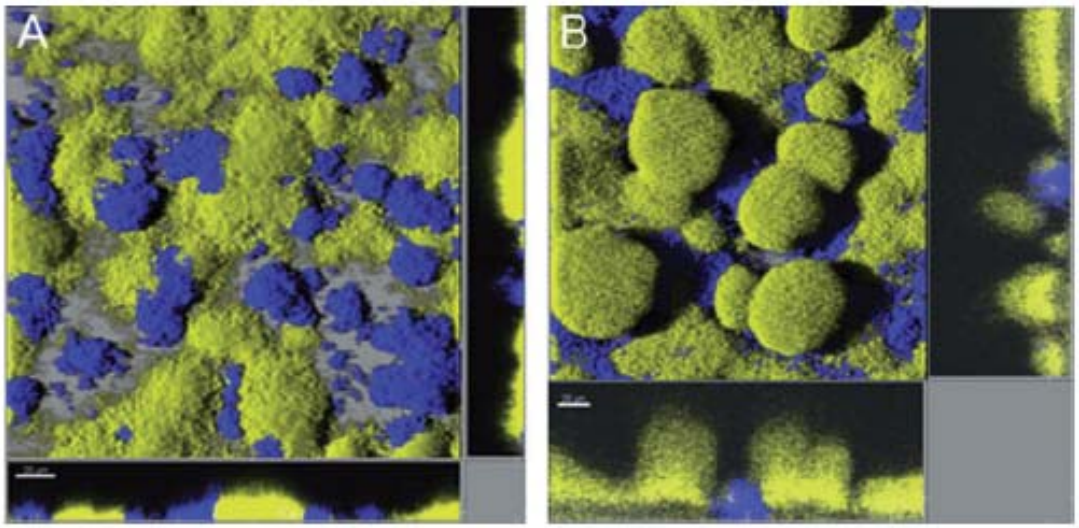

Fig. 15. Confocal laser scanning micrographs of a 2-day-old (A) and 4-day-old (B) biofilms formed by a mixture of *P. aeruginosa pilAlasRrhlR* Cfp and wild-type Yfp. The central images show top-down views and the flanking images show vertical optical sections (Barken *et al.*, 2008). The bars represent 20 μM.

The above example shed some light on how eDNA release is involved in *P. aeruginosa* biofilm development. However, there are still many mechanisms about this phenomenon remaining unknown. For example, which subpopulation (*pilA* or wild type) in this *pilA* Cfp/ wild type Yfp mixed biofilm contributes to the eDNA release? Why wild type subpopulation will associate with *pilA* subpopulation since the competition for nutrition will be high when they stay together?

2.2.2 Quorum sensing

In biofilms, bacterial cells encounter much higher local cell densities than their planktonic counterparts. An obvious consequence of this is the elevated levels of local nutrition competition, metabolic by-products, secondary metabolites and other secreted or excreted microbial factors (Parsek & Greenberg, 2005). How these activities are coordinated and contribute together to *P. aeruginosa* biofilm development is still largely unknown. Quorum sensing was shown to play an important role on regulating gene expression in a density dependent manner in many organisms (Henke & Bassler, 2004). There is no doubt it can regulate many essential pathways and products for *P. aeruginosa* biofilm development (Kirisits & Parsek, 2006; Parsek & Greenberg, 2005).

As a high level regulator, quorum sensing was shown to be able to regulate other group activities such as biosurfactant rhamnolipid synthesis, swarming motility, and iron siderophore pyoverdine synthesis (Wagner *et al.*, 2003), which are necessary for *P. aeruginosa* biofilm development (Banin *et al.*, 2005; Lequette & Greenberg, 2005; Pamp & Tolker-Nielsen, 2007). Rhamnolipid synthesis

deficient *P. aeruginosa rhlA* mutant could only form flat biofilms in flow chamber system (Davey *et al.*, 2003). A *rhlA* mutant could only weakly associate with a non-motile *pilA* mutant and formed reduced mushroom like biofilm structures in mixed biofilm cocultures (Fig. 16A) (Pamp & Tolker-Nielsen, 2007). Rhamnolipid may have several functions on the development of the mushroom like biofilm structures. Rhamnolipid is required for detachment of the *P. aeruginosa* cells from the substratum (Davey *et al.*, 2003). It also mediates the flagellum mediated swarming motility, which was further reported to be necessary for the association of non-motile and motile subpopulations (Fig. 16B) (Barken *et al.*, 2008). The *P. aeruginosa* flagellum deficient *fliM* mutant and the flagellum mediated chemotaxis deficient *cheA* mutant could not associate with a non-motile *pilA* mutant in mixed biofilm cocultures (Barken *et al.*, 2008). These studies indicated that *P. aeruginosa* type IV pili while not pili mediated motility were necessary for biofilm structure development and both flagellum and flagellum mediated chemotaxis were necessary for biofilm structure development. It is high possible the requirement of type IV pili for *P. aeruginosa* biofilm development is through its ability to bind with eDNA since it was reported that *P. aeruginosa* type IV pili could bind with DNA at a very high efficiency (van Schaik *et al.*, 2005).

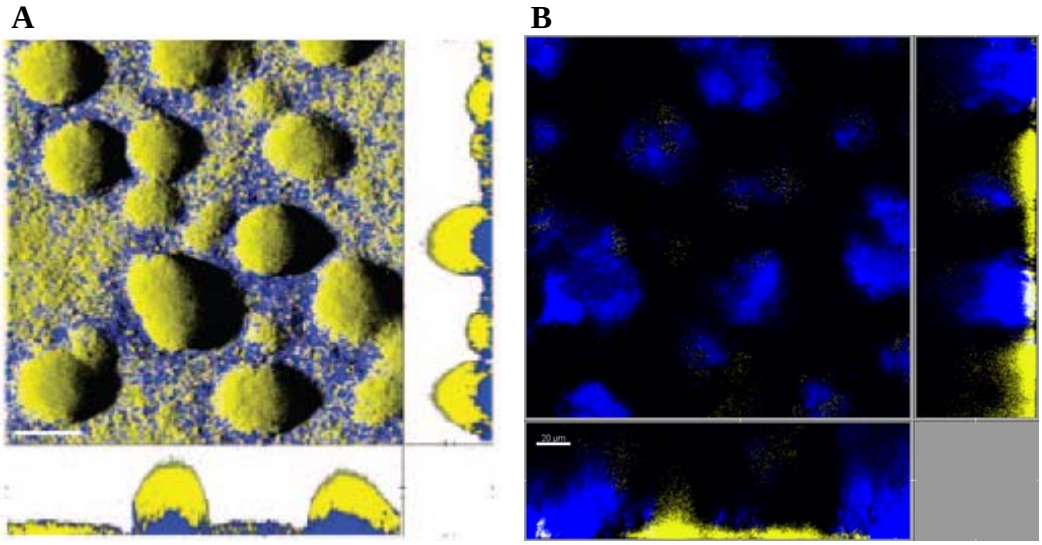

Fig. 16. Confocal laser scanning micrographs of 4-day-old biofilms formed by a mixture of *P. aeruginosa pilA* Cfp and *rhlA* Yfp mutant (A) and a mixture of *P. aeruginosa pilA* Cfp and *fliM* Yfp mutant. The bar represents 40 μM for A and 20 μM for B (Barken *et al.*, 2008; Pamp & Tolker-Nielsen, 2007).

To investigate the role of type IV pili and eDNA interactions during *P. aeruginosa* biofilm development, we have fed eDNA in the medium of *P. aeruginosa* biofilms cultivated in our standard flow chamber system after 2 days of cultivation. The *P. aeruginosa* wild type PAO1 formed giant mushroom like structures compared to non-fed control (Fig. 17A), while the *pilA* mutant did not change significantly compared its non-fed control (Fig. 17B). The eDNA fed mixed *pilA* Cfp and wild type Yfp biofilms also develop to giant mushroom like structures compared to their non-fed control (Fig. 17C). Considering the existence of large amounts of eDNA in many environmental samples as well as the lungs of CF patients (Dell'Anno & Danovaro, 2005; Kirchner *et al.*, 1996), the interaction between type IV pili and eDNA might help bacteria to build stable biofilm structures.

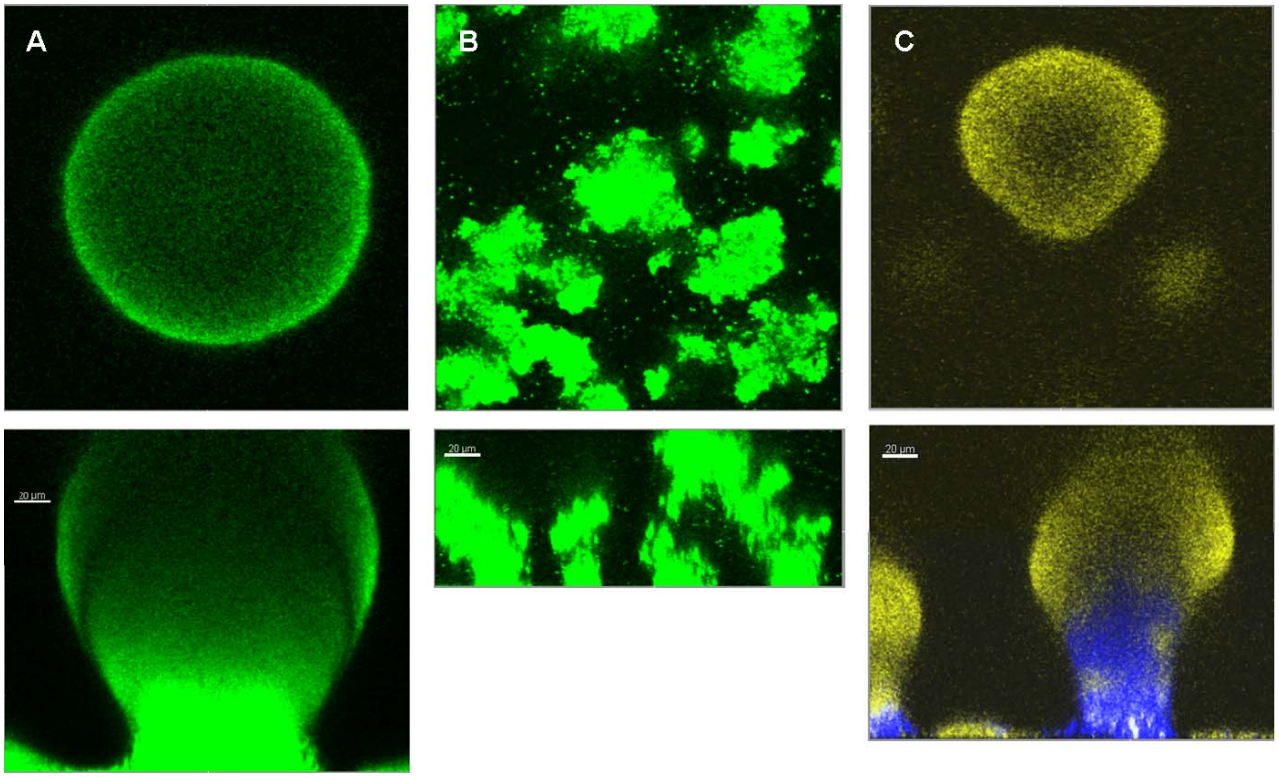

Fig. 17. Confocal laser scanning micrographs of 4-day-old biofilms formed by wild type Gfp (A), *pilA* Gfp mutant (B) and a mixture of *pilA* Cfp and wild type Yfp. The bars represent 20 µM. (Liang Yang, unpublished).

2.2.3 Iron siderophore production

We have mentioned in the above text that another quorum sensing regulated phenomenon, iron siderophore pyoverdine synthesis, are reported to be necessary for the *P. aeruginosa* biofilm development (Banin *et al.*, 2005). Unlike rhamnolipid, the synthesis of pyoverdine was only partially regulated by quorum sensing system, and a quorum sensing *lasI* mutant produced half amount of the pyoverdine compared to its isogenic parent strain (Stintzi *et al.*, 1998). Due to its key role in *P. aeruginosa* growth, the regulation of pyoverdine synthesis is very complicated (Oglesby *et al.*, 2008). The synthesis and transportation of pyoverdine to its major receptor fpvA was summarized in a review (Poole & McKay, 2003) and is shown in Fig. 18. Recently, it was reported that pyoverdine has a second receptor fpvB, which played a relatively less important role compared to fpvA and was essential for growth under iron limited condition when fpvA was deficient (Ghysels *et al.*, 2004). *P. aeruginosa* also synthesis another siderophore pyochelin (Heinrichs *et al.*, 1991) and can uses ferric citrate uptaking pathway when ferric citrate is the iron resource (Cox, 1980). A pyoverdine deficient *pvdA* mutant formed a flat biofilm in iron limited medium and supplement of ferric citrate to this medium could restore its ability to develop mushroom like biofilm structures (Banin *et al.*, 2005). What is more, iron limitation can also induce expression of genes involved in production of virulence factors in *P. aeruginosa* (Cornelis & Aendekerk, 2004).

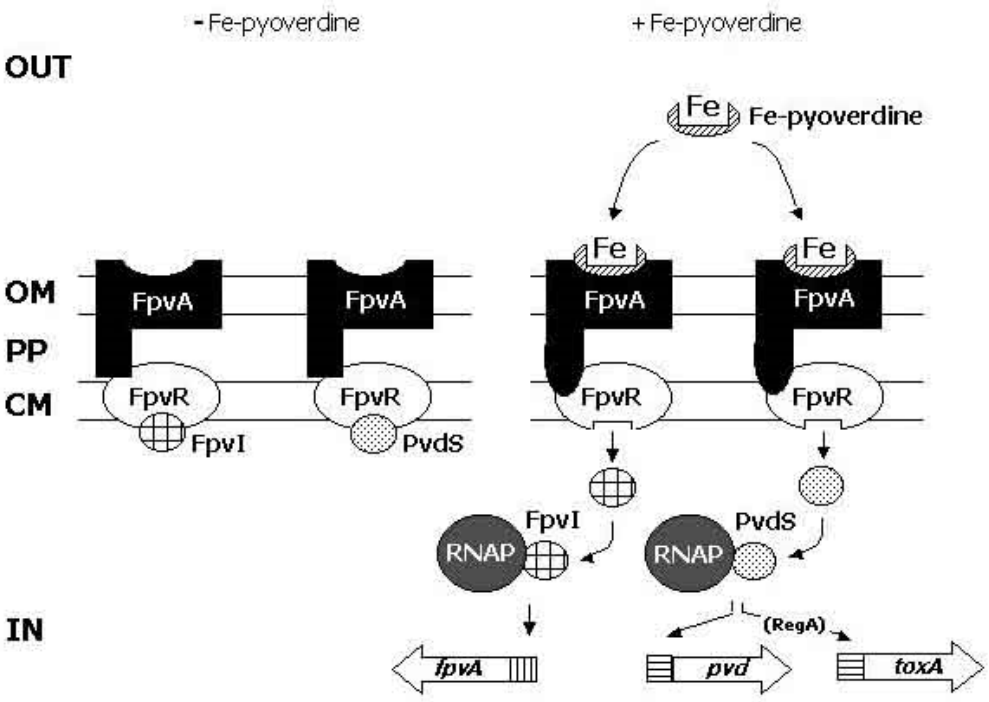

Fig. 18. Illustration of synthesis of pyoverdine and its major receptor fpvA in *P. aeruginosa*. OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane (Poole & McKay, 2003).

Under iron replete conditions (+Fe) the Fur repressor binds to Fur box in the promoter regions and blocking gene expression of ECF sigma factor genes (*pvdS*, *fpvI*), which are required for siderophore (*pvd*) and receptor (*fpvA*) gene expression. Under iron-limiting conditions (-Fe), Fur is released from the promoters and permit expression of the ECF sigma factors (*pvdS, fpvI*). (Poole & McKay, 2003). Under iron replete conditions, the Fur repressor also represses the expression of two regulatory RNAs (sRNAs) encoded by *prrF1* and *prrF2*, which are needed for PQS production (Oglesby *et al.*, 2008). Thus, under iron-limiting conditions, Fur can activate production of a series of virulence factors through PQS regulation.

It is clear that eDNA release, rhamnolipid synthesis, type IV pili structure, flagellum mediated motility and chemotaxis, and iron siderophore pyoverdine synthesis are involved in the association of non-motile and motile subpopulations during *P. aeruginosa* biofilm development. However, there are still many unknown gaps between these different events. For example, why there will be subpopulation differentiation during *P. aeruginosa* biofilm development? Which subpopulation mainly contributes to the matrix material eDNA release and siderophore synthesis, since these activities cost a lot of energy? Understanding these mechanisms will help us develop novel treatment strategies for biofilm related infections caused by *P. aeruginosa* and other bacteria.

3 *P. aeruginosa* virulence inhibition

Biofilms and the emergence of antibiotic resistance have become a major threat for current medical treatment for bacterial infection diseases (Costerton *et al.*, 1999; Neu, 1992). Due to the extensive using of antibiotics, clinically significant antibiotic resistance has arisen against virtually every antibiotic developed (Fig. 19) (Clatworthy *et al.*, 2007). It is a big challenge for us to develop novel classes of antibiotics to catch up our growing need for such drugs. Recently, it was proposed by many scientists that rather than focusing on therapeutics that target *in vitro* viability, much like conventional antibiotics, we should try an alternative approach which target functions essential for infection, such as virulence factors required to cause host damage and disease (Clatworthy *et al.*, 2007). Also, novel strategies to inhibit bacterial biofilm formation and disrupt mature biofilms are obviously in need. In the following text, the concept of anti virulence strategy, anti biofilm strategy and computer aided drug design will be introduced.

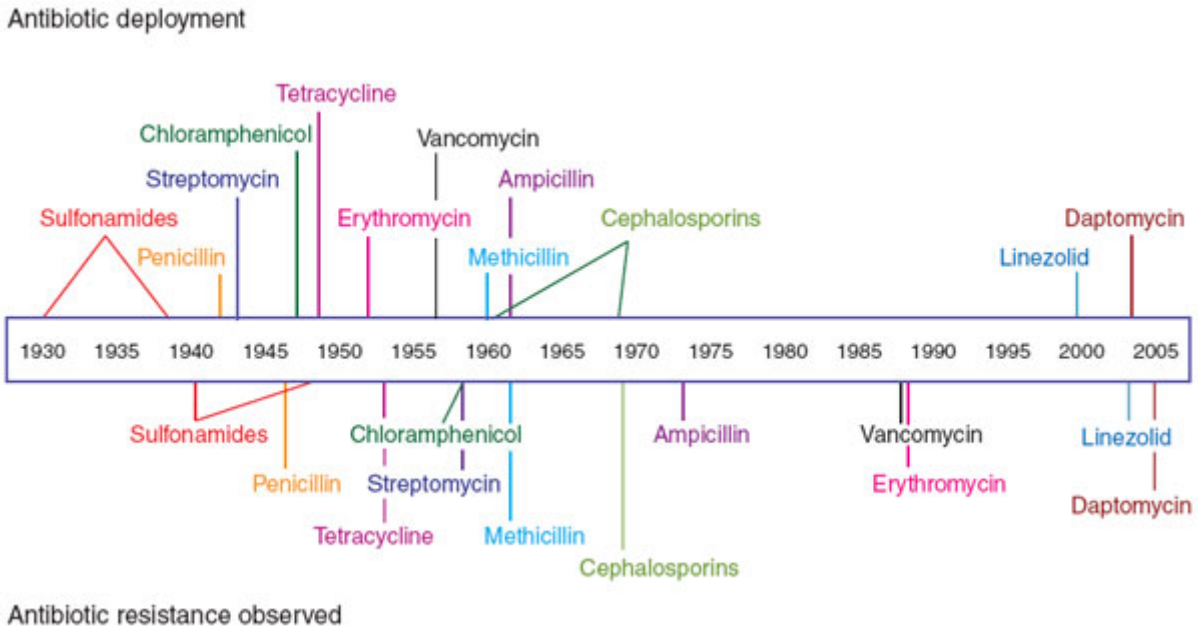

Fig. 19. Schedule of antibiotic deployment and the rise of antibiotic resistance. The year each antibiotic was deployed is depicted above the timeline, and the year resistance to each antibiotic was observed is depicted below the timeline (Clatworthy et al., 2007).

3.1 Anti virulence strategy development

Since the discovery of penicillin in 1928, regular antibiotics have routinely been identified based on their ability to kill bacteria (bacteriocidal) or inhibit growth (bacteriostatic). These regular antibiotics could inhibit a range of bacterial functions that are essential for *in vitro* growth such as cell wall synthesis (Beta-lactams), DNA replication (Fluoroquinolones), and protein synthesis (Aminoglycosides). However, the high mutation rate of bacterial genome has made it possible for bacteria to evolve resistant populations towards almost all classes of regular antibiotics (Maki & Schuna, 1978; Martinez & Baquero, 2000). Considering the current gap between our current effort to develop novel antibiotics and the real need for them, we must consider developing antimicrobials that have novel modes of action in addition to target only *in vitro* cell growth.

Pathogenic bacteria usually possess a wide range of virulence factors to enable themselves to successfully invade host tissues and cause disease. These virulence factors include adhesion, extracellular toxins, iron chelating siderophores and so on. For example, *P. aeruginosa* mutants which lack type IV pili or flagellum were seriously deficient in causing infections based on animal models (Feldman *et al.*, 1998; Tang *et al.*, 1995). Therefore, development of anti virulence therapies is aiming at inhibiting the invasion abilities of the pathogenic bacteria. The bacteria will then eventually be cleared by the host immune response with little to no impact on the normal human microbiota. It is proposed by some researchers that a potential (though yet unproven) advantage of this approach is that new anti virulence compounds may impose weaker selective pressure for the development of antibiotic resistance compared to the conventional antibiotics (Clatworthy *et al.*, 2007).

Table 3 summarized some of the recent strategies that target various pathways related to virulence, including inhibiting toxin function, toxin delivery, virulence expression regulator (quorum sensing), and adhesin (Clatworthy *et al.*, 2007).

Targets	Inhibitors (examples)	Modes of action *in vitro*	Effect on *in vivo* (or *ex vivo*) infection
Toxin function			
B. anthracis lethal factor (LF)	(2*R*)-2-[(4-fluoro-3-methylphenyl) sulfonylamino]-*N*-hydroxy-2-(tetrahydro-2*H*-pyran-4-yl) acetamide (LFI)	Binds LF active site and inhibits LF protease activity	Protects mice from spore infection when used in combination with ciprofloxacin; provides complete protection in mice immunized with LF and PA
	Peptide analogs derived from the optimal peptide substrate sequence	Inhibit LF cleavage of MKKs *in vitro*	Protect macrophages from LF-induced cytolysis *ex vivo*
B. anthracis protective antigen (PA)	Cisplatin	Inhibits LF translocation into the host cytosol	Protective when administered simultaneously with anthrax lethal toxin in murine models
	Hexa-D-arginine	Inhibits PA processing	Delays lethal toxin-induced toxemia in rodent models and cultured macrophages
Toxin delivery			
Type III secretion system (T3SS)	Acylated hydrazones of different salicylaldehydes (for example, INP0400)	Prevent effector molecule translocation	Attenuate *Y. pseudotuberculosis* infection and inhibit intracellular replication of *C. trachomatis* and *C. pneumoniae ex vivo*; preincubation of compound with bacteria before infection suppresses secretory and inflammatory responses in a bovine intestinal loop model of *S. enterica* serovar Typhimurium infection
Regulators of virulence expression			
V. cholerae ToxT	Virstatin	Prevents expression of the toxin-coregulated pilus and cholera toxin in *V. cholerae*	Protects infant mice from intestinal colonization with *V. cholerae*
Quorum sensing: LuxR homologs	Structural analogs of AHLs (for example, halogenated furanones)	Accelerate turnover of LuxR homologs; inhibit expression of quorum sensing regulated genes; inhibit the production of carbapenem in *E. carotovora* and virulence factors in *P. aeruginosa*	Promote clearance of *P. aeruginosa* from the lungs of mice in a pulmonary infection model; increase the survival time of mice in a lethal *P. aeruginosa* lung infection model
Quorum sensing: *S. aureus* AgrC	Inhibitory autoinducing peptides (AIPs)	Inhibits *agr* locus activation	Administration of inhibitory AIP to mice during *S. aureus* infection inhibits abscess formation
Adhesion			
Pilin chaperones (for example, PapD in UPEC)	Pilicides: bicyclic 2-pyridones and N-substituted amino acid derivatives	Inhibit pilus assembly	Bicyclic 2-pyridones inhibit adhesion of *E. coli* to bladder carcinoma cells *ex vivo*

Table 3. Targets of anti virulence inhibitors, modes of action and effects on infection *in vivo* (Clatworthy *et al.*, 2007).

3.2 Anti biofilm strategy development

Once the biofilms developed to a mature stage, it is extremely difficult to eradicate them with traditional antimicrobial agents (Costerton *et al.*, 1999). As our knowledge about the importance of biofilms in infections is increasing, researchers have started to develop anti biofilm strategies (Danese, 2002). Different anti biofilm strategies have been tried that were targeting different biofilm development mechanisms. These include nonbactericidal and bactericidal approaches.

For example, a wide range of novel catheter surface coating materials and catheter lock solutions were tried to reduce the initial attachment of bacterial cells to the catheter (Chiang *et al.*, 2009; Francolini *et al.*, 2004; Liedberg & Lundeberg, 1989; Shanks *et al.*, 2006; Stewart, 2003). In general, these approaches are similar to the antibiotic based approaches as far as their development is concerned. However, there is a lack of consistent demonstration of efficiency for the clinical usage of these antibacterial catheters (Danese, 2002). This is probably due to the fact that the dead cells of the initial attached bacteria will form a layer on the anti bacterial surface and provide a new surface for the attachment of the additional bacteria.

Digestion of EPS matrix materials may be another way to reduce biofilm formation. Without these 'house of biofilm cells', bacteria will only loosely attach to each other and will be sensitive towards the treatments of antimicrobial agents and host immune attack. Recombinant human DNase I was shown to be able to reduce the viscosity of the sputum of CF patients (Shak *et al.*, 1990; Shak, 1995). This is probably because that a large amount of DNA in the airways of CF patients can provide scaffolds for biofilm formation by the CF bacteria. It was reported that alginate lyase could enhance antibiotic killing of mucoid *P. aeruginosa* in biofilms (Alkawash *et al.*, 2006). Azithromycin was reported to be able to block EPS material alginate formation and quorum sensing (Hoffmann *et al.*, 2007) and it was further shown to improve lung function of CF patients, especially in the subgroup colonized with *Pseudomonas* (Florescu *et al.*, 2009).

Targeting the iron up taking pathways may also be a good choice to battle biofilm infections. Singh and colleagues has used an innate immunity component, lactoferrin, to prevent *P. aeruginosa* biofilm formation (Singh *et al.*, 2002). By chelating iron, lactoferrin could stimulate the type IV pili mediated twitching motility of *P. aeruginosa* thus block it to settle down and form biofilms (Singh *et al.*, 2002). Further more, Kaneko and colleagues have investigated a "Trojan horse" strategy by using the transition metal gallium to disrupt *P. aeruginosa* iron metabolism and exploit the iron stress of *in vivo* environments (Kaneko *et al.*, 2007). Due to its chemical similarity to iron, gallium can substitute for iron in many biologic systems and inhibit Fe-dependent processes. They have found that gallium could inhibit *P. aeruginosa* growth and biofilm formation (Kaneko *et al.*, 2007). What is more, too high concentration of iron was also found to be unfavorable for bacterial biofilm development (Johnson *et al.*, 2005; Musk *et al.*, 2005). Must and colleagues used iron salts such as ferric ammonium citrate to treat fully developed *P. aeruginosa* biofilms and found that iron salts could not only perturb biofilm formation but also disrupt existing biofilms by *P. aeruginosa* (Musk *et al.*, 2005).

Even though biofilm cells are very resistant toward conventional antimicrobial agents, there are still approaches to kill biofilm cells. One way is to treat biofilms with different combinations of antimicrobial agents. For example, several antibiotics targeting fast growing cells such as tobramycin, ciprofloxacin could only kill the surface cells of *in vitro P. aeruginosa* biofilms (Bjarnsholt *et al.*, 2005; Pamp *et al.*, 2008), while antimicrobial agents such as EDTA and colistin

were reported to prefer to kill slow growing cells inside the biofilm (Banin *et al.*, 2006; Haagensen *et al.*, 2007). A combination of antimicrobial agents from these above two classes could almost kill all the cells of *in vitro P. aeruginosa* biofilms (Pamp *et al.*, 2008).

Besides synergy of conventional antimicrobial agents, compounds targeting biofilm specific pathways can be used alone and together with conventional antimicrobial agents to eradicate biofilms. It was showed that garlic extract containing quorum sensing inhibitors could repress genes involved in oxidative stress response (Bjarnsholt *et al.*, 2005; Passador *et al.*, 1993) and could in combination with hydrogen peroxide efficiently eradicate *P. aeruginosa* biofilms. Barraud and colleagues showed that nitric oxide (NO) could cause dispersal of *P. aeruginosa* biofilm bacteria (Barraud *et al.*, 2006). They have found that exposure to NO donor sodium nitroprusside (SNP) (500 nM) greatly enhanced the efficacy of antimicrobial compounds (tobramycin, hydrogen peroxide, and sodium dodecyl sulfate) in removal of established *P. aeruginosa* biofilms (Fig. 20) (Barraud *et al.*, 2006).

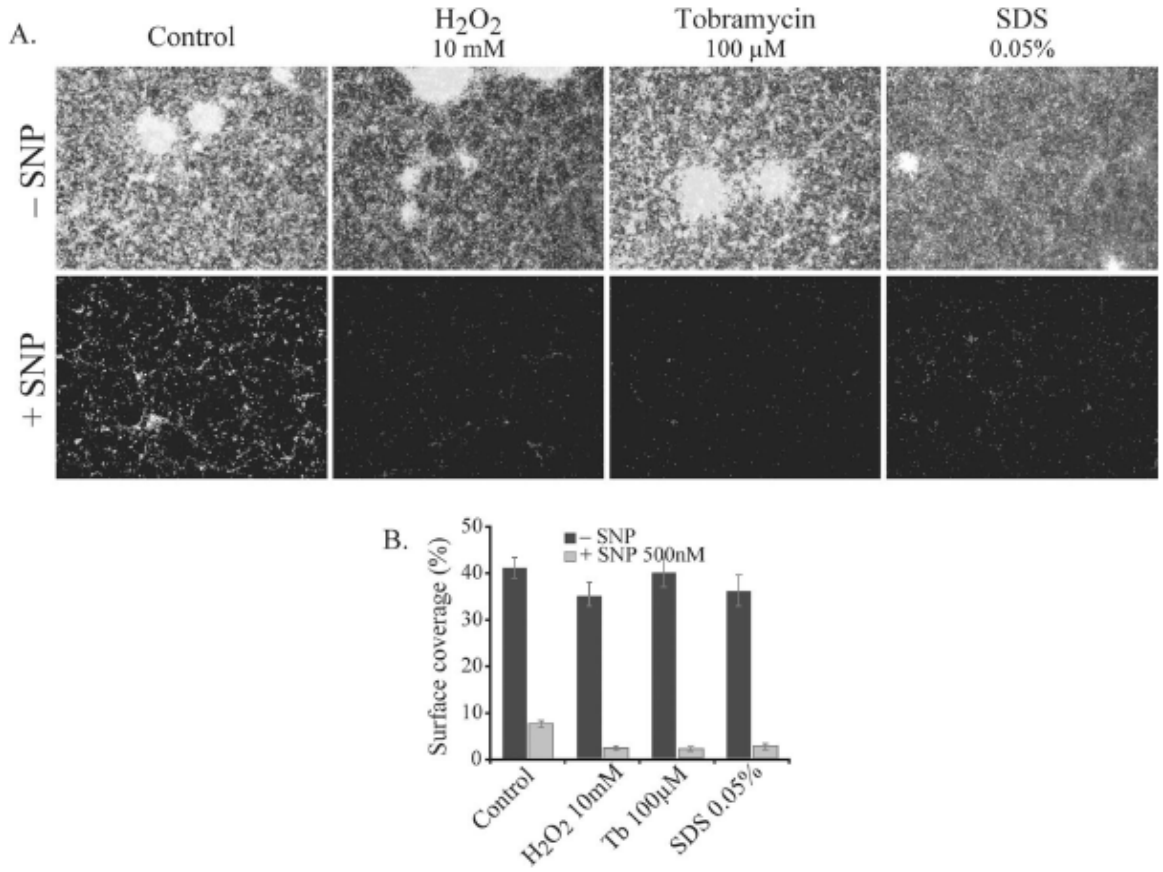

Fig. 20. SNP treatment disperse biofilm formed by *P. aeruginosa*. Cells remaining on the surface after SNP treatment could be easily removed by various antimicrobials (tobramycin, hydrogen peroxide, and SDS). (A) Microscopic pictures of the biofilms on the glass slides after the combinatorial treatments. (B) Levels of biofilm biomass after antimicrobial treatment when grown with or without 500 nM SNP, and error bars indicate standard errors (Barraud *et al.*, 2006).

3.3 Computer aided drug discovery

It is a trend to convert biological research from a qualitative, descriptive science to quantitative, predictive science. This process requires the integration of modern high-throughput experimental technologies with computational data-intensive analysis and high-performance modeling and simulation. There are numerous methods for identification of drug like or lead like compounds (Fig. 21) (Bleicher *et al.*, 2003). We have learned from many textbooks that the identification and optimization of new leads in the drug development process are mainly rely on experimental screening of either synthetic or natural large chemical libraries against a relevant therapeutic target (High-Throughput Screening, HTS). However, it has come to an age that computer aided drug discovery methods are playing a more and more important role in the drug discovery process (Hibert *et al.*, 1988; Marshall, 1987). This strategy has been accelerated in recent years with the developments of novel biologically validated target identification, protein production and purification methods, 3D structural determination methods (e.g. X-ray and NMR), homology modeling methods, *in silico* ligand docking and structure based virtual screening (SBVS) programs (Barril *et al.*, 2004; Kitchen *et al.*, 2004). Computer aid methods are extensively used in the lead identification process including target model building, pharmacophore analysis, chemical database management, virtual screening, (absorption, distribution, metabolism, and excretion) ADME/Tox prediction and so on. Bioinformatics and cheminformatics are two rapid developing disciplines with powerful theoretic supports for computer aided drug discovery.

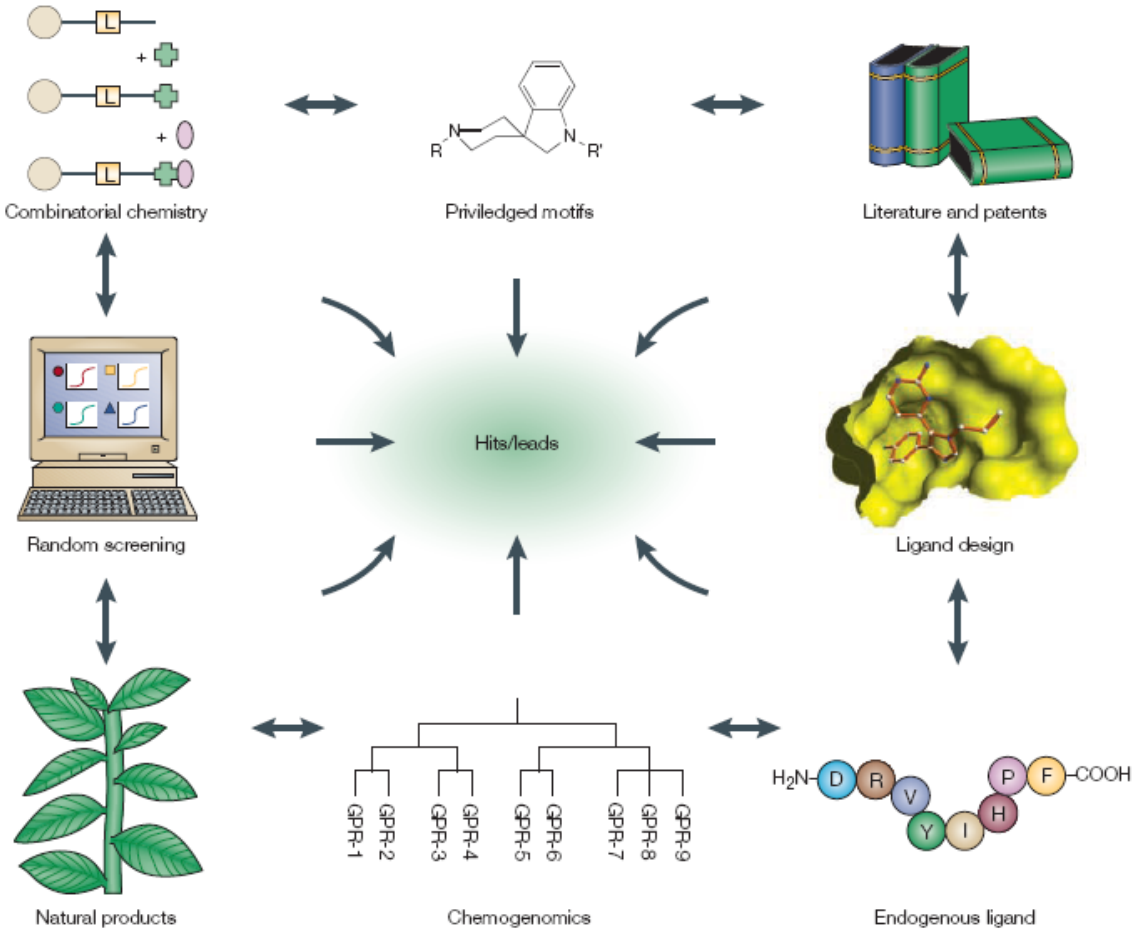

Fig. 21. Drug like or lead like compound identification strategies (Bleicher *et al.*, 2003).

Chemoinformatics is the mixing of those information resources to transform data into information and information into knowledge for the intended purpose of making better decisions faster in the area of drug lead identification and optimization (Brown, 2005). While bioinformatics approaches mainly deal with the early stages of target selection and structure identification, cheminformatics mainly deal with the later stage problems such as lead identification and lead optimization. Combining bioinformatics and cheminformatics tools, a new method termed as structure based virtual screening (SBVS), that screens virtual libraries of compounds by computational means, has been developed (Barril *et al.*, 2004). SBVS is conceptually and economically attractive since it makes possible the evaluation of an unlimited number of chemical structures *in silico*, and only a subset of which will be selected and subsequently assayed in lab screening experiments.

In the present, all drugs that are on the market are estimated to target less than 500 biomolecules. These targets include nucleic acids to enzymes, G-protein-coupled receptors (GPCRs), ion channels and so on (Fig. 22) (Barril *et al.*, 2004; Drews, 2000). Among these targets, enzymes and receptors represent the largest part. Thus, studying and predicting the protein-ligand interactions is obviously the primary methods for SBVS, which is also referred to as Docking.

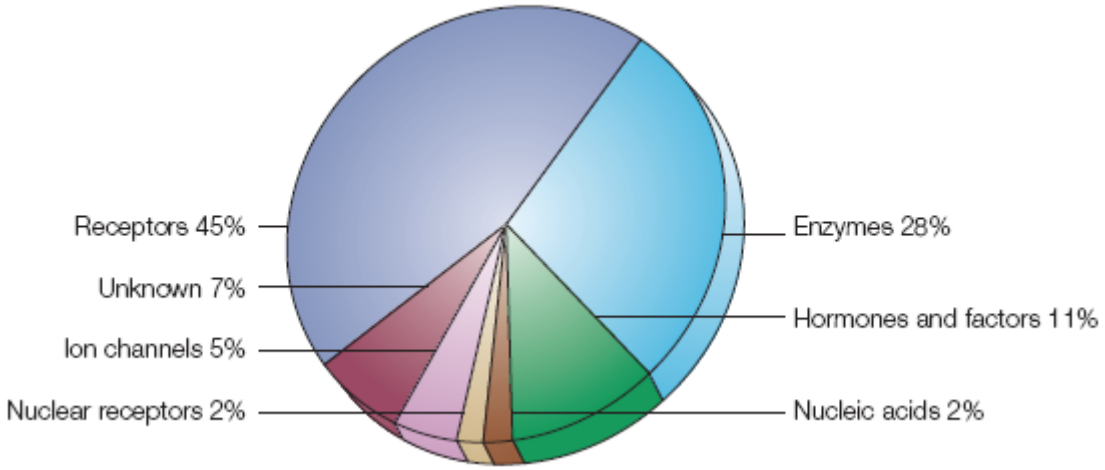

Fig. 22. Therapeutic target classes (Bleicher *et al.*, 2003).

When the 3D structures (either NMR or X-ray) of the protein are available, docking methods can be employed to predict the binding energies of small molecules with the target proteins (Fig. 23).

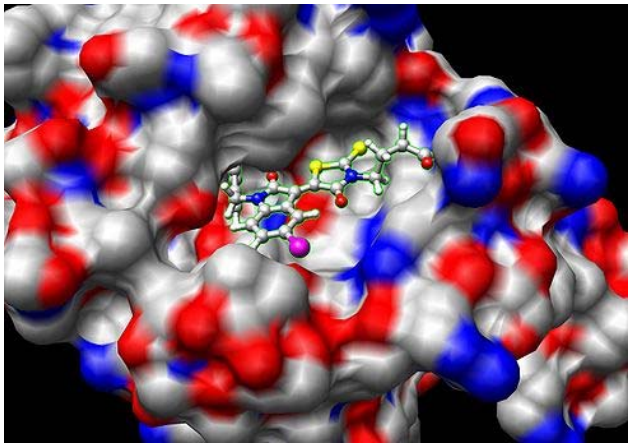

Fig. 23. Small molecule docked into a protein. The docking process can be thought of as a problem of *"lock-and-key"*, where the protein is a "lock" and the ligand is a "key" (http://en.wikipedia.org/wiki/Docking). Even though the basic principles remain the same as *"lock-and-key"* mode, many new algorithms and scoring functions have been developed in recent years. However, unlike the "key" of a "lock", there are many structurally different ligands which can fit in the same protein since ligands can usually induce conformation changes of the proteins.

Fig. 24 illustrate the key steps of molecular docking based virtual screening process for lead compounds identification (Cavasotto & Orry, 2007). After properly preparation (e.g. 3D confirmation generation) of the virtual ligand libarary, the ligands will be docked to the potential active binding site of a target protein one by one. Then the binding energy and pose of each ligand to the target will be calculated and ranked by specific scoring functions of the docking programs.

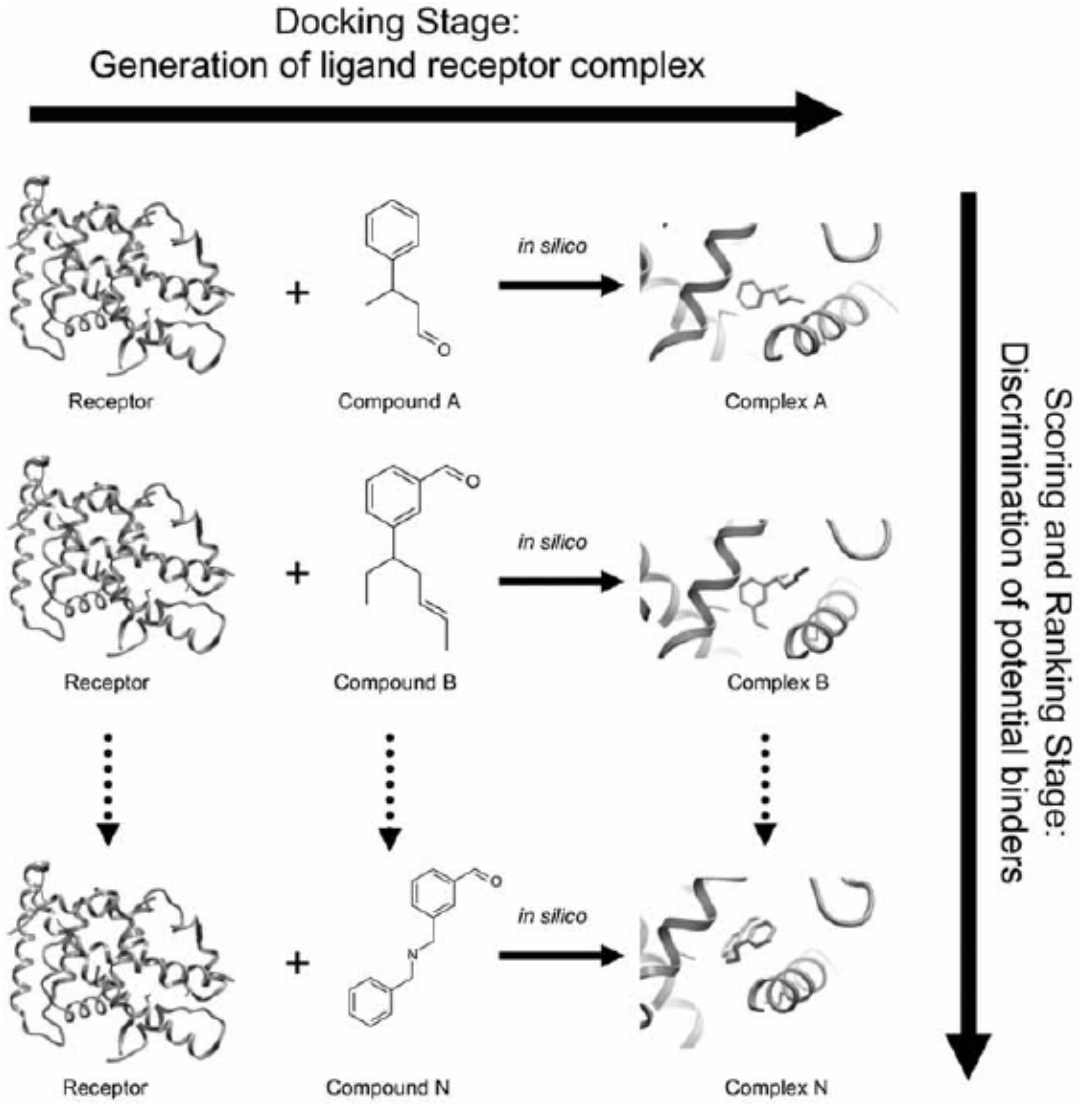

Fig. 24. Key steps of molecular docking based virtual screening process for lead compounds identification (Cavasotto & Orry, 2007).

When the 3D structural data of the target proteins are unknown, quantitative structure-activity relationship (QSAR) (Fig. 25) and pharmacophore modeling (Fig. 26) methods could be applied to search for the potential drugs (Kubinyi, 1998). These methods are based on some previously reported active molecules or drugs of the targets. Firstly, the topological, physical and/or chemical properties (also called molecular descriptors) of known ligands will be calculated and 2D or 3D pharmacophore models can be generated. Then the models can be used to search virtual chemical libraries in order to find new structural classes, or as a tool for lead optimization.

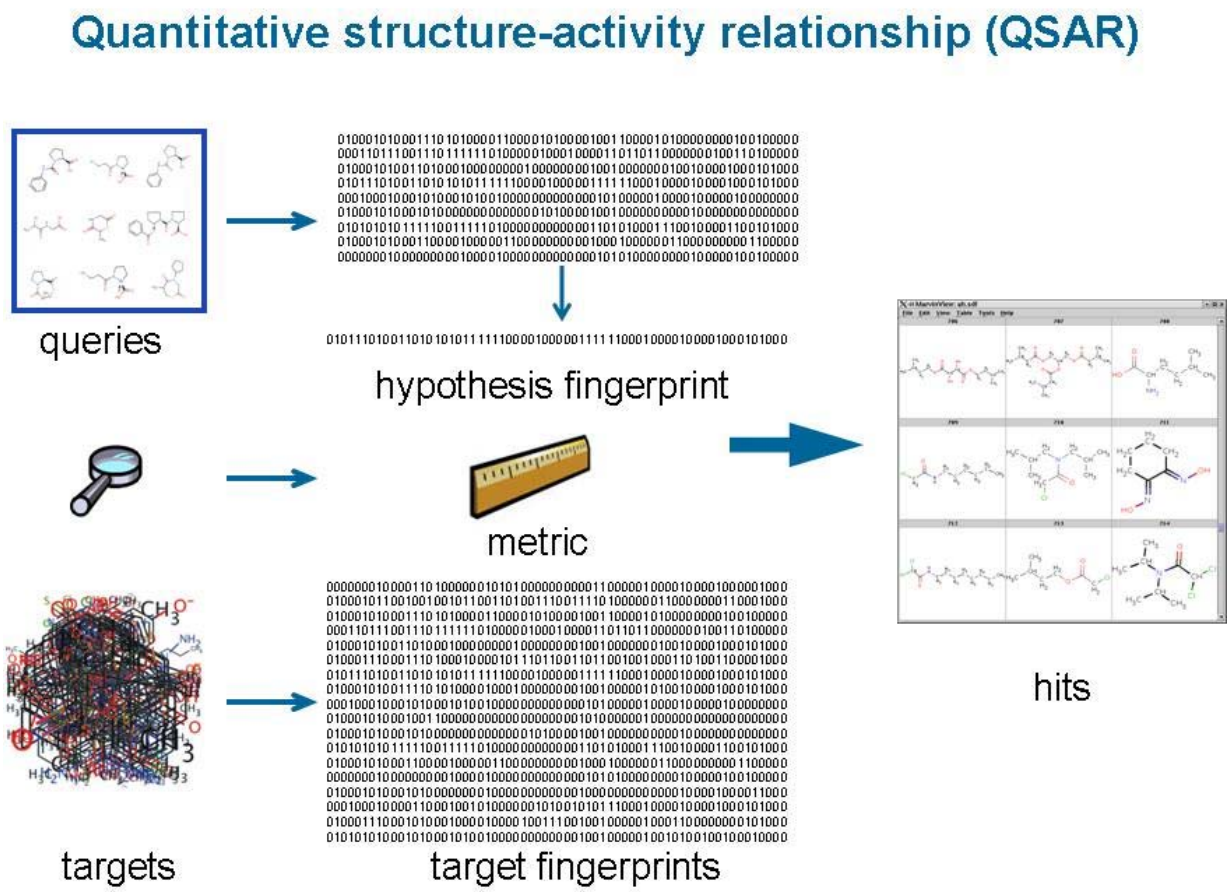

Fig. 25. QSAR based virtual screening methods (http://www.chemaxon.com/).

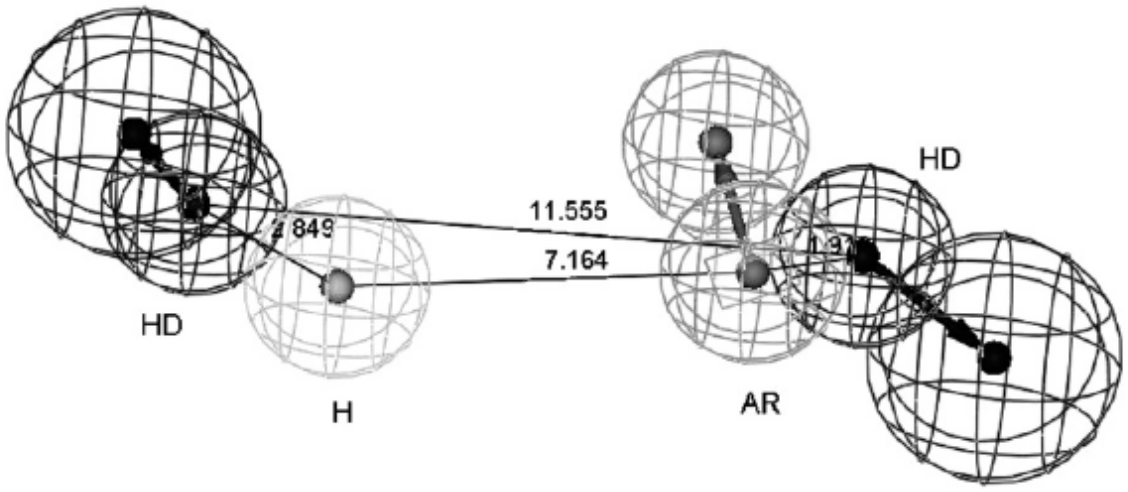

Fig. 26. Estrogen receptor beta (ERβ) agonist pharmacophore. Pharmacophore model of ERβ agonist consisting of two hydrogen bond donor (HD), one aromatic (AR) and one hydrophobic (H) descriptors, was generated by using Catalyst 4.11 tool (http://www.accelrys.com/products/catalyst/) based on published IC50 data from Chemical Sciences Group at Wyeth Research (Kapetanovic, 2008).

When the 3D structural data of target proteins are unknown and there is also no previously reported active molecule or drug, one can still have the chance to perform docking based virtual screening to identify potential drugs. This is highly depending on the development of techniques of protein homologue modeling. The fast growing speed of Protein Data Bank (PDB) has enhanced our capabilities to building good structural models of our target proteins with the help of a wide range of protein modeling tools.

Computer aided structural based virtual screening has been successfully applied to identify drug like compounds in microbiology fields. For example, Qin and colleagues used a SBVS method and identify seven potential inhibitors of YycG/YycF two component system (TCS) of *Staphylococcus epidermidis* (Qin *et al.*, 2006). 7 out of 70 highly scored compounds were shown to be able to bind with YycG proteins at high specificity and not affect the stability of mammalian cells. What is more, these identified compounds were shown to be able to efficiently kill *S. epidermidis* cells living as biofilms (Qin *et al.*, 2006). This study reminds us that searching antimicrobial agents targeting biofilm dependent pathways might provide better therapy strategies for biofilm related infections.

Computer aided drug design was also applied to identify *P. aeruginosa* quorum sensing inhibitors recently. Three-dimensional quantitative structure-activity relationships (3D-QSAR) analyses using CoMFA and CoMSIA were applied by Woodard and colleagues to investigate structural requirements for improving potency and selectivity of AHL as a quorum sensing inhibitors (Woodard & Saleh, 2008). They have build a pharmacophore model of quorum sensing inhibitors and revealed that electrostatic and steric properties play a significant role in potency and selectivity (Woodard & Saleh, 2008). However, AHL structural similar compounds might be very toxic and not good for further development as drugs (Hentzer & Givskov, 2003). Further more, *P. aeruginosa* quorum sensing AHL molecule was reported to inhibit T-Cell differentiation and cytokine production by a mechanism involving an early step in T-Cell activation (Ritchie *et al.*, 2005). Thus, molecular docking based screening might find better candidates of potential quorum sensing inhibitors with different structures as AHLs.

Soulere and colleagues have done an initial molecular docking study basing on the crystallized structure of quorum sensing receptor TraR and some already know quorum sensing inhibitors such as carboxamides, sulfonamides and ureas (Soulere *et al.*, 2007). They showed that AHL dependent quorum sensing transcriptional regulators (TraR, SdiA, LuxR and LasR) had a high structural homology of their active site (Soulere *et al.*, 2007). Their molecular docking studies indicated that conserved residues Tyr53, Tyr61 and Asp70 were implicated in both the development of additional hydrogen bonds and attractive interactions with the N-sulfonyl homoserine lactones and AHLs derived ureas antagonists (Fig. 27) (Soulere *et al.*, 2007).

Recently, the crystallized structure of *P. aeruginosa* quorum sensing receptor lasR protein was published and available via RCSB Protein Data Bank (entry code 2UV0) (Fig. 28) (Bottomley *et al.*, 2007). This structure was compared with previously published TraR structure and many active AHL binding sites were found to be conserved (Bottomley *et al.*, 2007). A virtual screening of lasR specific binding compounds will provide many potential quorum sensing inhibitors for the further development of anti virulence drugs.

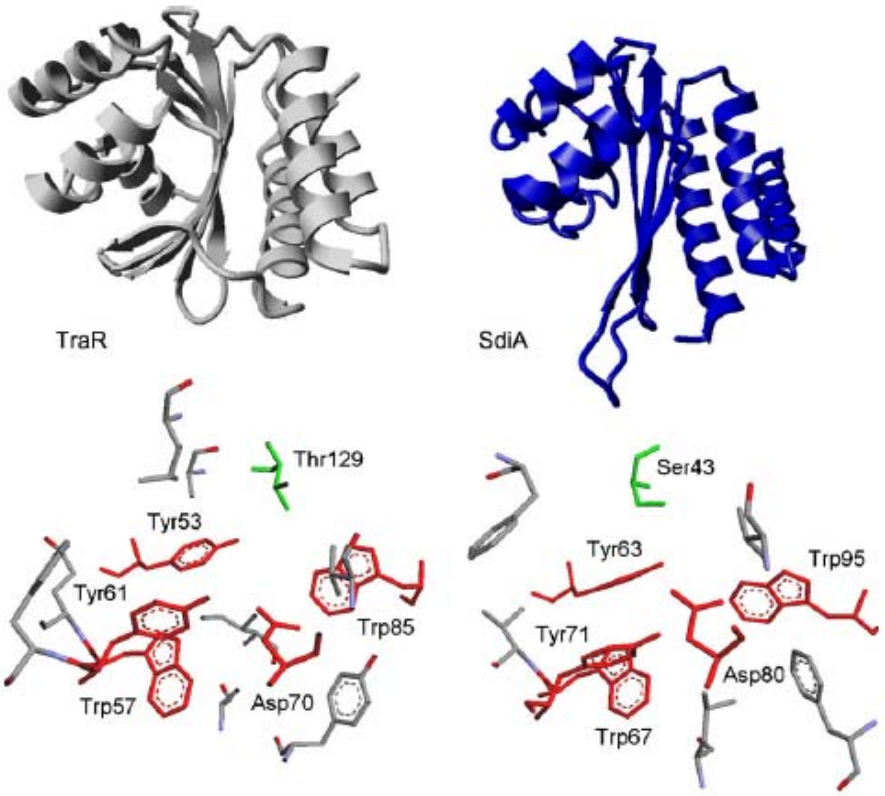

Fig. 27. Representation of the overall folding with secondary structure of the domain receiver and of the active site for the AHL mediated quorum sensing receptor proteins TraR and SdiA (conserved and homologous residues are indicated in red and green, respectively) (Soulere *et al.*, 2007).

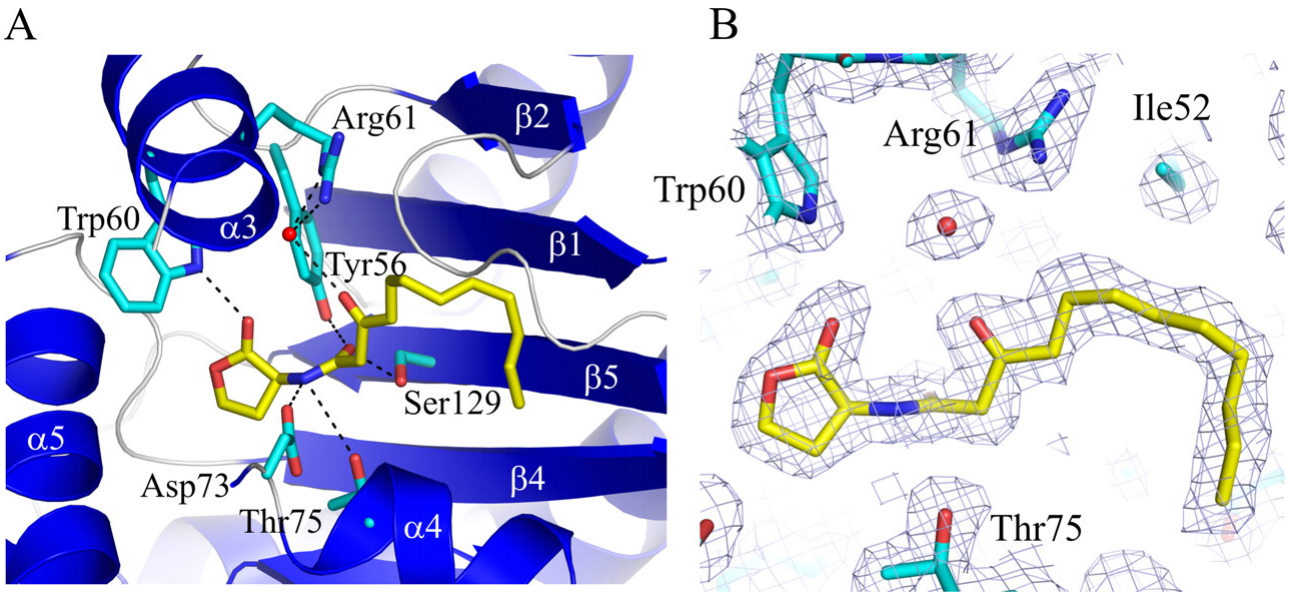

Fig. 28. The AHL binding site in LasR protein. A, LasR side chains make many H-bonds (*dotted lines*) to the autoinducer 3-oxo-C_{12}-HSL. These binding networks include Tyr-56-OH with the amide 1-oxo, Ser-129-OG with the 1-oxo, Trp-60-NE with the lactone carbonyl, Asp-73-OD2 with the amide NH, Thr-75-OG1 and then Arg-61 NE1 and NH1. The conformation of Asp-73 is stabilized by an H-bond with Tyr64. The 12-carbon AHL is entrapped by a large hydrophobic pocket formed by surrounded residues. B, part of the density-modified electron density map ($2F_o$ - F_c) contoured at 1.5 sigma, showing that the density is well defined for the AHL in the ligand binding site and also for the water molecule (*red sphere*) mediating an H-bond between Arg-61 and the 3-oxo group (Bottomley *et al.*, 2007).

4. Concluding remarks

Group activities exist in a wide range of microbes of the microbial kingdom. Bacteria tend to form biofilm communities as an adaptive strategy towards stressful environmental factors. These biofilm complexes consist of different bacterial cells and their self produced sticky EPS matrix materials. The formation of mature biofilms is a dynamic process with many development stages. Many other group activities are also involved in the biofilm development such as iron siderophore synthesis and quorum sensing. Understanding how these different activities are coordinated during biofilm development will provide useful information for the development of anti biofilm strategies.

In this thesis work, a large amount of screening based methods was used to investigate both the mechanisms of biofilm formation and anti biofilm methods. The opportunistic pathogen *P. aeruginosa* was used as a model organism for biofilm study since this bacterium causes many biofilm related infections, and some of these infections are fatal (e.g. cystic fibrosis lung infections). Also, the genome sequence of *P. aeruginosa* is available via NCBI, which enable us to construct mutation library for screening of different phenotypes.

In this thesis work, the quorum sensing molecule *Pseudomonas* quinolone signal (PQS) synthesis pathway was found to directly regulate eDNA release in *P. aeruginosa* biofilms. The dynamics of eDNA release and PQS synthesis were found to be correlated with each other very well during flow chamber biofilm development. Iron was found to be able to manipulate PQS synthesis pathway, which provides some clues and ideas for development of iron based therapy strategies (**paper I**). To further investigate genes involved in eDNA release, a transposon insertion mutant library was constructed for high-throughput screening. To our surprise, the majorities of the eDNA release deficient mutants we found were mutated in genes that directly involved in the PQS synthesis pathway. A *tatA* mutant which was defective in the twin-arginine translocation (Tat) system was found to synthesize the same amount of PQS as its parent strain while fail to release PQS to the extracellular environment. These studies indicated that PQS synthesis and its releasing process caused eDNA release (**paper II**).

To better understand biofilm development, two different group activities, PQS mediated eDNA release and siderophore pyoverdine mediated iron up taking were studied together to observe subpopulation interactions in *P. aeruginosa* biofilms. Both eDNA and pyoverdine are important shareable sources in *P. aeruginosa* biofilms. Type IV pili was shown to be necessary for interaction of cells with eDNA while pyoverdine major receptor fpvA was shown to be necessary for cells to up take iron. The non-motile stalk subpopulation in *P. aeruginosa* biofilms was shown to be the major provider of both eDNA and pyoverdine sources, while the motile subpopulation can utilize these sources and exploit the non-motile subpopulation. These studies clearly shown how a diversified biofilm population was maintained and these might provide some cues for the mechanisms of antibiotics tolerance rendered by *P. aeruginosa* biofilms (**paper III**).

Finally, as the last part of the thesis work, a computer aided structure based virtual screening (SBVS) was performed to identify novel class of quorum sensing inhibitors as anti biofilm drug leads. Chemical compounds were searched by similarity to quorum sensing signal molecule and their affinities to quorum sensing receptor lasR protein were predicted through molecular docking studies. Several recognized drugs were found to be efficient quorum sensing inhibitors and could significant reduce quorum sensing regulated reduce virulence factors and biofilm development *in vitro* (**paper IV**).

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Paper I

Paper I

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Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*

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Extracellular DNA is one of the major matrix components in *Pseudomonas aeruginosa* biofilms. It functions as an intercellular connector and plays a role in stabilization of the biofilms. Evidence that DNA release in *P. aeruginosa* PAO1 biofilms is controlled by the *las-rhl* and *pqs* quorum-sensing systems has been previously presented. This paper provides evidence that DNA release in *P. aeruginosa* PAO1 biofilms is also under iron regulation. Experiments involving cultivation of *P. aeruginosa* in microtitre trays suggested that *pqs* expression, DNA release and biofilm formation were favoured in media with low iron concentrations (5 μM $FeCl_3$), and decreased with increasing iron concentrations. Experiments involving cultivation of *P. aeruginosa* in a flow-chamber system suggested that a high level of iron (100 μM $FeCl_3$) in the medium suppressed DNA release, structural biofilm development, and the development of subpopulations with increased tolerance toward antimicrobial compounds. Experiments with *P. aeruginosa* strains harbouring fluorescent reporters suggested that expression of the *pqs* operon was induced in particular subpopulations of the biofilm cells under low-iron conditions (1 μM $FeCl_3$), but repressed in the biofilm cells under high-iron conditions (100 μM $FeCl_3$).

INTRODUCTION

Biofilms are sessile populations of micro-organisms that are surrounded by the slime they produce, and may be attached to an inert or living surface. Biofilm formation is described as a developmental sequence that varies to some extent between bacterial species but often results in the formation of mature microbial communities with tower- or mushroom-shaped microcolonies (Davey & O'Toole, 2000; Sauer *et al.*, 2002; Klausen *et al.*, 2003a). The bacteria in biofilms in medical settings often display increased resistance toward host immune responses and enhanced tolerance toward antibiotic treatment (Costerton *et al.*, 1999). Knowledge about the molecular mechanisms that are involved in biofilm development, and the factors that lead to the formation of resistant subpopulations, may be useful for creating strategies to control biofilm formation and eradicate persistent infections.

One of the most distinctive features of biofilms is the extracellular polymeric substances that surround the bacteria and constitute the biofilm matrix. The composition of biofilm matrices varies depending upon the bacterial species and the environmental conditions, but in general they contain components such as polysaccharides, proteins and extracellular DNA (Sutherland, 2001; Whitchurch *et al.*, 2002).

Evidence for a role of extracellular DNA as matrix component in *Pseudomonas aeruginosa* biofilms has been presented both for the *P. aeruginosa* PAO1 reference strain and for clinical *P. aeruginosa* isolates (Whitchurch *et al.*, 2002; Nemoto *et al.*, 2003). *P. aeruginosa* PAO1 biofilm development in the wells of microtitre plates was attenuated by the presence of DNase I, and biofilm formation by *P. aeruginosa* PAO1 in flow-chambers was almost absent when the flow-chambers were irrigated with medium containing DNase I (Whitchurch *et al.*, 2002). In addition, young *P. aeruginosa* PAO1 biofilms that had been grown in flow-chambers irrigated with DNase-free medium dispersed rapidly after addition of DNase I to the flowing medium, whereas mature *P. aeruginosa* PAO1 biofilms were not dispersed by DNase I treatment, suggesting that components other than extracellular DNA stabilize mature *P. aeruginosa* PAO1 biofilms (Whitchurch *et al.*, 2002). Matsukawa & Greenberg (2004) investigated the composition of the extracellular matrix of mature *P. aeruginosa* PAO1 biofilms and found that extracellular DNA was by far the most abundant polymer, although exopolysaccharide appeared to be the most critical structural matrix component. In contrast to the finding that extracellular DNA may not be the primary cell-to-cell interconnecting compound in mature *P. aeruginosa* PAO1 biofilms, Nemoto

Abbreviations: AHL, *N*-acylhomoserine lactone; CLSM, confocal laser scanning microscope/microscopy; DDAO, 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one; PQS, *Pseudomonas* quinolone signal.

et al. (2003) found that mature biofilms formed by four different clinical *P. aeruginosa* isolates could be dispersed by DNase treatment, suggesting that extracellular DNA is the critical matrix component in mature biofilms formed by these *P. aeruginosa* strains.

Quorum sensing, a mechanism by which bacteria can monitor their cell population density through the extracellular accumulation of signalling molecules, has been shown to play a role in structural development and stabilization of *P. aeruginosa* biofilms (Davies *et al.*, 1998). *P. aeruginosa* employs the three interconnected quorum-sensing systems *las*, *rhl* and *pqs* to control expression of a wide range of virulence factors (Passador *et al.*, 1993; Latifi *et al.*, 1995, 1996; Pesci *et al.*, 1999). The major signal molecules involved are 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL) in the case of the *las* system, C4-HSL in the case of the *rhl* system, and 2-heptyl-3-hydroxy-4-quinolone, designated the *Pseudomonas* quinolone signal (PQS), in the case of the *pqs* system (Latifi *et al.*, 1995, 1996; Pesci *et al.*, 1999). Synthesis of PQS depends on several putative enzymes encoded by the *pqsABCDE*, *pqsL*, *phnAB* and *pqsH* genes, as well as on a LysR-type regulator encoded by the *pqsR* gene (also known as *mvfR*) (Cao *et al.*, 2001; Gallagher *et al.*, 2002; D'Argenio *et al.*, 2002). Evidence has been presented that the quorum-sensing systems play a role in the formation of extracellular DNA in *P. aeruginosa* biofilms (Allesen-Holm *et al.*, 2006). Quorum-sensing-regulated DNA release from *P. aeruginosa* populations presumably involves lysis of a subpopulation of the cells (or lysis of DNA-containing vesicles), as experiments with *lacZ*-containing *P. aeruginosa* strains indicated that the formation of extracellular β-galactosidase correlated with the formation of large amounts of extracellular DNA (Allesen-Holm *et al.*, 2006).

P. aeruginosa colonizes the lungs of cystic fibrosis (CF) patients and is a major cause of lung deterioration, health decline and death of these patients (Høiby *et al.*, 2001). Several studies indicate that *P. aeruginosa* forms biofilms in the CF lung (e.g. Lam *et al.*, 1980; Baltimore *et al.*, 1989; Worlitzsch *et al.*, 2002; Høiby *et al.*, 2001), and the biofilm mode of growth is considered the major reason that these bacteria can not be eradicated by host defences or antibiotic treatment (Costerton *et al.*, 1999; Høiby *et al.*, 2001). The conditions that infecting bacteria encounter in the CF lung is of great interest, and artificial CF sputum media have been developed to enable experiments to be carried out under relevant and controllable conditions (Sriramulu *et al.*, 2005; Palmer *et al.*, 2005). *P. aeruginosa* cells in the CF lung are believed to be in intense competition for iron with the host (Haas *et al.*, 1991; Ratledge & Dover, 2000). Low iron concentrations were shown to promote biofilm formation in artificial CF sputum medium (Sriramulu *et al.*, 2005). In addition to low iron levels, the presence of extracellular DNA was shown to be important for *P. aeruginosa* biofilm formation in artificial sputum medium (Sriramulu *et al.*, 2005).

Knowledge about the factors that affect DNA release from *P. aeruginosa* is important for understanding the environmental cues that can lead to biofilm formation and increased pathogenicity of this organism. In this paper we present evidence that the level of iron in the medium affects *P. aeruginosa* DNA release and biofilm formation. The iron level was shown to have an effect on the amount of biofilm formed in the wells of microtitre trays, as well as on structural biofilm development in flow chambers, and on the development of subpopulations with increased tolerance toward antimicrobial compounds. Evidence is presented that iron affects *P. aeruginosa* DNA release and biofilm development by affecting the PQS signalling pathway.

METHODS

Bacteria and growth conditions. *P. aeruginosa* PAO1 was obtained from the Genetic Stock Center (strain PAO0001). The *lasI rhlI* derivative was constructed by allelic displacement in PAO1 as described by Hentzer *et al.* (2003). The *pqsA*, *pqsR* and *pqsL* mutants were constructed by D'Argenio *et al.* (2002) via transposon insertion in PAO1. The *gfp*-tagged PAO1 strain was constructed by insertion of a mini-Tn*7*-eGFP-Gm^r cassette as described by Klausen *et al.* (2003b). *Escherichia coli* strains MT102 and MC1000 were used for standard DNA manipulations. LB medium (Bertani, 1951) was used to cultivate *E. coli* strains. Batch cultivation of *P. aeruginosa* was carried out at 30 °C in AB minimal medium (Clark & Maaløe, 1967) supplemented with 30 mg glucose l^{-1}. *P. aeruginosa* biofilms were cultivated at 30 °C in flow-chambers irrigated with FAB medium (Heydorn *et al.*, 2000) supplemented with 0.3 mM glucose. $FeCl_3$ was added to the media to give the final ferric iron concentrations described in the text. Selective media were supplemented with ampicillin (Ap; 100 mg l^{-1}), gentamicin (Gm; 60 mg l^{-1}), kanamycin (Km; 100 mg l^{-1}), streptomycin (Sm; 100 mg l^{-1}), or tetracycline (Tc; 20 mg l^{-1}). AB medium supplemented with 0.05 mg propidium iodide ml^{-1} was used in the microtitre trays for quantification of extracellular DNA. DDAO [7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one] was used at a concentration of 1 μM to stain extracellular DNA in flow-chamber-grown biofilms. Propidium iodide was used at a concentration of 0.01 mg ml^{-1} to stain dead cells in flow-chamber-grown biofilms. Syto62 was used at a concentration of 1 μM to stain bacteria in flow-chamber-grown biofilms.

Measurements of *pvdD* expression. A classical β-galactosidase assay, described by Miller (1972), was used to measure expression of the *pvdD–lacZ* fusion in *P. aeruginosa* PAO1 cells transformed with the low-copy-number plasmid pPM190::*pvdD* (Rombel *et al.*, 1995), which carries the *pvdD* promoter fused to the *E. coli lacZ* gene.

Construction of the *pqsA*::*gfp(ASV)* reporter. The promoter-probe plasmid pAC37, containing a P_{pqsA}::*gfp(ASV)* transcriptional fusion, was constructed using standard techniques for DNA manipulations (Sambrook *et al.*, 1989). A *P. aeruginosa* DNA region containing the P_{pqsA} promoter was PCR amplified by use of the primers PA0996F (5′-GGAATTCTGATCAATGCCGTCGCCCCCTTGGA-3′) and PA0996R (5′-GGGTACCCATGACAGAACGTTCCCTCTT-3′). A *P. aeruginosa* DNA region containing the P_{rhlR} promoter and the *rhlR* gene was PCR amplified by use of the primers PA3477F (5′-CGTGATCAAACGGCTGGTCCGGCTCG-3′) and PA3477R (5′-GGAATTCAAAAAGGCCATCCGTCAGGATGGCCTTCTAGATTA-TCTACTCAGATGAGACCCAGCGCC-3′). An *E. coli* T2 transcriptional terminator and a unique *Xba*I cloning site were introduced in the PCR fragment through use of the PA3477R primer set. pJBA100, consisting of a *Not*I fragment with the *gfp(ASV)* gene from pJBA89

(Andersen *et al.*, 2001) cloned in the shuttle vector pUCP22*Not*I, was used as scaffold. pJBA100 was digested with *Kpn*I and *Eco*RI; the PCR product containing the P_{pqsA} promoter was digested with *Bcl*I and *Kpn*I; and the PCR product containing the *rhlR* gene was digested with *Bcl*I and *Eco*RI. The expression monitor pAC36 was constructed by ligating the three DNA fragments in one reaction. Plasmid pAC37 was constructed by deleting the *rhlR* gene on pAC36, via digestion with *Xba*I and *Bcl*I, Klenow treatment, and religation. The pAC37 plasmid was electroporated into *P. aeruginosa* PAO1 and the *P. aeruginosa lasI rhlI* mutant.

Measurement of biofilm and extracellular DNA in microtitre trays. The microtitre tray biofilm formation assay was performed essentially as described by O'Toole & Kolter (1998). Overnight cultures were grown in AB medium and diluted to OD_{600} 0.001 with fresh AB medium. The diluted cultures were transferred to the wells of polystyrene microtitre trays (150 µl per well) and incubated for 24 h at 30 °C. The medium was removed from the wells and they were washed twice with 0.9 % NaCl, stained with 0.1 % crystal violet and washed twice with 0.9 % NaCl; the crystal violet-stained biofilms were then resuspended in 96 % ethanol, and biofilm cell-associated dye was measured as A_{590}. Measurements of extracellular DNA in *P. aeruginosa* microtitre tray cultures was done as described by Allesen-Holm *et al.* (2006). Microtitre tray cultures were started by diluting *P. aeruginosa* overnight cultures to OD_{600} 0.001 in AB-glucose medium supplemented with 0.05 mM propidium iodide and $FeCl_3$ (as indicated) in wells of polystyrene microtitre plates (150 µl per well). The cultures were incubated for 24 h at 37 °C, after which propidium iodide absorbance was measured at 480 nm and cell density was measured at 600 nm by the use of a Wallac microplate reader.

DNase I treatment of biofilms in microtitre trays. Twenty-four-hour-old microtitre-tray-grown biofilms of PAO1 were washed twice with 0.9 % NaCl and treated with 0.9 % NaCl containing 100 µg DNase I ml^{-1} for 3 h, after which the amount of biofilm was measured as described above.

Cultivation of biofilms in flow-chambers. Biofilms were grown in flow-chambers with individual channel dimensions of 1×4×40 mm. The flow system was assembled and prepared as described previously (Sternberg & Tolker-Nielsen, 2005). The flow-chambers were inoculated by injecting 350 µl overnight culture diluted to an OD_{600} of 0.001 into each flow channel using a small syringe. After inoculation, the flow channels were left without flow for 1 h, after which medium flow was started using a Watson Marlow 205S peristaltic pump. The mean flow velocity in the flow-chambers was 0.2 mm s^{-1}, corresponding to laminar flow with a Reynolds number of 0.02.

Microscopy and image acquisition. All microscopy observations and image acquisitions were done with a Zeiss LSM510 confocal laser scanning microscope (CLSM) equipped with detectors and filter sets for monitoring of Gfp, propidium iodide Syto62 and DDAO fluorescence. Images were obtained using a 63×/1.4 objective or a 40×/1.3 objective. Simulated three-dimensional images and sections were generated using the IMARIS software package (Bitplane AG).

Assessment of extracellular DNA in flow-chamber-grown biofilms. DDAO was added to the medium to a final concentration of 1 µM to fluorescently stain extracellular DNA in flow-chamber-grown biofilms. DDAO fluorescence in different biofilms was recorded with fixed settings of the CLSM.

Antimicrobial treatment. Biofilm tolerance to EDTA or ciprofloxacin was assessed by irrigating 4-day-old flow-chamber-grown *P. aeruginosa* biofilms with medium containing 50 mM EDTA or 50 µg ciprofloxacin ml^{-1} for 24 h, followed by staining of the dead cells with propidium iodide, and CLSM image acquisition. To quantify survival rates, the bacteria from flow-chamber biofilms were recovered after EDTA treatment, ciprofloxacin treatment, or no antimicrobial treatment, by pumping bead-containing (Sigma glass-beads 212–300 µm) medium rapidly in and out of the flow-chambers using syringes. The bead-containing bacterial suspensions were vortex mixed, and the bacteria were plated on LB plates; c.f.u. were determined and survival rates calculated.

COMSTAT image analysis. CLSM images were analysed by use of the computer program COMSTAT (Heydorn *et al.*, 2000). A fixed threshold value and connected volume filtration were used for all image stacks.

RESULTS

We initially performed experiments to determine the iron status of *P. aeruginosa* in our AB-glucose medium supplemented with different concentrations of iron. Expression of the *pvdD* gene in *P. aeruginosa* is known to be suppressed by high levels of iron (Ochsner *et al.*, 2002). We measured β-galactosidase activity in microtitre tray cultures of *P. aeruginosa* harbouring the low-copy-number plasmid pPM190::*pvdD*, which carries the *pvdD* promoter fused to the *E. coli lacZ* gene (Rombel *et al.*, 1995). The β-galactosidase activity (Miller units) was 7757, 4944, 2773 and 2206 (three replicates, SD 581, 458, 347 and 58) in cultures supplemented with 5, 10, 50 and 100 µM $FeCl_3$. Accordingly, the selected range of iron concentrations in our medium evidently affected the iron status of the *P. aeruginosa* cells.

When *P. aeruginosa* PAO1 was cultivated in microtitre trays, a high cell density was reached in wells containing AB-glucose medium supplemented with 100 or 50 µM iron, whereas a slightly lower cell density was reached in wells containing AB-glucose medium supplemented with 10 µM iron, and a significantly lower cell density was reached in wells containing AB-glucose medium supplemented with 5 µM iron (Fig. 1A). However, the lowest iron concentration used did not limit growth dramatically, and the highest iron concentration used did not inhibit growth.

Iron affects *P. aeruginosa* DNA release and biofilm formation in microtitre trays

High concentrations of iron salts were reported to inhibit *P. aeruginosa* biofilm formation (Musk *et al.*, 2005). Because extracellular DNA is a major matrix component of *P. aeruginosa* biofilms (Whitchurch *et al.*, 2002; Nemoto *et al.*, 2003; Matsukawa & Greenberg, 2004; Allesen-Holm *et al.*, 2006), we found it of interest to investigate whether the presence of high levels of iron might inhibit the generation of extracellular DNA from *P. aeruginosa*. In accordance with the work of Musk *et al.* (2005), we found that biofilm formation by *P. aeruginosa* PAO1 in microtitre trays decreased with increasing iron concentrations (Fig. 1B). In order to quantify the extracellular DNA generated in the microtitre tray cultures (from planktonic as well as from biofilm cells), we supplemented the medium with propidium iodide (which fluoresces when it is bound to DNA,

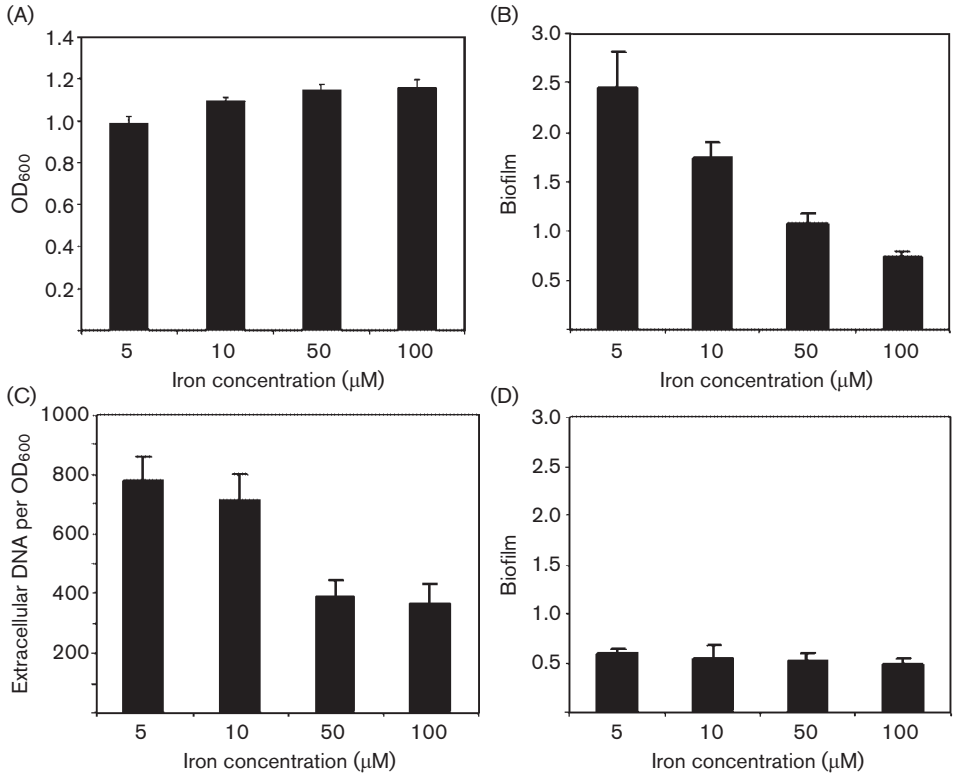

Fig. 1. *P. aeruginosa* PAO1 was grown for 24 h in microtitre tray wells containing AB-glucose medium with different concentrations of $FeCl_3$, then culture densities, biofilm amounts and extracellular DNA amounts were determined. (A) Final OD_{600} of the cultures. (B) Relative amounts of biofilm in the wells determined via a crystal violet staining assay. (C) Relative amounts of extracellular DNA in the cultures determined via a propidium iodide staining assay. (D) Relative amounts of biofilm in the wells after 3 h of DNase I treatment. Means and standard deviations of eight replicates are shown.

and does not penetrate live bacteria) and measured the level of fluorescence in the cultures after 1 day of growth. The amount of extracellular DNA in *P. aeruginosa* PAO1 microtitre tray cultures was high in medium supplemented with 5 μM iron, and decreased with increasing iron concentrations (Fig. 1C). Treatment of 1-day-old *P. aeruginosa* PAO1 biofilms with DNase I led to dispersal of large amounts of the biofilms (Fig. 1D), showing that extracellular DNA plays a critical role in biofilm formation in the experimental setup used.

Iron affects the *Pseudomonas* quinolone signal (PQS) pathway

In a previous report, we presented evidence that release of extracellular DNA from *P. aeruginosa* populations was regulated through the *pqs* quorum-sensing system (Allesen-Holm *et al.*, 2006). To investigate whether the observed effect of iron on DNA release from *P. aeruginosa* populations occurs through the PQS system, we initially tested DNA release from different PQS mutants in microtitre tray cultures supplemented with different concentrations of iron. The mutants *pqsA* and *pqsR* were found to release less extracellular DNA than the wild-type in an iron-independent manner, whereas a *pqsL* mutant was found to release more extracellular DNA than the wild-type via a pathway that was suppressed by high iron concentrations (Fig. 2). These results are in agreement with evidence reported by D'Argenio *et al.* (2002) that the *pqsA* and *pqsR* mutants were deficient in PQS production and autolysis, whereas the *pqsL* mutant overproduced PQS and was hyper-autolysing.

To further investigate whether the observed effect of iron on DNA release and biofilm formation might be caused by an effect on the PQS pathway, we constructed a plasmid-based *pqsA*::*gfp(ASV)* transcriptional reporter (as detailed in Methods). The *gfp(ASV)* gene encodes an unstable version of Gfp (Andersen *et al.*, 1998) and the *pqsA*::*gfp(ASV)* reporter-construct is thus useful for monitoring *pqsABCDE* expression. The plasmid with the reporter-construct was transferred to the *P. aeruginosa* wild-type as well as to an isogenic *P. aeruginosa lasI rhlI* quorum-sensing mutant, and *pqsA*::*gfp(ASV)* expression was measured in microtitre tray cultures. The experiments confirmed that the *pqsABCDE* operon is under *N*-acylhomoserine lactone (AHL) quorum-sensing control, as expression of the *pqsA*::*gfp(ASV)* reporter consistently was lower in the *lasI rhlI* quorum-sensing mutant

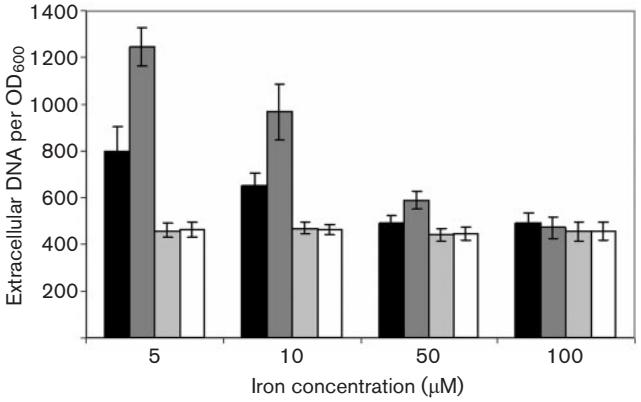

Fig. 2. Relative amounts of extracellular DNA, determined via a propidium iodide staining assay, in microtitre tray cultures of PAO1 wild-type (black bars), *pqsL* (dark grey bars), *pqsA* (light grey bars) and *pqsR* (white bars) grown in AB-glucose medium containing different amounts of $FeCl_3$. Means and standard deviations of eight replicates are shown.

than in the wild-type (Fig. 3A). Transcription of the *pqsABCDE* operon in the *P. aeruginosa* wild-type was found to be high in medium supplemented with 5 μM iron, and decreased with increasing iron concentrations (Fig. 3A). In the *lasI rhlI* quorum-sensing mutant there was also an inverse relation between *pqsA*::*gfp(ASV)* expression and the level of iron in the medium (Fig. 3A), suggesting that the effect of iron on *pqsABCDE* expression to some extent is independent of the AHL quorum-sensing system. DNA release in the microtitre tray cultures was found to correlate with the expression of the *pqsABCDE* operon (Fig. 3B). Based on these results, we suggest that iron affects *P. aeruginosa* DNA release and biofilm formation by affecting the PQS signalling pathway.

In order to address whether the observed effects of ferric iron supplementation could be due to induction of an oxidative stress response in the *P. aeruginosa* cells, we investigated microtitre tray cultures supplemented with different concentrations (ranging from 0 to 10 mM) of H_2O_2, and found no reduction in *pqsA*::*gfp(ASV)* expression, DNA release, or biofilm formation (data not shown), indicating that the observed effects of iron are not due to an oxidative stress response.

Iron affects *P. aeruginosa* DNA release and structural biofilm development in flow-chambers

In order to examine the effect of iron concentration on DNA release in *P. aeruginosa* biofilms, we grew the PAO1 wild-type in flow-chambers irrigated with normal FAB medium (containing 1 μM iron) or FAB100 medium (containing 100 μM iron). Because of the continuous supply of medium, the amount of iron available to the bacteria will be higher in the flow-chamber setup than in the microtitre tray setup, and we therefore chose to use FAB, containing 1 μM iron, as the medium with the lowest iron concentration in the investigations. After 4 days of growth, the extracellular DNA in the biofilms was visualized by staining with the compound DDAO followed by CLSM. In agreement with our previous study (Allesen-Holm *et al.*, 2006), the biofilms grown in FAB medium contained large amounts of extracellular DNA in the part of the biofilm close to the substratum and in the outer region of the stalk portions of the mushroom-shaped multicellular structures (Fig. 4A). In agreement with the quantifications of extracellular DNA and biofilm formation in the microtitre tray assays (described above), the biofilms grown in FAB100 medium contained less extracellular DNA and less biomass than the biofilms grown in normal FAB medium (Fig. 4B). In addition, biofilms grown in FAB100 medium appeared less spatially

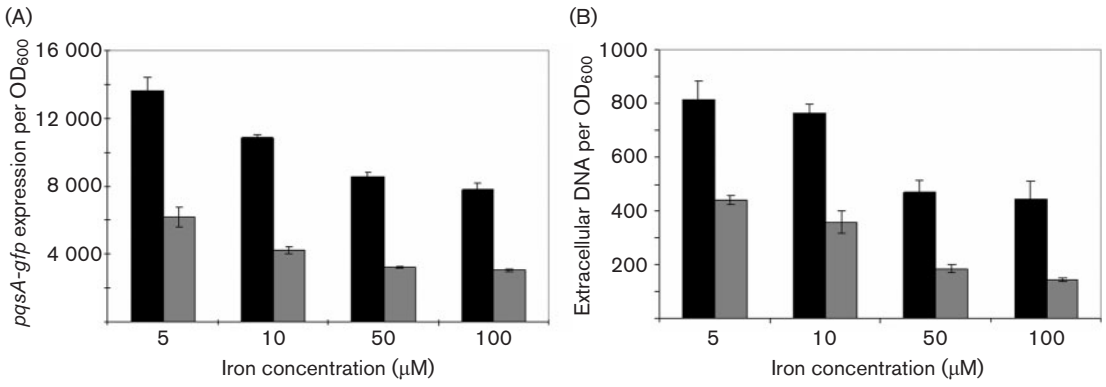

Fig. 3. (A) Expression of a plasmid-borne *pqsA*::*gfp(ASV)* fusion in microtitre tray cultures of the PAO1 wild-type (black bars) and a *lasI rhlI* mutant (grey bars) grown in AB-glucose medium with different concentrations of $FeCl_3$. (B) Relative amounts of extracellular DNA, determined via a propidium iodide staining assay, in microtitre tray cultures of the PAO1 wild-type (black bars) and *lasI rhlI* mutant (grey bars) grown in AB-glucose medium containing different amounts of $FeCl_3$. Means and standard deviations of eight replicates are shown.

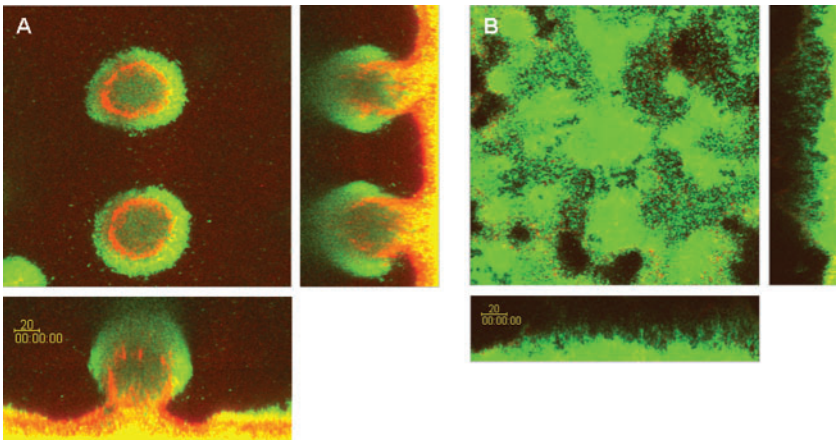

Fig. 4. Four-day-old biofilms of *gfp*-tagged PAO1 grown in FAB medium (A) or FAB100 medium (B) were stained with DDAO and images visualizing cells (green) and extracellular DNA (appearing red or yellow) were acquired by CLSM. The central pictures show horizontal optical sections, and the flanking pictures show vertical optical sections. Bars, 20 μm.

organized and did not contain the mushroom-shaped multicellular structures typical of the biofilms grown in normal FAB medium (Fig. 4). The difference in biomass and spatial structure between biofilms grown in normal FAB medium and FAB100 medium was confirmed by COMSTAT analysis (Table 1).

Iron affects expression of the *pqsABCDE* operon in flow-chamber-grown *P. aeruginosa* biofilms

In order to investigate expression of the *pqsABCDE* operon in *P. aeruginosa* biofilms, we grew PAO1 containing the *pqsA*::*gfp(ASV)* reporter plasmid in flow-chambers irrigated with FAB medium or FAB100 medium. In FAB medium, expression of the *pqsA*::*gfp(ASV)* fusion was detectable at day 1 (Fig. 5A) and reached the highest expression level at day 2 where it was localized mainly in the outer part of the microcolonies (Fig. 5C). After day 2, expression of the *pqsA*::*gfp(ASV)* fusion decreased, and at day 4 only a weak green signal could be detected in the stalk portion of the mushroom-shaped multicellular structures (Fig. 5E). In FAB100 medium, there was significantly less expression of the *pqsA*::*gfp(ASV)* fusion, and this expression appeared less coordinated in time and space (Fig. 5B, D and F).

Iron affects tolerance to antimicrobial compounds in flow-chamber-grown *P. aeruginosa* biofilms

Since we had found that the level of iron in the medium may control whether a structured or an unstructured *P. aeruginosa* biofilm is formed, and because tolerance to antimicrobial compounds in biofilms may be related to the spatial structure, we found it of interest to investigate tolerance to the antibiotic ciprofloxacin and to the antimicrobial compound EDTA in *P. aeruginosa* biofilms grown in FAB medium or FAB100 medium. After 4 days of growth, the biofilms were treated with either ciprofloxacin or EDTA, after which the dead bacteria in the biofilms were visualized by staining with propidium iodide. In 4-day-old *P. aeruginosa* PAO1 biofilms grown in FAB medium, ciprofloxacin treatment caused killing of the bacteria located in the outer part of the cap portion of the mushroom-shaped structures (Fig. 6C), whereas EDTA treatment caused killing of the cells located within the mushroom-shaped structures (Fig. 6E). In 4-day-old *P. aeruginosa* PAO1 biofilms grown in FAB100 medium, treatment with ciprofloxacin or EDTA appeared to kill a large proportion of the bacteria, and there was no special area for the ciprofloxacin- or EDTA-mediated killing (Fig. 6D, F). Propidium iodide staining of biofilms that had not been treated with antibiotic showed that virtually no dead cells

Table 1. Quantification of biofilm architecture

The results are means of datasets obtained from analysis of eight CLSM images acquired at random positions in each of the biofilms. Standard deviations are shown in parentheses.

Biofilm medium	Total biomass ($\mu m^3\ \mu m^{-2}$)	Mean thickness (μm)	Roughness coefficient	Surface area covered (μm^2)	Surface/volume ($\mu m^2\ \mu m^{-3}$)	Max. thickness (μm)
FAB (containing 1 μM $FeCl_3$)	40.06 (1.97)	42.82 (2.74)	0.39 (0.08)	8.71×10^5 (2.72×10^5)	0.41 (0.12)	96.63 (3.42)
FAB100 (containing 100 μM $FeCl_3$)	13.08 (4.76)	17.56 (5.41)	0.59 (0.18)	1.21×10^6 (3.30×10^5)	1.81 (0.36)	59.75 (2.12)

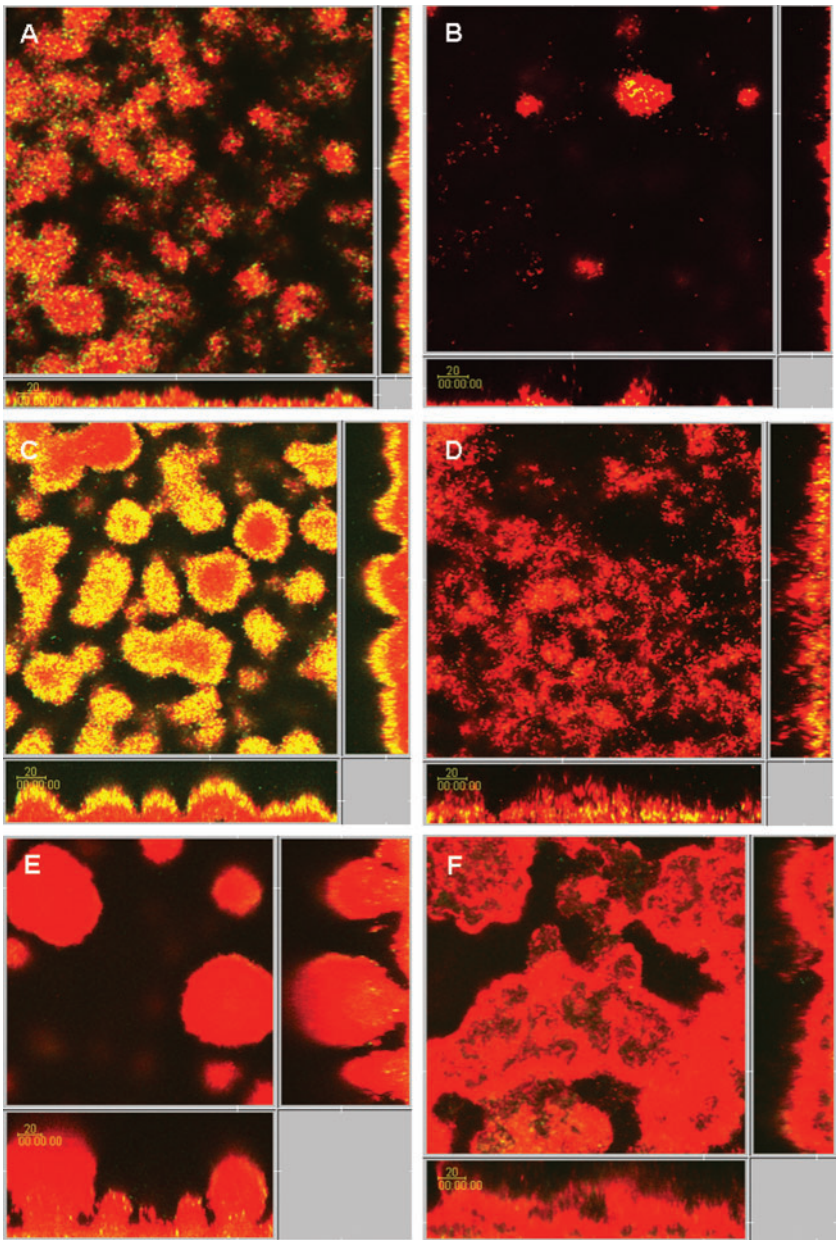

Fig. 5. Biofilms of PAO1 containing a *pqsA-gfp(ASV)* reporter were grown for 1 (A, B), 2 (C, D) and 4 (E, F) days in flow chambers irrigated with FAB medium (A, C, E) or FAB100 medium (B, D, F). The cells were stained with Syto62, and images visualizing cells (red) and *pqsA-gfp(ASV)* expression (appearing yellow) were acquired by CLSM. The central pictures show horizontal optical sections, and the flanking pictures show vertical optical sections. Bars, 20 μm.

were present in the biofilms before antibiotic treatment, and that the propidium iodide staining did not visualize extracellular DNA with the setting of the CLSM that was used (Fig. 6A, B). (Propidium iodide does not give as high a signal as DDAO, and with normal settings of the CLSM it will therefore mainly visualize dead cells where the concentration of DNA is high compared to in the biofilm matrix.)

Assessment of survival in the biofilms through recovery of the bacteria from the flow-chambers, after EDTA treatment, ciprofloxacin treatment, or no antimicrobial treatment, followed by plating and c.f.u. counting, confirmed that the structured *P. aeruginosa* biofilms grown in FAB medium displayed higher tolerance to the ciprofloxacin and EDTA treatment than the unstructured *P. aeruginosa* biofilms grown in FAB100 medium. C.f.u. from ciprofloxacin-treated biofilms divided by c.f.u. from untreated biofilms was 0.22 (three replicates, SD 0.13) for the biofilms grown in FAB, and 0.02 (three replicates, SD 0.001) for the biofilms grown in FAB100. C.f.u. from EDTA-treated biofilms divided by c.f.u. from untreated biofilms was 0.55 (three replicates, SD 0.095) for the biofilms grown in FAB and 0.21 (three replicates, SD 0.082) for the biofilms grown in FAB100.

DISCUSSION

A number of recent reports suggest that iron affects biofilm formation by *P. aeruginosa*. Experiments using iron-chelating lactoferrin suggested that a minimum concentration of free

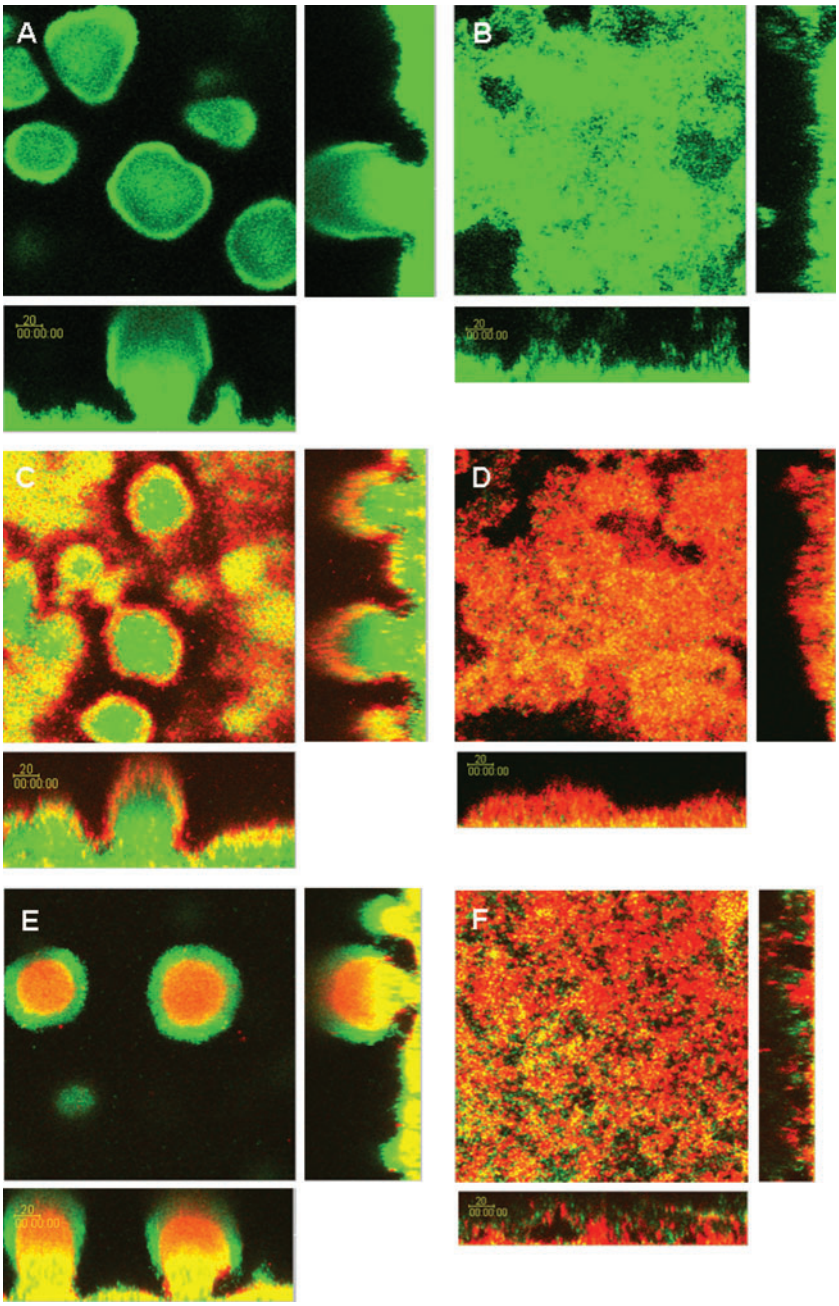

Fig. 6. Four-day-old biofilms of *gfp*-tagged PAO1 grown in FAB medium (A, C, E) or FAB100 medium (B, D, F) were either not treated (A, B), treated with ciprofloxacin (C, D), or treated with EDTA (E, F), after which they were stained with propidium iodide and images were acquired by CLSM. Live cells appear green and dead cells appear yellow or red. The central pictures show horizontal optical sections, and the flanking pictures show vertical optical sections. Bars, 20 μm.

iron is necessary for the formation of structured *P. aeruginosa* biofilms in a flow-chamber system (Singh *et al.*, 2002; Banin *et al.*, 2005). On the other hand, high concentrations of iron were shown to suppress *P. aeruginosa* biofilm formation in both microtitre tray and flow-chamber systems (Musk *et al.*, 2005), and low iron concentrations were shown to be required for *P. aeruginosa* biofilm (microcolony) formation in artificial sputum medium (Sriramulu *et al.*, 2005). In agreement with these previous studies, we found in the present study that in our microtitre tray and flow-chamber setups low iron levels promoted biofilm formation while high iron levels suppressed biofilm formation. Experiments involving DNase I treatment of biofilms in microtitre trays (this study) or flow-chambers (Allesen-Holm *et al.*, 2006) suggested that extracellular DNA is an important constituent of the biofilm matrix. Furthermore, evidence was presented that the formation of extracellular DNA in *P. aeruginosa* biofilms is regulated via the PQS quorum-sensing system (Allesen-Holm *et al.*, 2006). In this paper, we present evidence that low iron concentrations promote *P. aeruginosa* biofilm development via up-regulation of the *pqs* genes and the formation of extracellular DNA, and that high levels of iron suppress *P. aeruginosa* biofilm development via down-regulation of the *pqs* genes and the formation of extracellular DNA. In agreement with our present and previous work, a study

comparing different microarray results identified the PQS regulator gene *pqsR* as being among genes that are induced at low iron levels and also positively controlled by quorum sensing (Cornelis & Aendekerk, 2004).

Under specific conditions in flow-chamber systems (e.g. the glucose FAB medium used in the present study), *P. aeruginosa* forms heterogeneous biofilms with mushroom-shaped multicellular structures via a process that involves formation of microcolonies in the initial phase and subsequent formation of cap-shaped structures on top of the microcolonies in the later phase (e.g. Klausen *et al.*, 2003a). In agreement with the present study, the extracellular DNA in such *P. aeruginosa* biofilms was shown to be present in high concentrations close to the substratum and in the outer part of the microcolonies in the initial phase of biofilm development, and in the outer region of the stalk portion of the mushroom-shaped multicellular structures in the later phase of biofilm development (Allesen-Holm *et al.*, 2006). In the present study we observed that when *P. aeruginosa* formed biofilms in flow-chambers irrigated with FAB medium, expression of a *pqsA*::*gfp(ASV)* fusion was detectable in 1-day-old biofilms and reached the highest expression level at day 2, where it was localized mainly in the outer part of the microcolonies. After day 2, expression of the *pqsA*::*gfp(ASV)* fusion in the biofilms decreased, and at day 4 only a weak green signal could be detected in the stalk portion of the mushroom-shaped multicellular structures. This spatio-temporal expression pattern of *pqsABCDE* is in accordance with the location of extracellular DNA in *P. aeruginosa* biofilms reported here and by Allesen-Holm *et al.* (2006). In the medium containing a high iron concentration, there was significantly less expression of the *pqsA*::*gfp(ASV)* fusion, and this expression appeared less coordinated in time and space. In agreement with the quantifications of extracellular DNA and biofilm formation in the microtitre tray assays, and in agreement with the role of the *pqs* genes in DNA release (Allesen-Holm *et al.*, 2006), the biofilms grown at high iron concentration contained less extracellular DNA and less biomass than the biofilms grown in normal FAB medium. In addition, biofilms grown at high iron concentration appeared less spatially organized and did not develop mushroom-shaped multicellular structures.

The cap-forming and the stalk-forming subpopulation of the mushroom-shaped multicellular structures in *P. aeruginosa* biofilms in many cases display differential tolerance to antimicrobial compounds. For example, the antibiotic tobramycin was shown to kill preferentially bacteria located in the outer part of the cap portion of the mushroom-shaped structures, whereas the antibiotic colistin, the detergent SDS and the chelator EDTA were shown to kill preferentially bacteria in the stalk portion of the mushroom-shaped structures (Bjarnsholt *et al.*, 2005; Banin *et al.*, 2006; Haagensen *et al.*, 2007). Since we had found that the level of iron in the medium may control whether a structured or an unstructured *P. aeruginosa* biofilm is formed, we found it of interest to investigate tolerance to antimicrobial compounds in *P. aeruginosa* biofilms grown in FAB medium or in FAB100 high-iron medium. Ciprofloxacin treatment and EDTA treatment caused killing of cells located in the cap portion and stalk portion, respectively, of the mushroom-shaped structures of 4-day-old *P. aeruginosa* biofilms grown in FAB medium, whereas there was no special area for ciprofloxacin- or EDTA-mediated killing in 4-day-old biofilms grown in high-iron medium. In addition, the structured *P. aeruginosa* biofilms, grown in FAB medium, displayed higher tolerance to the antimicrobial treatment than the unstructured *P. aeruginosa* biofilms, grown in high-iron medium.

The formation of *P. aeruginosa* extracellular DNA is evidently regulated by both quorum sensing and the level of available iron. Other processes that appear to be subject to regulation by the same cues include elastase production and Mn-superoxide dismutase (SOD) production, which were both shown to be quorum-sensing controlled (Hassett *et al.*, 1999; Winson *et al.*, 1995), and also regulated by iron so that they were induced under low-iron conditions but repressed under high-iron conditions (Bjorn *et al.*, 1979; Hassett *et al.*, 1997; Sokol *et al.*, 1982; Bollinger *et al.*, 2001). The fact that iron regulation of these processes to some extent is independent of quorum-sensing regulation makes nutritional override of quorum sensing possible. Other examples of possible nutritional override of quorum sensing include pyocyanin production, which is positively controlled by quorum sensing (Brint & Ohman, 1995), but can also be induced by phosphate starvation (Hassett *et al.*, 1992). The possibility of nutritional override of quorum sensing should be taken into account when possible strategies for manipulating quorum sensing for controlling bacterial infections are considered.

Evidence was recently presented that biofilm formation by *Staphylococcus aureus* is induced at low iron concentrations and repressed at high iron concentrations via a mechanism that does not affect expression of polymeric *N*-acetylglucosamine, which is the major exopolysaccharide implicated in *S. aureus* biofilm formation (Johnson *et al.*, 2005). Our unpublished results suggest that extracellular DNA plays a role in *S. aureus* biofilm formation, and that formation of *S. aureus* extracellular DNA occurs at high levels in low-iron medium and at low levels in high-iron medium. It is possible, therefore, that there is a connection between iron concentration, extracellular DNA and biofilm formation also for the Gram-positive organism *S. aureus*.

The finding that high amounts of iron may inhibit *P. aeruginosa* biofilm formation raises the question whether iron might be used to treat biofilm infections in medical settings. However, because the lack of free iron in the human body in most cases is limiting bacterial growth (Weinberg, 1999), treatment of biofilm-based infections with iron might not be feasible. Nevertheless, the possibility exists that a combination treatment involving antibiotics and iron might be useful. The finding that iron can suppress biofilm

formation may motivate a search for similar compounds that can suppress biofilm formation but do not stimulate bacterial growth.

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Paper II

This paper is in preparation and experiments are still in progress.

A two-column layout has been applied for clarity.

Effects of the twin-arginine translocation pathway on PQS-mediated DNA release, virulence factor production and biofilm development in *Pseudomonas aeruginosa.*

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen and can cause a wide range of persistent human infections, which are to a large extent due to its ability to form biofilms. We have previously provided evidence that extracellular DNA is an important constituent of the extracellular matrix in *P. aeruginosa* biofilms, and is released via a process that requires the Pseudomonas Quinolone Signal (PQS) system. In the present study, we have constructed a *P. aeruginosa* transposon insertion mutant library and performed a screen for mutants defective in DNA-release. *tatA* and *tatB* mutants, defective in the twin-arginine translocation pathway, were among the mutants that displayed a reduced level of DNA release. Characterization of *P. aeruginosa tatA* showed that the defect in the *tatA* gene attenuated PQS secretion, but did not affect the level of PQS synthesis. In addition to PQS-mediated DNA-release, the *P. aeruginosa tatA* mutant was also deficient in virulence factor production and biofilm development.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen which can cause a wide range of human infections such as pulmonary infections, medical-device-related infections, urinary tract infections, wound infections and bacteremia (Bodey *et al.*, 1983). *P. aeruginosa* is notorious for its tolerance to antimicrobial agents and this is to a large extent due to its ability to form biofilms (Costerton *et al.*, 1995; Costerton *et al.*, 1999). Biofilms are agglomerates of microorganisms embedded in a self-produced polymeric matrix (Costerton *et al.*, 1995). Bacterial cells living in biofilms were shown to be 1000-times more resistant to antibiotic treatment than their planktonic counterparts (Costerton *et al.*, 1999).

P. aeruginosa is a model organism for biofilm research. Intercellular communication (quorum-sensing), cell surface appendages, extracellular polymeric substances, biosurfactants, and motility were reported to be involved in *P. aeruginosa* biofilm development (Davies *et al.*, 1998; Donlan, 2002; Allesen-Holm *et al.*, 2006;

Barken *et al.*, 2008; Klausen *et al.*, 2003a; Klausen *et al.*, 2003b; Pamp & Tolker-Nielsen, 2007; Flemming *et al.*, 2007). *P. aeruginosa* biofilms were reported to contain distinct subpopulations which are tolerant to different antibiotics (Haagensen *et al.*, 2007; Pamp & Tolker-Nielsen, 2007).

Recently, extracellular DNA was recognized as one of the major matrix components of bacterial biofilms (Allesen-Holm *et al*., 2006; Qin *et al.*, 2007; Rice *et al.*, 2007; Whitchurch *et al.*, 2002). In *P. aeruginosa*, release of extracellular DNA is regulated through the quorum-sensing systems (Allesen-Holm *et al.*, 2006; Yang *et al.*, 2007). Evidence has been provided that *Pseudomonas* Quinolone Signal (PQS)-mediated cell lysis or membrane vesicle release is a major cause of DNA release in *P. aeruginosa* biofilms (Allesen-Holm *et al.*, 2006). Membrane vesicles containing DNA and other virulence factors were shown to be released from the outer membrane of *P. aeruginosa* cells dependent on PQS (Mashburn *et al.*, 2005), and they were found to be a common particulate feature of the matrix of *Pseudomonas aeruginosa* biofilms (Schooling & Beveridge, 2006). Recently, PQS was reported to act as a pro-oxidant which sensitizes the bacteria towards oxidative and other stresses (Haussler & Becker, 2008), which may provide another explanation for PQS-mediated cell lysis. However, to better understand *P. aeruginosa* biofilm development, more studies are needed on PQS-mediated DNA release.

In this study, we identified genes which are involved in the release of extracellular DNA from *P. aeruginosa*. A *P. aeruginosa* mutant library was constructed by the use of Mariner transposon mutagenesis, and a screen for DNA release defective mutants was carried out. In addition to mutants that were inactivated in genes involved in PQS synthesis, *tat* mutants, defective in the twin-arginine translocation pathway, were identified to have reduced levels of DNA release. Further studies showed that a *tatA* mutation did not affect the level of PQS synthesis but blocked PQS secretion, virulence factor production and biofilm structure development.

Materials and Methods

Bacterial strains and growth conditions

P. aeruginosa PAO1 (Holloway & Morgan, 1986) was used as the wild-type strain in this study. *Escherichia coli* strains MT102 and DH5a were used for standard DNA manipulations. Luria–Bertani (LB) medium (Bertani, 1951) was used to cultivate *E. coli* strains. *P. aeruginosa* biofilms were cultivated at 30^{o}C in flow-chambers irrigated with FAB medium (Heydorn *et al.*, 2000) supplemented with 0.3 mM glucose. Selective media were supplemented with ampicillin (Ap; 100mg/liter), gentamicin (Gm; 60mg/liter), or streptomycin (Sm; 100mg/liter).

Mutant library construction and screening

The *P. aeruginosa* mutant library was constructed by using the Mariner transposon vector pBT20 as previously described (Kulasekara *et al.*, 2005). Transconjugants carrying transposon insertion were picked from the selective plates and inoculated into microtiter tray wells containing ABTG medium supplemented with propidium iodide (PI) (Allesen-Holm *et al.*, 2006) by using a Qpix2 robot (Genetix). The red fluorescence originating from propidium bound to DNA was measured in a Wallac Victor 2 1420 Multilabel Counter (Perkin Elmer), and it was used as a measure of the amount of extracellular DNA produced by each mutant. Mutants that produced reduced levels of extracellular DNA were selected and saved for further analysis.

PqsA::gfp(ASV) expression assay

For measuring *pqs* operon expression in *P. aeruginosa*, a reporter plasmid harbouring a *pqsA::gfp(ASV)* fusion (Yang *et al.*, 2007) was transformed into the *P. aeruginosa* wild-type PAO1 strain and different mutants by electroporation, and Gfp fluorescence was

measured as previously reported (Yang *et al.*, 2007).

Sequencing of transponson mutants

The sequence flanking the Mariner transponson in mutants was identified by arbitrary PCR essentially as previously described (Friedman & Kolter, 2004) but with the following TnM specific primers: Rnd1-TnM 5'-GTG AGC GGA TAA CAA TTT CAC ACA G, Rnd2-TNm 5'-ACA GGA AAC AGG ACT CTA GAG G. Sequencing was performed by Macrogen, Seoul, Korea with the TnM specific primer: TnMseq 5'-CAC CCA GCT TTC TTG TAC AC.

Construction of the *tatA* complementation plasmid

A full length *tatA* fragment was PCR amplified by use of the primers tatAF 5'-GGAATTCCCCTGAACCTACACATTGCCA-3' and tatAR 5'-GGGGTACCCCATTCCGAACATCGATGGC TA-3'. The PCR product was digested with *EcoR*I and *Kpn*I and then ligated into *EcoR*I/*Kpn*I digested pUCP22Not vector (Herrero *et al.*, 1990). The ligation product was electroporated into the *P. aeruginosa tatA* mutant. Transformants were selected on LB plates containing 200 µg/ml carbenicillin. Lack of pyoverdine production and different types of motility was observed for the *P. aeruginosa tatA* mutant as previously reported (Ochsner *et al.*, 2002), and these defects were found to be restored in the complemented strain *P. aeruginosa tatA*/pUCP22::*tatA*

PQS assay

The *P. aeruginosa* PAO1 wild-type, *P. aeruginosa tatA* mutant and *P. aeruginosa tatA*/pUCP22::*tatA* complemented mutant were grown in LB at 37°C with vigorous shaking. Samples were obtained at OD_{600} values 0.3, 0.8 and 2.0 unless otherwise indicated, and were prepared as previously published (Gallagher *et al.*, 2002) with modifications. At each time point, 300 µl culture was added to 900 µl acidified ethyl acetate, and samples were vortexed for 2 min and centrifuged for 2 min at 13.000 *g* (Gallagher *et al.*, 2002). A portion of the ethyl acetate layer (450 µl) was removed to a clean tube and allowed to dry down at 37°C. Extracts were resuspended in 50 µl of 1:1 acidified ethyl acetate: acetonitrile by vortexing for 10 min at low speed. Thin layer chromatography (TLC) was used to quantify PQS production by *P. aeruginosa* strains as previously described (Gallagher *et al.*, 2002). Aluminum TLC sheets (EMD Chemicals, silica gel 60 F254) were activated in a 0.5% KH2PO4 solution, air dried, and baked at 100°C for 1 h prior to use. Samples (2µl) were applied to each plate using 1µl glass capillaries, and synthetic PQS (25µg) was loaded for comparison. The TLC solvent used was 17:2:1 methylene chloride:acetonitrile:dioxane. Plates were visualized using a handheld long-wave UV light, and photographs were taken using a digital camera. Fold changes in spot intensity were determined with Image J (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

Rhamnolipid assay

An emulsification activity assay was used to measure the production of rhamnolipid from *P. aeruginosa* strains as previously described (Cooper & Goldenberg, 1987) with slight modification. Simply, 1 ml of (18 hours) over night culture supernatant and 1 ml of n-hexadecane was mixed in an eppendorf tube and vortexed at high speed for 1 min. The mixture was allowed to stand for 1 hour before inspection. The emulsification activity was defined as the height of the emulsion layer divided by the total height of the liquid column.

Cultivation of static biofilms, and analysis of them by atomic force microscopy (AFM)

P. aeruginosa overnight cultures were diluted 100 times in ABTG medium and incubated in eppendorf tubes with glass cover slips at 37°C for 24 hours without shaking. After incubation the biofilms formed at the air-liquid interface on the glass cover slips were dipped 3 times into

double distilled water, and air-dried at room temperature for AFM analysis. The air-dried *P. aeruginosa* biofilms were imaged with a DualScope DS 95-50/200 AFM scanner (DME - Danish Micro Engineering A/S, Copenhagen/Herlev, Denmark). The instrument was operated using the tapping mode (Jalili & Laxminarayana, 2004). Images were obtained using SenseMode™ AC probes (DME) with a spring constant of 42 N/m. At least three, and up to five high-quality images were captured for each bacterial strain. After images were collected, the data were analyzed with the SPM program (DME) to gain information on biofilm topography. AFM images were also analyzed with SPIP 4.8.1 (Image Metrology A/S, Hørsholm, Denmark) to gain detailed information such as 3D image and height profile. For each strain, the cell length and cell width of 20 different bacteria were measured on topographic images acquired with the SPIP 4.8.1 software.

Cultivation of flow-chamber biofilms

Biofilms were grown in flow-chambers with individual channel dimensions of 1 x 4 x 40 mm. The flow system was assembled and prepared as described previously (Heydorn *et al.*, 2000). The flow-chambers were inoculated by injecting 350 µl of overnight culture diluted to an OD_{600} of 0.001 into each flow channel using a small syringe. After inoculation, the flow channels were left without flow for 1 h, after which medium flow was started using a Watson Marlow 205S peristaltic pump. The mean flow velocity in the flow-chambers was 0.2 mm/s, corresponding to laminar flow with a Reynolds number of 0.02. After 4-days of cultivation 10 µM of tobramycin was added to the biofilm medium, and after 24-hours of tobramycin treatment 10 µM propidum iodide (Invitrogen) was added to the flow cells to visualize the dead cells.

Microscopy and image processing of flow cell biofilms

All microscopy observations and image acquisitions of flow-chamber-grown biofilms were done with a Zeiss LSM510 confocal laser scanning microscope (CLSM) (Carl Zeiss, Jena, Germany) equipped with detectors and filter sets for monitoring of green fluorescent protein and propidium iodide. Images were obtained using a 63x/1.4 objective or a 40 x/1.3 objective. Simulated 3-D images and sections were generated using the IMARIS software package (Bitplane AG, Zürich, Switzerland).

Results

Tn Mariner mutagenesis and mutant library screening

In order to identify genes involved in DNA-release in *P. aeruginosa*, we generated a transposon mutant library by the use of Mariner transposon mutagenesis. After screening of 10.000 mutants, 84 mutants that displayed low levels of DNA-release were selected. The mutants were subjected to a second screening for low levels of DNA-release by inoculation of each of them into 8 microtiter tray wells followed by quantification of extracellular DNA. The mutants that consistently showed reduced levels of DNA release were subjected to Southern blotting to eliminate double insertion mutants. After that, the regions flanking the Mariner transposon in 34 DNA-release mutants were sequenced and the specific genes interrupted by the transposon were identified.

Many of the DNA-release deficient mutants had the transposon insertion in PQS synthesis genes (e.g. *pqsC*, *pqsH*, *pqsR*) (Fig. 1, and data not shown). The DNA-release deficient phenotypes of these mutants were in accordance with our previous report (Allesen-Holm *et al.*, 2006), and were verified with related defined mutants (data not shown). In addition, some of the DNA-release deficient mutants had the Mariner transposon insertion in the *tatA* or *tatB* gene (Fig. 1), which encode part of a membrane protein involved in the twin-arginine translocation (TAT) pathway (Robinson & Bolhuis, 2001).

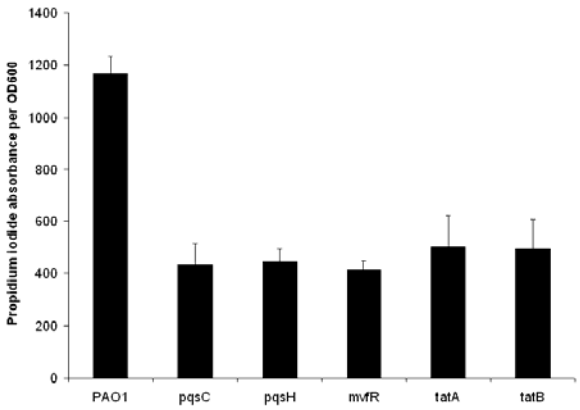

Fig. 1. *P. aeruginosa* wild-type and mutants were grown for 24 h in microtitre tray wells containing AB-glucose medium, after which the relative levels of extracellular DNA in the cultures were determined via a propidium iodide staining assay. Averages and standard deviations of eight replicates are shown.

Because we previously have obtained evidence that DNA-release by *P. aeruginosa* is connected to PQS synthesis (Allesen-Holm *et al.*, 2006), we measured expression of the *pqs* operon in the *P. aeruginosa tatA* mutant and some of the *pqs* mutants by using a reporter plasmid carrying a *pqsA::gfp(ASV)* fusion. Unlike the *pqs* synthesis defective mutants, the *tatA* mutant expressed the *pqsA::gfp(ASV)* reporter at a level similar to the wild-type strain (Fig. 2), suggesting that the Tat pathway might have an indirect effect on PQS-mediated DNA-release in *P. aeruginosa*.

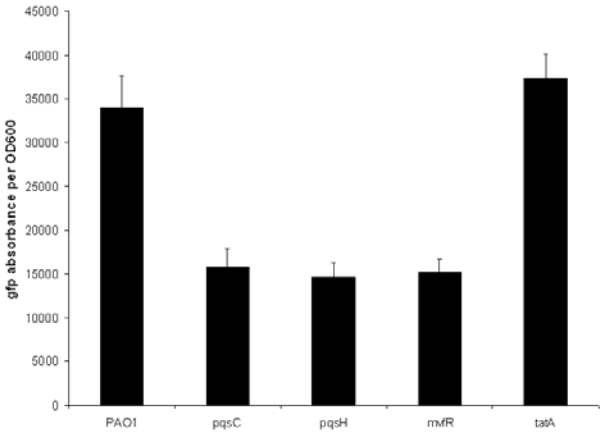

Fig. 2. *P. aeruginosa* wild-type and mutants were grown for 24 h in microtitre tray wells containing AB-glucose medium, after which the relative levels of *pqsA::gfp(ASV)* expression in the cultures were determined via a Gfp fluorescence assay. Averages and standard deviations of eight replicates are shown.

Regulation of PQS secretion by the twin-arginine translocation pathway

The Tat pathway is a secretion pathway originally found in plants and recently discovered in bacteria (Robinson & Bolhuis, 2001). The Tat pathway was shown to be essential for export of phospholipases, proteins involved in pyoverdine-mediated iron-uptake, anaerobic respiration, osmotic stress defense, motility, and biofilm formation in *P. aeruginosa* (Ochsner *et al.*, 2002). We therefore speculated that PQS might be synthesized in the *tat* mutants but could not be transported out of the cells. To test this hypothesis, we quantified PQS in the supernatant of *P. aeruginosa* wild-type, *tatA*, and *tatA*/pUCP22::*tatA* stationary-phase cultures by the use of TLC. The *tatA* mutant had reduced levels of PQS in the supernatant (Fig. 3A), whereas the *tatA*/pUCP22::tatA complemented strain had wild-type levels of PQS in the supernatant (Fig. 3A). If the cells were lysed prior to removal of the supernatant, the *tatA* mutant and the *tatA*/pUCP22::*tatA* complemented strain had approximately the same level of PQS in the supernatant as the wild-type PAO1 strain (Fig. 3B).

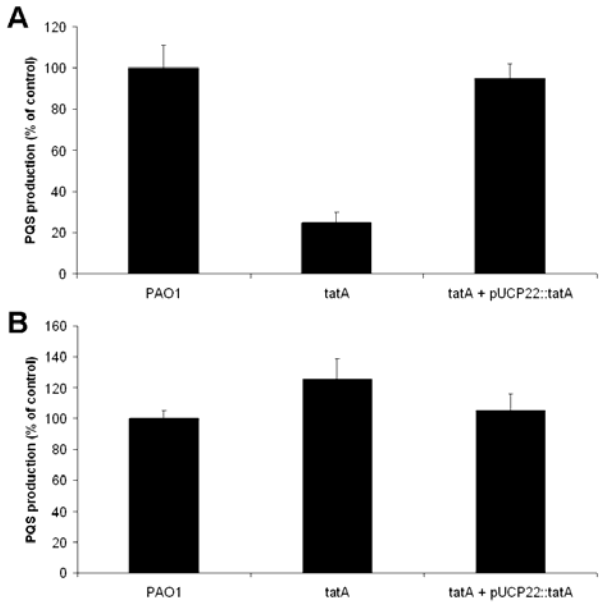

Fig. 3. The PQS level in the supernatant of un-lysed (A) or lysed (B) *P. aeruginosa* wild-type, *P. aeruginosa tatA* and *P. aeruginosa tatA*/pUCP22::*tatA* cultures determined by TLC analysis. Averages and standard deviations of three replicates are shown.

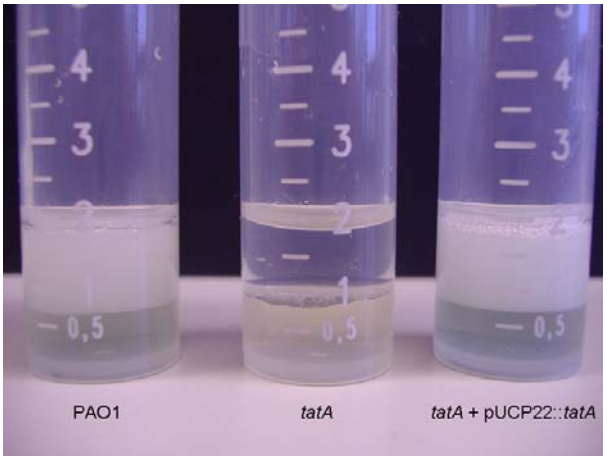

Fig. 4. Production of biosurfactant and pyocanine by *P. aeruginosa* wild-type (A), *P. aeruginosa tatA* (B) and *P. aeruginosa tatA*/pUCP22::*tatA* (C). N-hexadecane was added to culture supernatant and the mixture was vortexed at high speed and was then allowed to stand for 1 hour before inspection. Biosurfacatant activity is indicated by the extent of the top emulsification layer, and the amount of pyocanine is indicated by the level of blue color in the bottom layer.

Regulation of rhamnolipid and pyocanine production by the twin-arginine translocation pathway

As PQS mediated quorum sensing system was reported to regulate production of virulence factors such as rhamnolipid and pyocanine (Gallagher *et al.*, 2002; McKnight *et al.*, 2000; West *et al.*, 2006), we tested the production of rhamnolipid and pyocanine by the *P. aeruginosa* wild-type, *P. aeruginosa tatA* mutant, and *P. aeruginosa tatA*/pUCP22::*tatA* complemented strain. Unlike the wild-type and *tatA*/pUCP22::*tatA* complemented strain, the *tatA* mutant did not produce these two virulence factors (Figure 4).

Regulation of biofilm structure development by the twin-arginine translocation pathway

We used two different tools, CLSM and AFM, to analyse the structural features of biofilms formed by the *P. aeruginosa* wild-type, *P. aeruginosa tatA* mutant, and *P. aeruginosa tatA*/pUCP22::*tatA* complemented strain under different conditions.

CLSM was used to analyse biofilms formed in flow-chambers under continuous flow conditions. After 4-days cultivation, the *P. aeruginosa* wild-type and *P. aeruginosa tatA*/pUCP22::*tatA* complemented strain formed biofilms with mushroom-shaped structures, whereas the *P. aeruginosa tatA* mutant formed thin and flat biofilms (Fig. 5). We challenged the biofilms formed by the wild-type, *tatA* mutant and *tatA*/pUCP22::*tatA* strain with tobramycin, and found that the *tatA* mutant biofilm was more sensitive to tobramycin treatment than the wild-type and *tatA*/pUCP22::*tatA* biofilms (Fig. 5).

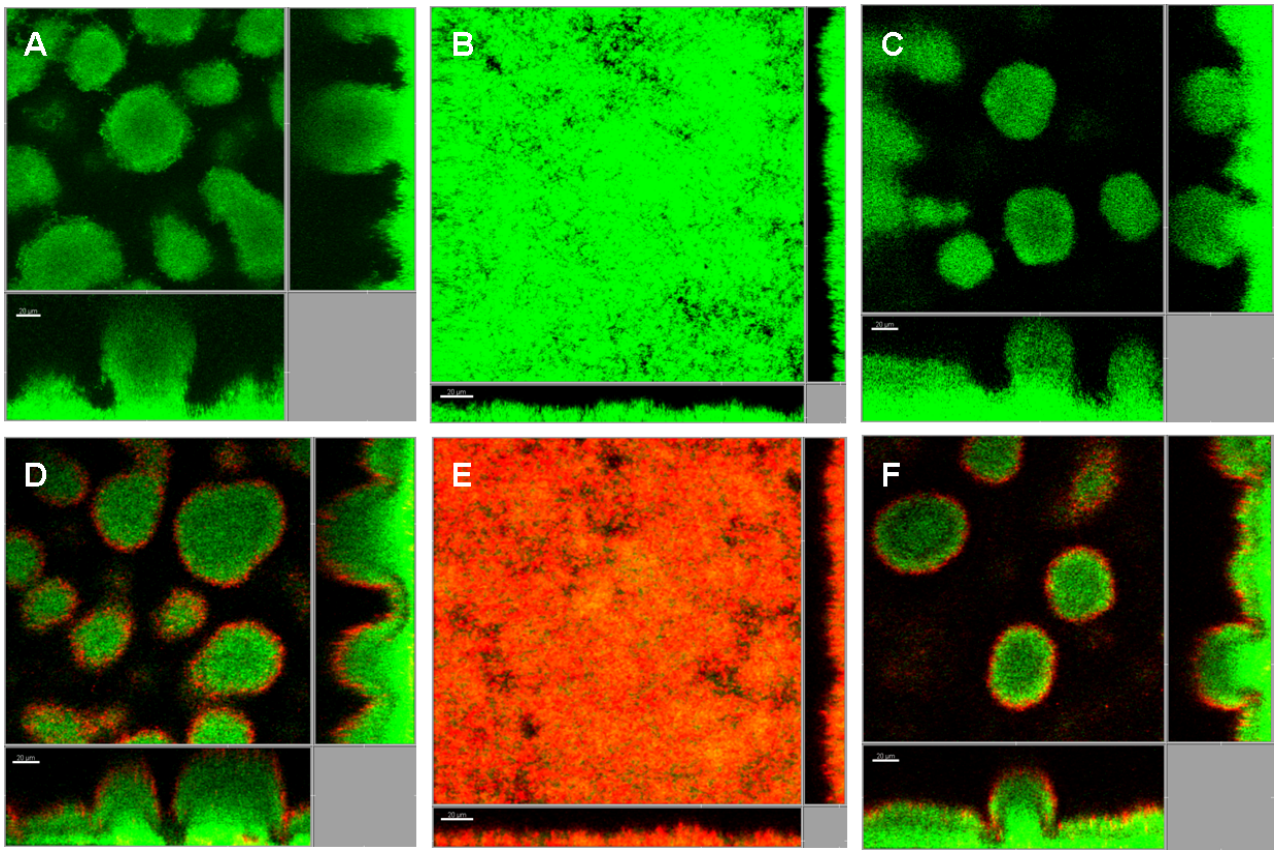

Fig. 5. CLSM micrographs showing four-day-old biofilms of *gfp*-tagged *P. aeruginosa* wild-type (A, D), *tatA* mutant (B, E) and *tatA*/pUCP22::*tatA* complemented strain (C, F) before (A, B, C) and after (D, E, F) treatment with tobramycin. The medium was supplemented with propidium iodide prior to CLSM, and therefore dead cells appear yellow or red while live cells appear green. The central pictures show horizontal optical sections, and the flanking pictures show vertical optical sections. Bars, 20 µm.

AFM was used to analyse biofilms formed on glass slides in static cultures. Judged from AFM topographic, height contrast, and 3D images, the wild-type and *tatA*/pUCP22::*tatA* biofilms showed a higher level of structural heterogeneity than the *tatA* biofilm (Fig. 6). The wild-type and *tatA*/pUCP22::*tatA* biofilms consisted of multiple layers and the thickness varied between different areas while the *tatA* biofilm was flat and thin with some loosely attached cell aggregates (Fig. 6). The cells in the wild-type biofilm and *tatA*/pUCP22::*tatA* biofilm were rounder and smaller in size than the cells in the *tatA* biofilm (Table 1). According to the height profiles, the edges around the cells in the wild-type and *tatA*/pUCP22::*tatA* biofilms were not as clear as those around the cells in the *tatA* biofilm (Fig. 6).

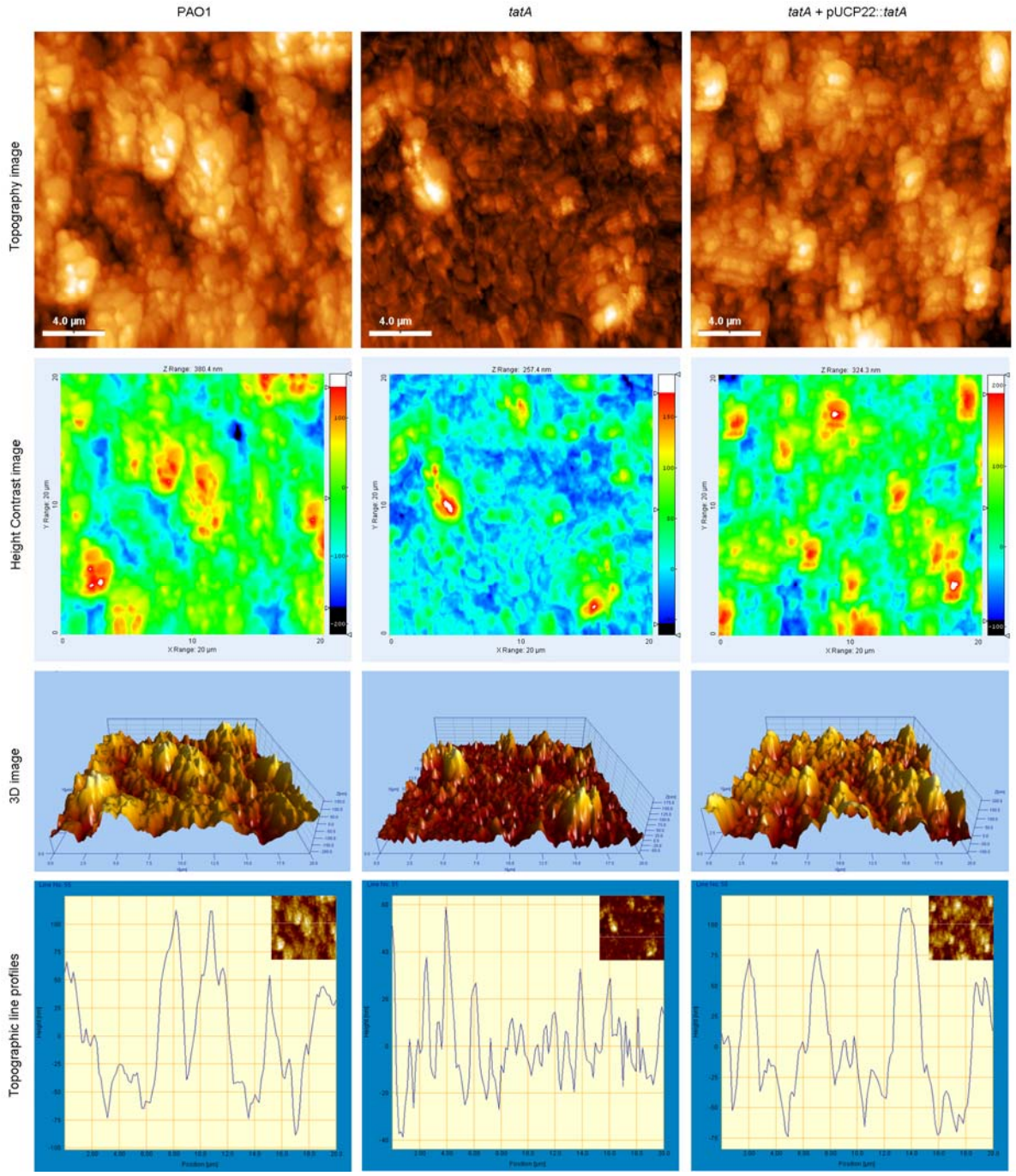

Fig. 6. AFM analysis of biofilms formed by the *P. aeruginosa* wild-type (first column), *tatA* mutant (second column) and *tatA* pUCP22::tatA complemented strain (third column) during static growth for 24 hours. The first row shows topography AFM images, the second row shows height contrast images, the third row shows 3D images, and the fourth row shows topographic line profiles obtained from the AFM images.

Discussion

Bacterial biofilm formation is a complicated process which needs coordination of different mechanisms such as quorum sensing, motilities, EPS material production, and iron up take (Davey & O'Toole G, 2000; O'Toole *et al.*, 2000; Klausen *et al.*, 2006; Parsek and Tolker-Nielsen, 2008). These mechanisms attribute

characters like biofilm subpopulation differentiation and antibiotic tolerance, which are important underlying causes of persistent infections (Costerton *et al.*, 1999). It is difficult to link genes into a hierarchically ordered genetic network that deterministically controls biofilm development. However, understanding these networks is expected to facilitate the development of anti-biofilm strategies. In this study, we have screened genes involved in the release of extracellular DNA biofilm matrix material from *P. aeruginosa*. We provide evidence that the Tat secretion pathway plays a role in PQS quorum sensing in *P. aeruginosa*, and control DNA release and the production of some virulence factors (e.g. rhamnolipid and pyocyanine). Together with an earlier report (Ochsner *et al.*, 2002) showing that the *tatABC* operon in *P. aeruginosa* regulates several virulence factors (e.g. phospholipase C and pyoverdine) as well as motilities, we have uncovered a role of the Tat pathway as a regulator in *P. aeruginosa* biofilm development.

Our group has previously provided evidence that the PQS synthesis pathway is necessary for DNA release in *P. aeruginosa* biofilms (Allesen-Holm *et al.*, 2006), and this process was also shown to be regulated by iron (Yang *et al.*, 2007). However, the detailed mechanisms concerning PQS mediated DNA release and its regulation networks are still poorly investigated. In the present study, we generated and screened a mutant library to identify genes involved in PQS mediated DNA release. We found that one group of DNA release mutants were inactivated in the *tat* genes which encode components of the twin-arginine translocase pathway. Measurements of the expression level of the *pqs* operon in the wild-type and *tatA* mutant, and quantification of the PQS level in the supernatant of un-lysed and lysed cultures of the wild-type and *tatA* mutant, provided evidence that the *tatA* mutant can synthesize PQS but can not secrete it. Together these findings suggest that DNA release from *P. aeruginosa* occurs in response to extracellular PQS. In accordance, membrane vesicles containing DNA and virulence factors were shown to be released from the outer membrane of *P. aeruginosa* cells in response to extracellular PQS (Mashburn *et al.*, 2005). In addition, PQS was reported to act as a pro-oxidant which sensitizes bacteria towards oxidative and other stresses (Haussler & Becker, 2008), which suggest that extracellular PQS may play a role in DNA release via cell lysis.

The Tat pathway is a transport system that enables transport of folded proteins across the cytoplasmic membrane and it is present in many bacterial pathogens (De Buck *et al.*, 2008; Robinson & Bolhuis, 2001). A twin-arginine motif is the typical characteristics of the signal peptide of Tat-dependant proteins and it is possible to predict putative Tat substrates by *in silico* analysis (De Buck *et al.*, 2008; Robinson & Bolhuis, 2001). Nevertheless, few of the predicted Tat substrates were confirmed to be actually transported by the Tat pathway (De Buck *et al.*, 2008). The exact working mechanism of Tat transport is not fully unraveled and it is still unclear how the Tat pathway is involved directly in virulence. There is no substrate identified to explain the deficiencies of the *P. aeruginosa tatA* mutant in motility and biofilm formation (De Buck *et al.*, 2008). However, our study show that the *P. aeruginosa tatA* mutant is unable to perform PQS mediated DNA release and produce rhamnolipid and pyocanine, and therefore provide an explanation for the swarming motility and biofilm formation deficiency of *P. aeruginosa* Tat pathway mutants. Together with a recent report showing that the Tat pathway is involved in the production of an unknown secreted signaling molecule in *Providencia stuartii* (Stevenson *et al.*, 2007), our study indicates that the Tat pathway might play a general role in bacterial cell-to-cell communication. However, our unpublished investigations suggest that the Tat pathway does not play a role in *las* quorum sensing (data not shown), which is in accordance with our finding of active expression of the *pqs* operon in the *tatA* mutant.

CLSM analysis of flow-chamber grown biofilms showed very clear differences between the *P. aeruginosa* wild-type and *tatA* biofilms. While the wild-type formed characteristic mushroom-shaped structures under these conditions, the *tatA* mutant formed flat and thin biofilms. In addition, the biofilms formed by the *tatA* mutant was more sensitive to tobramycin treatment than wild-type biofilms, presumably due to the inability of the *tatA* mutant to form differentiated multicellular structures. Formation of flat and thin biofilms by the *P. aeruginosa tatA* mutant is in agreement with previous reports showing that extracellular DNA, rhamnolipid, and bacterial motility all play roles in structural biofilm development by *P. aeruginosa* (Whitchurch *et al*., 2002; Klausen *et al*., 2003; Allesen-Holm *et al*., 2006; Pamp and Tolker-Nielsen, 2007; Barken *et al*., 2008).

Biofilm development often occurs along with the production of a tight network of EPS that facilitates dense adherence of cells to each other and leads to a differentiated three-dimensional biofilm architectures (Davey & O'Toole G, 2000; Sutherland, 2001). In the present study, we have characterized the impact of the Tat pathway on biofilm structure development at high resolution by the use of AFM. Bacterial cells were visible on the AFM images as tightly associated cellular unities in wild-type biofilm, but as a thin layer in *tatA* mutant biofilm. In addition the AFM analysis showed that the edges around the cells in the wild-type biofilms were not as clear as those around the cells in the *tatA* biofilm. In agreement with our finding that the *tatA* mutant is deficient in DNA release, Auerbach *et al.* (2000) found that EPS production in biofilms could lead to difficulties to visualize the edges of individual bacterial cells by the use of AFM.

In conclusion, the present study provided evidence that the twin-arginine translocation pathway in *P. aeruginosa* is involved in PQS release, generation of extracellular DNA, and production of rhamnolipid and pyocyanine. In accordance with these phenotypes the *P. aeruginosa tat* mutants displayed defects in biofilm formation.

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Paper III

This paper is submitted for publication.

A two-column layout has been applied for clarity.

Pyoverdine and PQS mediated subpopulation interactions involved in *Pseudomonas aeruginosa* biofilm development.

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Summary

The existence of physiologically distinct subpopulations in biofilms plays an important role in their tolerance towards environmental insult. The structured nature of biofilms is thought to enable buildup and maintenance of physiologically distinct subpopulations in different micro-niches. However, using flow-chamber-grown *Pseudomonas aeruginosa* biofilms as model system, we show in the present study that the development of heterogeneous biofilms may occur through mechanisms that involve complex subpopulation interactions. One example of this phenomenon is expression of the iron-siderophore pyoverdin in one subpopulation being necessary for development of another subpopulation which does not itself express the pyoverdin syntheses genes. Another example is quorum-sensing-controlled DNA-release in one subpopulation being necessary for development of another subpopulation which does not itself express the quorum-sensing genes. Mechanisms involved in the development of physiologically distinct subpopulations in biofilms may contribute to their innate tolerance towards biocides, protozoa, immune systems, and antibiotics, and therefore may be relevant as biofilm control targets.

Introduction

Microorganisms in medical, industrial and environmental settings often reside in biofilms (Costerton *et al*., 1995). Because bacteria in biofilms in many cases display tolerance to host immune systems, antibiotics and biocides they are often difficult or impossible to eradicate (Costerton *et al*., 1999). Biofilm formation therefore leads to various persistent infections in humans and animals, and to a variety of complications in industry where solid-water interfaces occur. Detailed knowledge about biofilm development processes is essential in order to create strategies to control biofilm formation.

Evidence has been provided that the existence of physiologically distinct cells in biofilms is an underlying cause of their tolerance towards adverse conditions as it often allows one or more subpopulations to survive environmental insult (e.g. see Pamp *et al*., 2008). It is generally assumed that physiologically distinct subpopulations arise and are maintained in biofilms because of the structured nature of the

biofilms which allow different subpopulations to occupy different micro-niches (see Stewart and Franklin, 2008) for a recent review). However, we show in the present report that development of heterogeneous biofilms may occur through mechanisms that involve complex subpopulation interactions. Biofilms in most medical, industrial and environmental settings undergo subpopulation succession and turnover, and mechanisms involved in the development and maintenance of physiologically distinct subpopulations may contribute to their innate tolerance towards adverse conditions.

The model system used in the present study is *Pseudomonas aeruginosa* biofilms grown in flow-chambers irrigated with glucose minimal medium. Under these conditions *P. aeruginosa* forms biofilms that contains mushroom-shaped multicellular structures. The stalk and cap subpopulations in these mushroom-shaped multicellular structures are physiologically distinct as the cells differ with respect to for example antibiotic tolerance properties (e.g. Banin *et al*., 2006; Bjarnsholt *et al*. 2005; Haagensen *et al*., 2007; Kaneko *et al*., 2007; Pamp *et al*., 2008; Yang *et al*., 2007). The formation of the mushroom-shaped structures evidently occurs in a sequential process involving a non-motile subpopulation which forms initial microcolonies by growth in certain foci of the biofilm, and a migrating subpopulation which initially forms a monolayer on the substratum, and subsequently forms caps by aggregating on top of the initial microcolonies (which are then termed stalks) (Haagensen *et al*., 2007; Klausen *et al*., 2003a). A large amount of extracellular DNA is produced in *P. aeruginosa* biofilms via a process that depends on the PQS quorum-sensing system (Allesen-Holm *et al*., 2006). The extracellular DNA functions as a biofilm matrix and has a stratified distribution in *P. aeruginosa* biofilms (Allesen-Holm *et al*., 2006; Whitchurch *et al*., 2002). In young glucose-grown *P. aeruginosa* biofilms the extracellular DNA was shown to be present in high concentrations specifically in the outer layer of the microcolonies, whereas in mature *P. aeruginosa* biofilms the extracellular DNA was shown to be present in high concentrations between the stalk and cap portion of the mushroom-shaped structures (Allesen-Holm *et al*., 2006).

Formation of the cap-portion of the mushroom-shaped structures in *P. aeruginosa* biofilms depends on type IV pili (Klausen *et al*., 2003a). In a biofilm consisting of a mixture of *P. aeruginosa* wild-type and *P. aeruginosa pilA* mutant (deficient in biogenesis of type IV pili) the *pilA* mutants can only form stalks whereas the wild-type bacteria can form both stalks and caps (Klausen *et al*., 2003a). The majority of the mushroom-shaped structures in a wild-type/*pilA* mixed biofilm therefore contain *pilA* mutants in the stalk and wild-type bacteria in the cap, and a minority of the mushroom-shaped structures contain wild-type bacteria in both the stalk and the cap (Klausen *et al*., 2003a). A model system consisting of cyan fluorescent protein-tagged *P. aeruginosa pilA* mutant as the stalk-former, and yellow fluorescent protein-tagged *P. aeruginosa* wild type as the cap-former, therefore enables a study of the roles of subpopulation interactions in structural biofilm development. We focus on the two genes, *pvdA* and *pqsA*, which have previously been shown to be important for the development of structured *P. aeruginosa* biofilms (Allesen-Holm *et al*., 2006, Banin *et al*., 2005; Yang *et al*., 2007). The *pvdA* gene encodes a synthase necessary for production of the pyoverdine siderophore which is involved in iron acquisition (Visca *et al*., 1994). Pyoverdine production in *P. aeruginosa* is derepressed under iron limiting conditions, and is in part regulated by quorum-sensing (Stintzi *et al*., 1998). The *pqsA* gene is part of an operon encoding the PQS quorum-sensing system which is under *las* quorum-sensing regulation (Calfee *et al*., 2001; Gallagher *et al*., 2002; McGrath *et al*, 2004; Wade *et al*., 2005). We provide evidence that: i) synthesis of the iron-siderophore pyoverdin occurs in the stalk-

portion of the mushroom-shaped structures and is necessary for development of the cap-forming subpopulation which does not express the pyoverdin syntheses genes but needs to express the *fpvA* gene encoding part of the ferric-pyoverdin uptake system; and ii) Pqs-controlled DNA-release in the stalk-portion of the mushroom-shaped structures is necessary for development of the cap-forming subpopulation which does not express the *pqs* genes.

Results

Initially we investigated growth in batch cultures of the *P. aeruginosa* PAO1 wild-type and mutants used in the present study. We found that planktonic growth of all the mutants was similar to planktonic growth of the wild-type (Fig. S1). Based on these results we exclude that the observed differences in biofilm structure can be explained due to growth defects of the mutants.

Pvd-mediated structure development in *P. aeruginosa* biofilms.

Using a *pvdA::gfp* fluorescent reporter, Kaneko *et al.* (2007) have previously shown that the *pvdA* gene is expressed specifically in the stalk-portion of the mushroom shaped structures in *P. aeruginosa* wild-type biofilms. Here, we used the *pvdA::gfp* reporter to localize expression of the *pvdA* gene in our *pilA*/wild-type model biofilms. In agreement with the results of Kaneko *et al.* (2007) we found that the *pvdA::gfp* reporter was expressed specifically in the stalk-portion of the mushroom-shaped structures that contained wild-type bacteria both in the stalk and the cap (data not shown). In addition, we found that the *pvdA::gfp* reporter was expressed specifically in the *pilA* stalk-forming subpopulation in the mushroom-shaped structures that contained *pilA* mutants in the stalk and wild-type bacteria in the cap (Fig. 1).

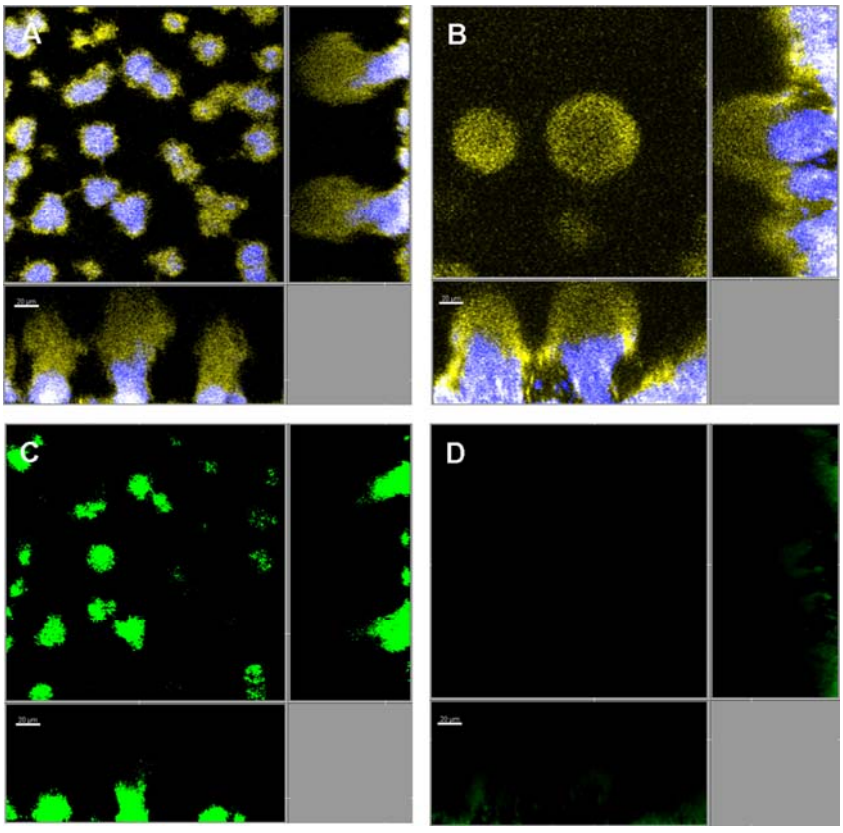

Fig. 1. 4-day-old *pilA*/wild-type biofilms with a *pvdA::gfp(L)* reporter in the *pilA* mutant (A, C) or in the wild-type (B, D). The *pilA* mutant was tagged with cfp (blue) and the wild-type was stained with cyto62 (converted to yellow). A and B visualize the cells, whereas C and D visualize *pvdA::gfp(L)* fluorescence. The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. The bars are 20 μm.

Subsequently, we investigated the requirement for *pvdA* expression in *P. aeruginosa* biofilm development by determining the morphology of biofilms formed by different mutants. The controls in the study confirmed in agreement with previous studies (Banin *et al*., 2005; Klausen *et al*., 2003a), that the *P. aeruginosa* wild-type formed biofilms with mushroom-shaped multicellular structures (Fig. 2A), whereas the *P. aeruginosa pilA*/wild-type mixture formed biofilms that contained mushroom-shaped structures with wild-type bacteria in the cap and *pilA* mutants in the stalk (Fig. 2B), and the *P. aeruginosa pvdA* mutant formed flat biofilms without mushroom-shaped structures (Fig. 2C). The biofilm formed by the *P. aeruginosa pvdA* mutant lacks the structural heterogeneity of the wild-type biofilm and the *pilA*/wild-type biofilm. Because the experiments described above had indicated that the *pvdA* gene is expressed specifically in the stalk-portion of the mushroom-shaped structures in heterogeneous *P. aeruginosa* biofilms, we wanted to examine whether the localised *pvdA* expression is important for structural biofilm development. We therefore investigated biofilm formation of a *pilA*/*pvdA* mixture and a *pilApvdA*/wild-type mixture. In the *pilA*/*pvdA* biofilm expression of the *pvdA* gene is blocked by mutation in the cap-formers, whereas in the *pilApvdA*/wild-type biofilm expression of the *pvdA* gene is blocked by mutation in the stalk-formers. The *pilA*/*pvdA* mixture formed biofilms with mushroom-shaped structures containing the *pilA* mutant in the stalk and the *pvdA* mutant in the cap (Fig. 2D), whereas such mushroom-shaped structures were not formed in the *pilApvdA*/wild-type biofilm which contained *pilApvdA* microcolonies that were not capped by the wild-type (Fig 2E). Although we searched extensively in numerous *pilApvdA*/wild-type biofilms we did not find mushroom-shaped structures formed by the wild-type alone, suggesting that formation of such structures is inhibited in the *pilApvdA*/wild-type biofilms. Complementation with a plasmid-borne *pvdA* gene restored the ability of the *pilApvdA* mutant to form stalks capped with the wild-type in mixed biofilms (Fig. S2). Altogether these results indicate that expression of the *pvdA* gene in the stalk is necessary for the cap to be formed.

The cell surface receptor proteins FpvA and FpvB are needed for uptake of ferric-pyoverdine complexes by *P. aeruginosa* (Ghysels *et al*., 2004; Poole *et al*., 1993). We investigated *pilA*/*fpvA* biofilms, *pilA*/*fpvB* biofilms and *pilA*/*fpvAB* biofilms, and found that the *fpvA* mutant (Fig. 2F) and *fpvAB* mutant (Fig. S3A) were unable to form normal caps on top of the *pilA* mutant, whereas the *fpvB* mutant (Fig. S3B) did form normal caps on the *pilA* stalks. Together the experiments indicate that the cap-forming subpopulation in *P. aeruginosa* biofilms is dependent on FpvA-mediated uptake of ferric-pyoverdine produced by the stalk-forming subpopulation. In addition, the experiments suggest that FpvB does not play a role in ferric-pyoverdine uptake in our *P. aeruginosa* biofilms. In agreement with these findings, Banin *et al*. (2005) reported that a *P. aeruginosa fpvA* mutant, unlike the wild-type but similar to a *pvdA* mutant, formed flat biofilms in flow-chambers, suggesting that FpvB does not contribute significantly to ferric-pyoverdine uptake in biofilms.

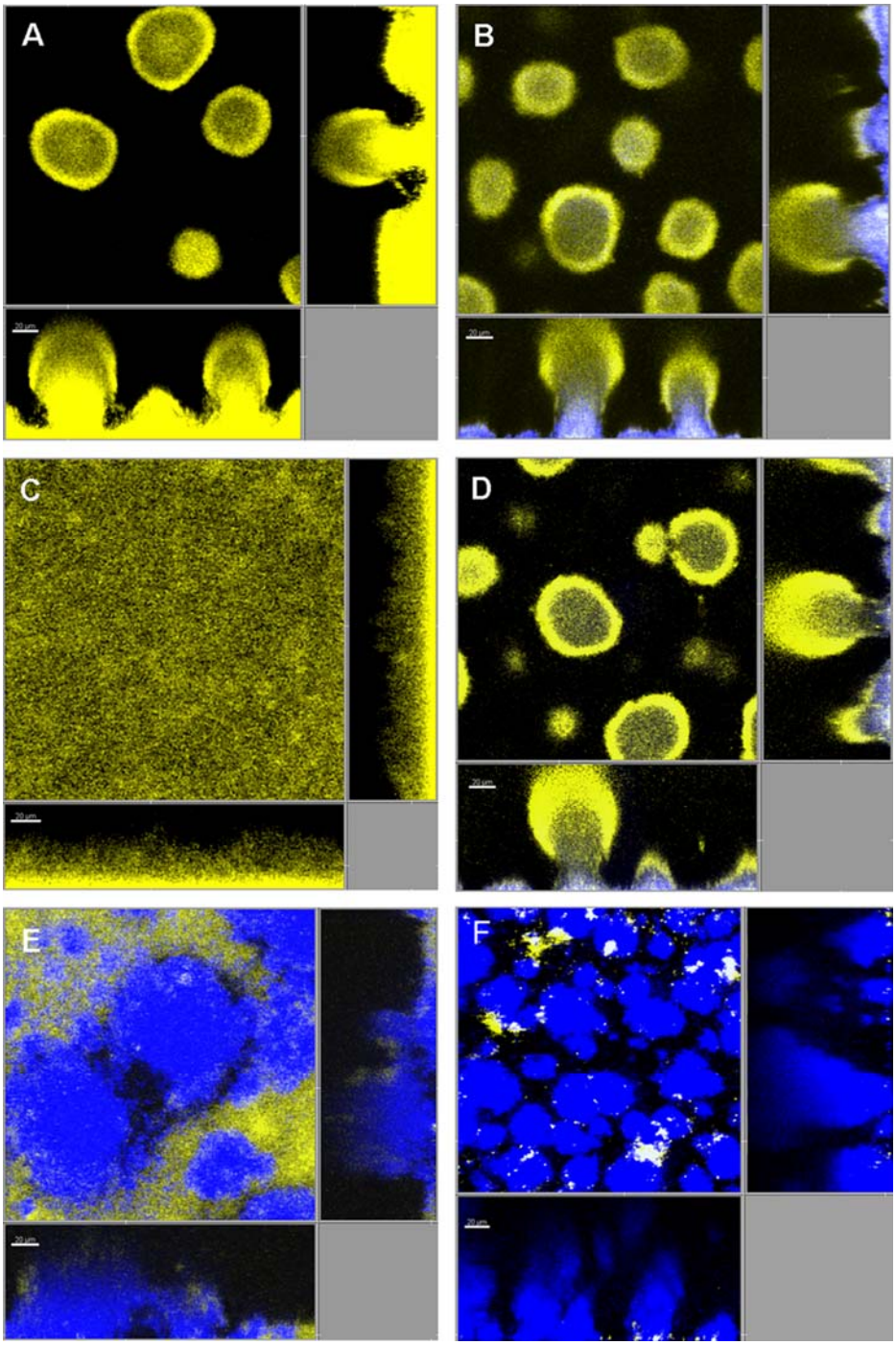

Fig. 2. 4-day-old wild-type biofilm (A), *pilA*/wild-type biofilm (B), *pvdA* biofilm (C), *pilA*/*pvdA* biofilm (D), *pilApvdA*/wild-type biofilm (E) and *pilA*/*fpvA* biofilm (F). The *pilA* mutant and *pilApvdA* mutant were tagged with cfp (blue), whereas the wild-type, *pvdA* mutant and *fpvA* mutant were tagged with yfp (yellow). The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. The bars are 20 µm.

Complementation of structure development in *P. aeruginosa* biofilms with Fe-citrate.

The experiments described above suggested that the wild-type bacteria do not form caps on top of the *pilApvdA* microcolonies in the *pilApvdA*/wild-type biofilm because of a lack of pyoverdine production in the *pilApvdA* microcolonies, whereas the *fpvA* bacteria do not form caps on top of the *pilA* microcolonies in the *pilA*/*fpvA* biofilm because they can not take up the ferric-pyoverdine produced by the *pilA* bacteria. We wanted to investigate how structure development would occur if the bacteria could acquire iron via an alternative pathway. Because *P. aeruginosa* can take up ferric-citrate independently of the pyoverdine system (e.g. see Banin *et al*., 2005) we used this compound as an alternative iron source. As shown in Fig. 3, cyan-stalk/yellow-cap mushroom-shaped structures were formed in *pilApvdA*/wild-type and *pilA*/*fpvA* biofilms when they were grown in medium supplemented with 5 μM ferric-citrate. These experiments support the conclusion that in the normal biofilm medium the wild-type does not form caps on top of *pilApvdA* microcolonies because they do not produce ferric-pyoverdine, whereas the *fpvA* mutant does not form caps on top of the *pilA* microcolonies because it can not take up ferric-pyoverdine. However, the experiments also indicate that the suggested pyoverdine-mediated metabolic interaction is not important *per se* for structure formation in the biofilms. Because we previously have provided evidence that extracellular DNA produced at distinct locations plays a key role in promoting association between the stalk-forming and cap-forming subpopulations in *P. aeruginosa* biofilms (Allesen-Holm *et al*., 2006; Barken *et al*., 2008; Yang *et al*., 2007), we subsequently investigated the role of extracellular DNA in biofilm structure formation.

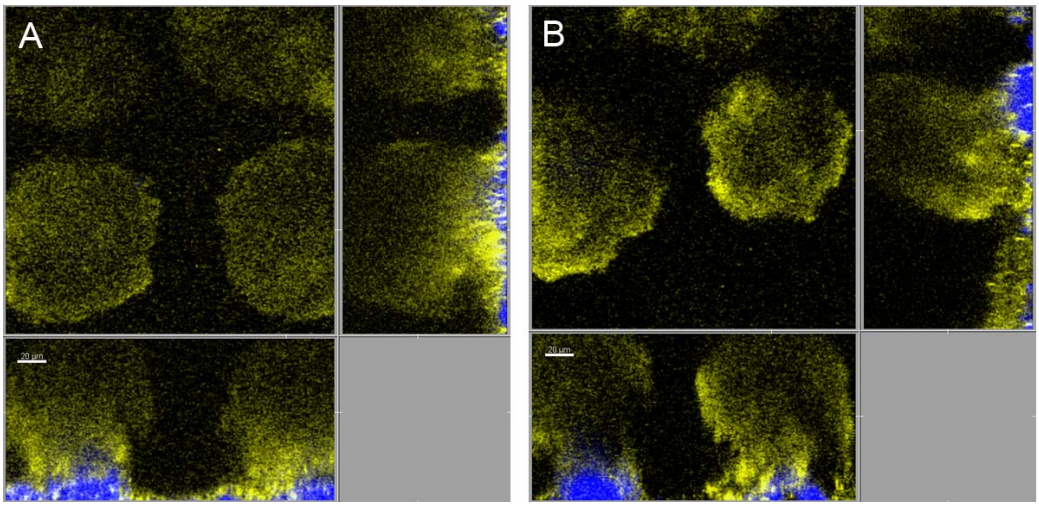

Fig. 3. 4-day-old *pilApvdA*/wild-type biofilm (A) and *pilA*/*fpvA* biofilm (B) grown in FAB medium supplemented with 5 μM Fe-citrate. The *pilApvdA* and *pilA* mutants were tagged with cfp (blue) and the wild-type and *fpvA* mutant were tagged with yfp (yellow). The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. The bars are 20 μm.

Pqs-mediated structure development in *P. aeruginosa* biofilms.

We have previously shown that PQS expression is involved in DNA release in *P. aeruginosa* biofilms, and that expression of the

pqsA::gfp(ASV) reporter occurs specifically in the microcolonies in the early phase of biofilm development (Allesen-Holm *et al*., 2006; Yang *et al*., 2007). Here, we monitored *pqsA::gfp(ASV)* expression in *pilA*/wild-type biofilms with the *pqsA::gfp(ASV)* reporter either in the *pilA* mutant or in the wild-type. Expression of the green fluorescent Gfp(ASV) protein was recorded in these biofilms after 2 days of cultivation since the *pqsA::ASV(gfp)* reporter was found to be expressed at a maximum level in 2-day-old biofilms (Yang *et al*., 2007). The 2-day-old microcolonies, consisting of a stalk of *pilA* bacteria covered with a small cap of wild-type bacteria, showed *pqsA::gfp(ASV)* expression when the reporter was present in the *pilA* mutant (Fig. 4A and 4C) but not when it was present in the wild-type (Fig. 4B and 4D), suggesting that the *pqsA* gene is expressed specifically in the stalk-forming subpopulation.

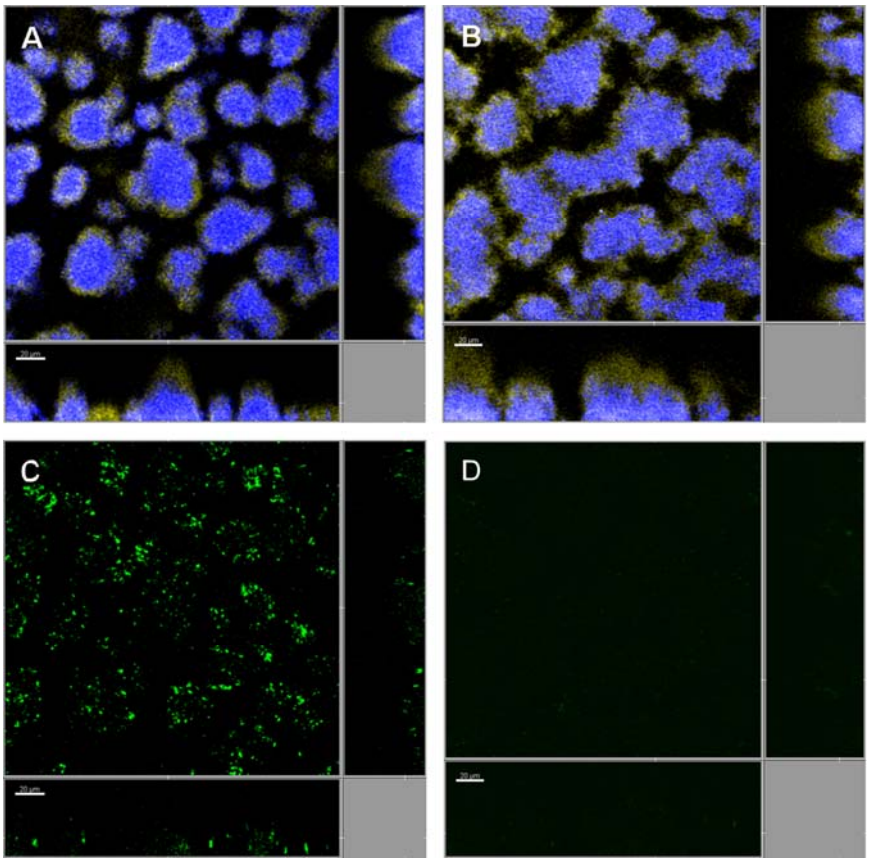

Fig. 4. 2-day-old *pilA*/wild-type biofilms with the *pqsA::gfp(ASV)* reporter in the *pilA* mutant (A, C) or in the wild-type (B, D). The *pilA* mutant was tagged with cfp (blue) and the wild-type was stained with cyto62 (converted to yellow). A and B visualize the cells, whereas C and D visualize *pqsA::gfp(ASV)* fluorescence. The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. The bars are 20 μm.

Subsequently, we investigated the requirement for *pqsA* expression in *P. aeruginosa* biofilm development. The *P. aeruginosa pqsA* mutant formed flat biofilms without mushroom-shaped structures (Fig. 5A), lacking the heterogeneity of the wild-type biofilm (Fig. 2A) and the *pilA*/wild-type biofilm (Fig. 2B). To examine a role of localised *pqs* expression in the

development of the heterogeneous *P. aeruginosa* biofilms, we investigated biofilm formation of a *pilA*/*pqsA* mixture and a *pilApqsA*/wild-type mixture. The *pilA*/*pqsA* mixture formed biofilms with mushroom-shaped structures containing the *pilA* mutant in the stalk and the *pqsA* mutant in the cap (Fig. 5B). The *pilApqsA*/wild-type mixture formed biofilms where the wild-type and *pilApqsA* cells were unassociated, so that the wild-type formed mushroom-shaped structures independent of the *pilApqsA* colonies (Fig. 5C). Complementation with a plasmid-borne *pqsA* gene restored the ability of the *pilApqsA* mutant to form stalks capped with the wild-type in mixed biofilms (Fig. S4). These results indicate that expression of the *pqsA* gene in the stalk is necessary for the cap to be formed.

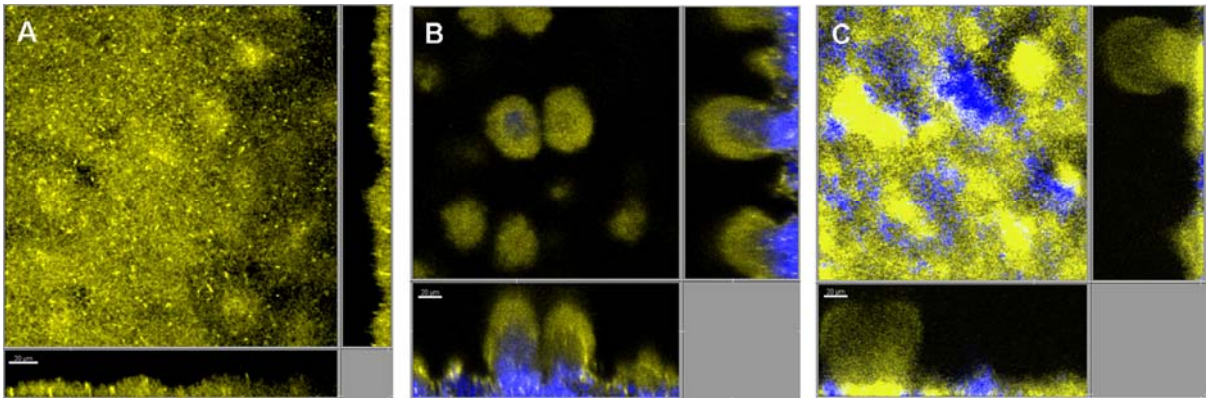

Fig. 5. 4-day-old *pqsA* biofilm (A), *pilA*/*pqsA* biofilm (B), and *pilApqsA*/wild-type biofilm (C). The *pilA* mutant and *pilApqsA* mutant were tagged with cfp (blue), whereas the wild-type and *pqsA* mutant were tagged with yfp (yellow). The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. The bars are 20 μm.

Complementation of structure development in *P. aeruginosa* biofilms with extracellular DNA.

Based on our present and previous findings (Allesen-Holm *et al*., 2006; Barken *et al*., 2008; Yang *et al*., 2007) we suggest that the wild-type bacteria do not form caps on top of the *pilApqsA* microcolonies in the *pilApqsA*/wild-type biofilm because the *pilApqsA* microcolonies do not produce extracellular DNA. In analogy with the experiments with complementation of the *pilApvdA*/wild-type and *pilA*/*fpvA* biofilms with ferric-citrate, we wanted to investigate if formation of cyan-stalk/yellow-cap mushroom-shaped structures would occur in *pilApqsA*/wild-type biofilm if it was supplemented with *P. aeruginosa* genomic DNA. We supplemented the medium irrigated to a *pilApqsA*/wild-type biofilm with DNA at day 2 where *pqs*-dependent DNA-release is known to occur (Allesen-Holm *et al*., 2006; Yang *et al*., 2007). Staining of DNA-supplemented biofilms with propidium iodide showed that the extracellular DNA associated with and covered the microcolonies (data not shown). As shown in Fig. 6A, cyan-stalk/yellow-cap mushroom-shaped structures were formed in the DNA-supplemented *pilApqsA*/wild-type biofilm. This result supports the conclusion that cyan-stalk/yellow-cap mushroom-shaped structures are normally not formed in the *pilApqsA*/wild-type biofilms due to a lack of extracellular DNA on the *pilApqsA* microcolonies, and it constitutes further evidence that extracellular DNA is an important factor in structure formation in our *P. aeruginosa* biofilms. Addition of DNA to *P. aeruginosa* wild-type biofilms led to the formation of huge mushroom shaped structures

much bigger than those found in wild-type biofilms without DNA addition (Fig. 6B). On the contrary, addition of DNA to *P. aeruginosa pilA* biofilms did not result in changed structure compared to *pilA* biofilms without addition of DNA (Fig. 6C), suggesting that extracellular DNA-promoted cap-formation requires type IV pili on the cap-forming bacteria.

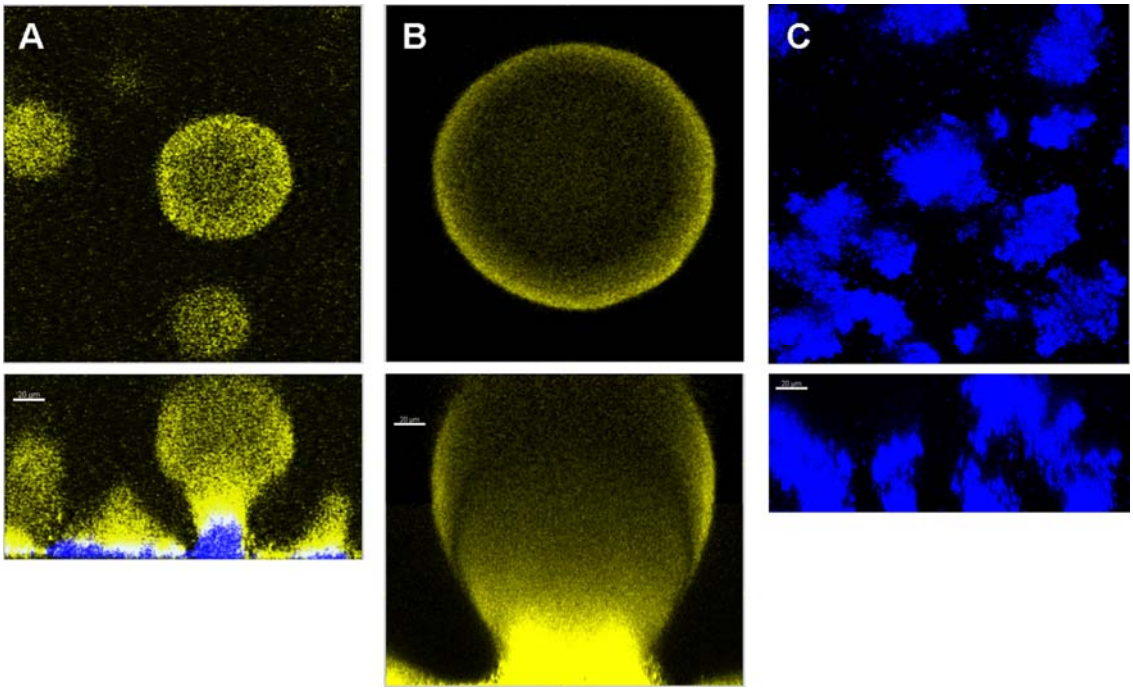

Fig. 6. 4-day-old *pilApqsA*/wild-type biofilm (A), wild-type biofilm (B), and *pilA* biofilm (C) grown in FAB medium supplemented with *P. aeruginosa* genomic DNA at day 2. The *pilApqsA* and *pilA* mutants were tagged with cfp (blue) and the wild-type was tagged with yfp (yellow). The upper pictures show horizontal CLSM optical sections, and the lower pictures show vertical CLSM optical sections. The bars are 20 μm.

Inhibition of structure development in *P. aeruginosa* biofilms.

The finding that pyoverdine and PQS production evidently are involved in mushroom structure formation in our *P. aeruginosa* biofilms, suggested that it should be possible to inhibit structure development by inhibiting pyoverdine and PQS production via chemical treatment. High concentrations of iron have previously been shown to inhibit both pyoverdine production (Ochsner *et al*., 2002) and PQS production (Bredenbuch *et al*., 2006; Yang *et al*., 2007), and it has been reported that high iron concentrations could perturb *P. aeruginosa* biofilm formation and structure development (Musk *et al*., 2005; Musk and Hergenrother, 2008; Yang *et al*., 2007). Experiments with *P. aeruginosa* microtiter tray cultures showed that addition of 100 μM $FeCl_3$ repressed expression of the *pvdA::gfp* and *pqsA::gfp* reporters without inhibiting growth of the *P. aeruginosa* bacteria (Fig. S5). In agreement with the suggestion that pyoverdine and PQS production are involved in mushroom structure formation in our *P. aeruginosa* biofilms, the *pilA*/wild-type biofilm, *pilA*/*pvdA* biofilm and *pilA*/*pqsA* biofilm all formed un-associated microcolonies in flow-chambers

irrigated with medium containing 100 μM $FeCl_3$ (Fig. 7).

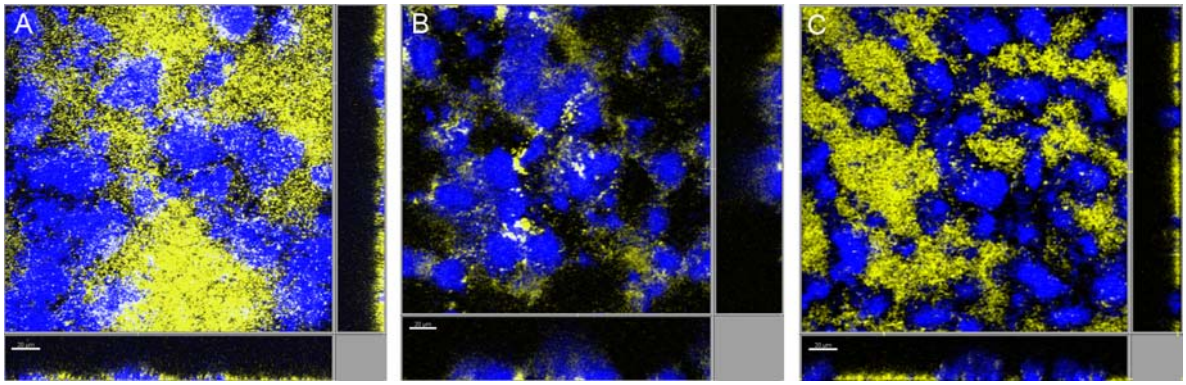

Fig. 7. 4-day-old *pilA*/wild-type biofilm (A), *pilA*/*pvdA* biofilm (B), and *pilA*/*pqsA* biofilm grown in FAB medium supplemented with 100 μM $FeCl_3$. The *pilA* mutant was tagged with cfp (blue) and the wild-type, *pvdA* and *pqsA* mutant were tagged with yfp (yellow). The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. The bars are 20 μm.

Discussion

In the present report we provide evidence that subpopulation interactions are involved in the development of mushroom-shaped multicellular structures in *P. aeruginosa* biofilms. Our results indicate that: i) synthesis of the iron-siderophore pyoverdin in the stalk-portion of the mushroom-shaped structures is necessary for development of the cap-forming subpopulation which does not express the pyoverdin syntheses genes but needs to express the *fpvA* gene encoding part of the ferric-pyoverdin uptake system; and ii) quorum-sensing-controlled DNA-release in the stalk-portion of the mushroom-shaped structures is necessary for development of the cap-forming subpopulation which does not express the quorum-sensing genes.

Our experiments suggest that the pyoverdine mediated interaction is not important for structure formation *per se*, but is a metabolic interaction where stalk cells provide Fe-pyoverdine to cap-forming cells. On the contrary, the available evidence suggests that the extracellular DNA produced by the stalk subpopulation is a critical component in structure formation. We suggest that the PQS mediated interaction plays a role in locating the cap-forming cells on the microcolonies, whereas the pyoverdin mediated interaction feeds the cap cells with Fe-pyoverdine so that they can form the cap. As an alternative to promoting growth of the cap-forming cells by supplying them with iron, the Fe-pyoverdin produced by the stalk cells may be required for settling the migrating bacteria on the cap, since iron limitation has been shown to induce twitching motility (Singh *et al*., 2002).

The present study, and our previous studies (e.g. Allesen-Holm *et al*., 2006; Yang *et al*., 2007; Barken *et al*., 2008), have shown that the microcolonies formed by stalk cells usually have much higher cell density than the structures formed by the cap-forming cells (e.g. see Fig. 2A). Expression of the Pvd system most likely occurs specifically in the stalk forming cells due to iron limitation arising in these dense cell populations. However, presently we can not explain why the cap-forming cells, that evidently need an iron source to form the cap, apparently do not produce

pyoverdine themselves. Expression of the quorum-sensing controlled Pqs system most likely also occurs specifically in the stalk subpopulation due to its high cell density. In agreement with our findings, de Kievit *et al*. (2001) provided evidence that the Las and Rhl quorum-sensing systems were maximally expressed in the portion of cells close to the substratum in *P. aeruginosa* biofilms.

The inability of the *P. aeruginosa* wild-type to form mushroom shaped structures on its own in the *pilApvdA*/wild-type biofilm may be because the *pilApvdA* bacteria do not produce pyoverdin but take up the Fe-pyoverdin produced by the wild-type bacteria. The wild-type bacteria that differentiate to become pyoverdin-producing stalk cells therefore can not deliver Fe-pyoverdin to potential cap-formers. In support of this suggestion, complementation with a plasmid-borne *pvdA* gene in the *pilApvdA* mutant not only restored the ability of the *pilApvdA* mutant to form stalks capped with the wild-type, but also restored the ability of the wild-type to form mushroom-shaped structures on its own in the *pilApvdA*(pPvdA)/wild-type biofilm (see Fig. S2). The finding that the *P. aeruginosa* wild-type does form mushroom-shaped structures on its own in the *pilApqsA*/wild-type biofilm may be because the extracellular DNA, unlike pyoverdin, does not diffuse but stays on the producing microcolonies. Even if the extracellular DNA can diffuse to some extent in biofilms, the piliated wild-type bacteria are expected to bind more efficiently to extracellular DNA than the non-piliated *pilApqsA* mutants, because type IV pili have been shown to bind with high affinity to DNA (Aas *et al*., 2002; van Schaik *et al*., 2005).

The finding that the inability of the *pilApqsA*/wild-type mixture to form blue-stalk/yellow-cap mushroom shaped structures could be complemented by addition of *P. aeruginosa* genomic DNA supports our previous findings that the *pqs* genes play a role in the generation of extracellular DNA in *P. aeruginosa* biofilms (Allesen-Holm *et al*., 2006; Yang *et al*., 2007), and constitutes additional evidence that this matrix component is important for structure formation in our *P. aeruginosa* biofilms. Addition of DNA to *P. aeruginosa* wild-type biofilms led to the formation of huge mushroom shaped structures much bigger than those found in wild-type biofilms without DNA addition. Because the biofilm biomass is determined by the opposing effects of bacterial growth and detachment, we believe that addition of DNA to the wild-type biofilm resulted in decreased detachment of the cap-forming piliated bacteria. Addition of DNA to *P. aeruginosa pilA* biofilms did not result in changed structure compared to *pilA* biofilms without addition of DNA, suggesting that DNA-promoted cap-formation requires type IV pili on the cap-forming bacteria.

The involvement of biosurfactant production in the formation of the mushroom-shaped structures in *P. aeruginosa* biofilms (Davey *et al*., 2003; Pamp and Tolker-Nielsen, 2007) may be another example of a role of subpopulation interactions in the development of structured biofilms. A *P. aeruginosa rhlA* mutant, which is deficient in biosurfactant production, was shown to form a flat biofilm under conditions similar to those used in the present study (Davey *et al*., 2003; Pamp and Tolker-Nielsen, 2007). Furthermore, a study which employed fluorescent reporter-genes indicated that biosurfactant production preferentially takes place in the stalk-portion of the mushroom-shaped structures in *P. aeruginosa* biofilms (Lequette and Greenberg, 2005). It appears, therefore, that formation of the heterogeneous *P. aeruginosa* biofilm with mushroom-shaped structures is dependent on biosurfactant production by the stalk-forming subpopulation.

Studies of flow-chamber-grown *P. aeruginosa* biofilms have provided evidence that the antibiotics tobramycin, ciprofloxacin, and tetracycline preferentially kill bacteria located in the cap-portion of the mushroom-shaped structures, whereas the antibiotic colistin, the

detergent SDS, and the chelator EDTA preferentially kill bacteria located in the stalk-portion of the mushroom-shaped structures (Bjarnsholt *et al*. 2005; Banin *et al*., 2006; Haagensen *et al*., 2007; Yang *et al*., 2007; Pamp *et al*., 2008). *P. aeruginosa* biofilms that were grown in the presence of high levels of iron or quorum-sensing inhibitors developed more flat biofilms than the untreated control biofilms, were much more sensitive to various antimicrobials including tobramycin, ciprofloxacin, and EDTA, and did not show the characteristic spatial distributions of tolerant and sensitive bacteria (e.g. Bjarnsholt *et al*. 2005, Yang *et al*., 2007). The existence of physiologically distinct subpopulations in heterogeneous biofilms therefore appears to function as an insurance mechanism against environmental insult, and the mechanisms involved in their formation and maintenance may be relevant as biofilm control targets.

Experimental procedures

Bacteria and growth conditions.

P. aeruginosa PAO1 (Holloway and Morgan, 1986) was used as the model organism in this study. The *pilA* and *pilApqsA* mutants were constructed by allelic displacement in the PAO1 wild-type and a *pqsA* mutant (D'Argenio *et al*., 2002) as described (Klausen *et al*., 2003b). The *pvdA*, *pilApvdA, fpvA, fpvB,* and *fpvAB* mutants were constructed by allelic displacement as described below. Fluorescence-tagged strains were constructed by insertion of a mini-Tn*7*-eCFP-Gm^r or mini-Tn*7*-eYFP-Sm^r cassette as described (Klausen *et al*., 2003b, Koch *et al*., 2001). *Escherichia coli* strains MT102, DH5a and S17-1 were used for standard DNA manipulations. LB medium (Bertani, 1951) was used to cultivate *E. coli* strains. *P. aeruginosa* biofilms were cultivated at 30^{o}C in flow-chambers irrigated with FAB medium (Heydorn *et al*., 2000) supplemented with 0.3 mM glucose. FAB medium contains approximately 1 μM ferric sulphate, but FAB medium supplemented with 5 μM ferric citrate or 100 μM ferric chloride was used where indicated in the text. Selective media were supplemented with ampicillin (Ap; 100mg/liter), gentamicin (Gm; 60mg/liter), streptomycin (Sm; 100mg/liter), or tetracycline (Tc; 60mg/liter). Cyto62 was used at a concentration of 1 μM to stain untagged cells in biofilms where three fluorescent colours (Yfp, Gfp, and cyto62) were needed.

Construction of *P. aeruginosa pvdA, pilApvdA, fpvA, fpvB,* and *fpvAB* mutants.

The *P. aeruginosa pvdA*, *pilApvdA, fpvA, fpvB,* and *fpvAB* mutants were constructed as follows: Knockout fragments containing a gentamicin (Gm) resistance cassette were generated by PCR overlap extension essentially as described by Choi and Schweizer (2005). Primers PvdA-UpF-GW, PvdA-UpR-Gm, PvdA-DnF-Gm, PvdA-DnR-GW, FpvA-UpF-GW, FpvA-UpR-Gm, FpvA-DnF-Gm, FpvA-DnR-GW, FpvB-UpF-GW, FpvB-UpR-Gm, FpvB-DnF-Gm, and FpvB-DnR-GW were used to amplify chromosomal regions of *pvdA*, *fpvA* and *fpvB*, and primer Gm-F and Gm-R were used to amplify a gentamicin resistance cassette with plasmid pPS856 (Hoang *et al*., 1998) as template. The PCR fragments were fused together and amplified with primers GW-attB1 and GW-attB2 incorporating the *att*B1 and *att*B2 recombination sites at either end of the knockout cassette. Using the Gateway cloning system (Invitrogen) the resulting knockout fragments were first transferred by the BP reaction into pDONR221 generating entry plasmids pDONR211pvdA, pDONR211fpvA, and pDONR211fpvB, and subsequently transferred by the LR reaction into pEX18ApGW generating the knockout plasmids pEX18AppvdA, pEX18ApfpvA, and pEX18ApfpvB. The knockout plasmids were transferred into the PAO1 wild-type and *pilA* mutant by two-parental mating using the donor strain *E. coli* S17-1 with selection done on AB-citrate plates supplemented with gentamicin. Resolution of single cross-over events was achieved by streaking on 5% sucrose plates via

the counter-selectable *sacB* marker on the knockout plasmid. In order to construct the *fpvAB* double mutant, Flp-mediated excision of the Gm resistance cassette in the *fpvA* mutant was performed using the pFLP2 plasmid as described by Choi and Schweizer (2005). The resulting double crossovers in the mutants were confirmed by PCR. Sequences of the primers will be supplied upon request.

Construction of a *pvdA* complementation plasmid.

A full length *pvdA* fragment was PCR amplified by use of the primers pvdAF and pvdAR. The PCR product was digested with *Eco*RI and *Kpn*I and then ligated into *Eco*RI/*Kpn*I digested pME6031 vector (Heeb *et al*., 2000). The ligation product was electroporated into the *P. aeruginosa pilApvdA* strain. Transformants were selected on LB plates containing 60 µg/ml tetracycline and later confirmed by checking the pyoverdine production as described (Meyer *et al*., 1998). Sequences of the primers will be supplied upon request.

Construction of a *pqsA* complementation plasmid.

A full length *pqsA* fragment was PCR amplified by use of the primers pqsAF and pqsAR. The PCR product was digested with *BamH*I and *Xba*I and then ligated into *BamH*I/*Xba*I digested pUCP22Not vector (Herrero *et al*., 1990). The ligation product was electroporated into the *P. aeruginosa pilApqsA* strain. Transformants were selected on LB plates containing 60 µg/ml gentamicin and later confirmed by checking the PQS production as described (Calfee *et al*., 2001). Sequences of the primers will be supplied upon request.

Cultivation of biofilms in flow-chambers.

Biofilms were grown in flow-chambers with individual channel dimensions of 1 x 4 x 40 mm. The flow system was assembled and prepared as described previously (Sternberg and Tolker-Nielsen, 2006). The flow-chambers were inoculated by injecting 350 µl of overnight culture diluted to an OD_{600} of 0.001 into each flow channel using a small syringe. After inoculation, the flow channels were left without flow for 1 h, after which medium flow was started using a Watson Marlow 205S peristaltic pump. The mean flow velocity in the flow-chambers was 0.2 mm/s, corresponding to laminar flow with a Reynolds number of 0.02. In one experiment the biofilm medium was supplemented with 0.25 µg/ml *P. aeruginosa* DNA after 48 hours of biofilm cultivation.

Measurements of *pvdA::gfp(L)* expression in flow-chamber biofilms.

For observing *pvdA::gfp(L)* expression in flow-chamber biofilms, we transformed the *P. aeruginosa* wild-type and *P. aeruginosa pilA* mutant with the *pvdA::gfp(L)* reporter plasmid (Kaneko *et al*., 2007). Expression of *pvdA::gfp(L)* was subsequently recorded via CLSM in 4-day-old *pilA*/wild-type mixed biofilms containing the reporter fusion in either the wild-type or the *pilA* mutant.

Measurements of *pqsA::ASV(gfp)* expression in flow-chamber biofilms.

For observing *pqsA::gfp(ASV)* expression in flow-chamber biofilms, we transformed the *P. aeruginosa* wild-type and *P. aeruginosa pilA* mutant with the *pqsA::ASV(gfp)* reporter plasmid (Yang *et al*., 2007). Expression of *pqsA::gfp(ASV)* was subsequently recorded via CLSM in 2-day-old *pilA*/wild-type two-strain biofilms containing the reporter fusion in either the wild-type or the *pilA* mutant.

Measurements of *pvdA::gfp(L)* and *pqsA::ASV(gfp)* expression in microtiter tray cultures.

Cultures in wells of polystyrene microtitre trays (150 µl per well) were started by diluting *P. aeruginosa*(*pvdA::gfp*) or *P. aeruginosa*(*pqsA::gfp*) overnight cultures to

OD_{600} = 0.001 in AB medium supplemented with 0.05% glucose and 10 μM $FeCl_3$ or 100 μM $FeCl_3$. The cultures were incubated for 24 hours at 37°C, and Gfp-fluorescence and cell density were measured regularly by the use of a Wallac microplate reader.

Microscopy and image acquisition.

All microscopy observations and image acquisitions were done with a Zeiss LSM510 confocal laser scanning microscope (CLSM) (Carl Zeiss, Jena, Germany) equipped with detectors and filter sets for monitoring of Gfp, Cfp, Yfp, and cyto62 fluorescence. Images were obtained using a 63x/1.4 objective or a 40 x/1.3 objective. Simulated 3-D images and sections were generated using the IMARIS software package (Bitplane AG, Zürich, Switzerland).

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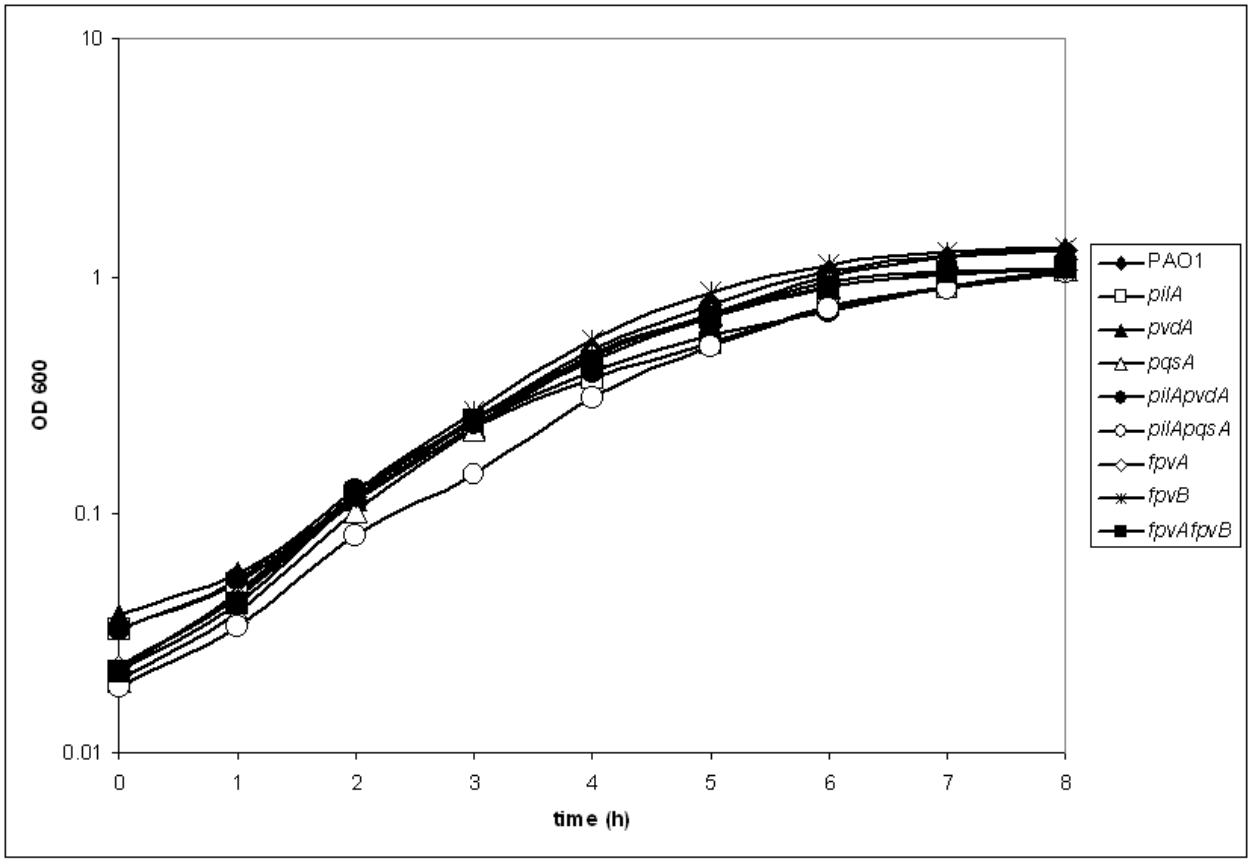
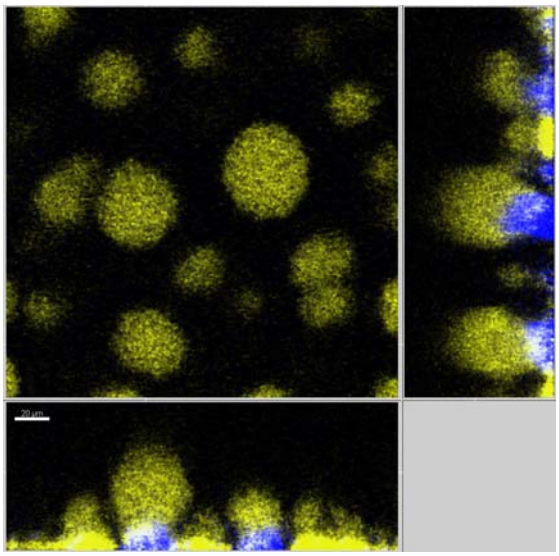
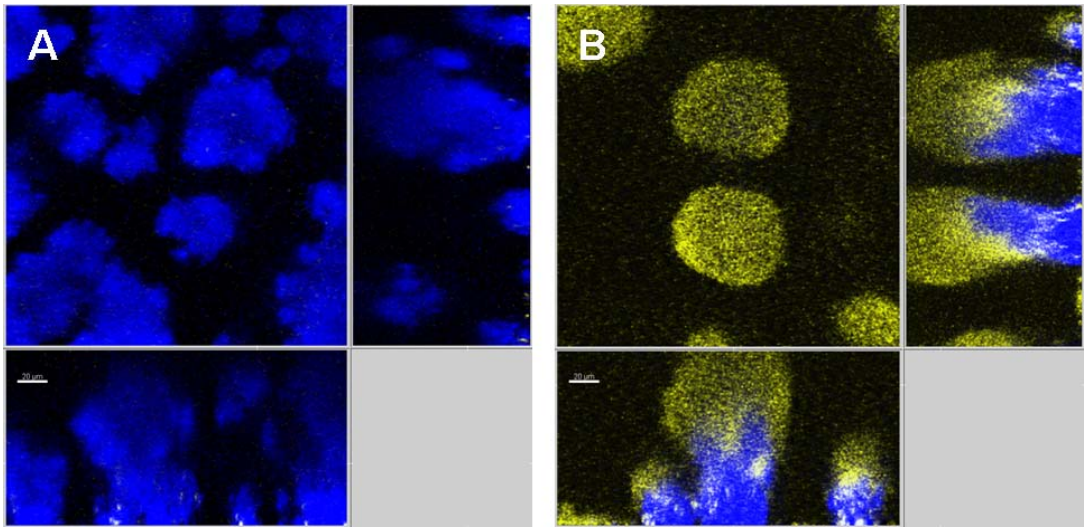
Supplementary figures



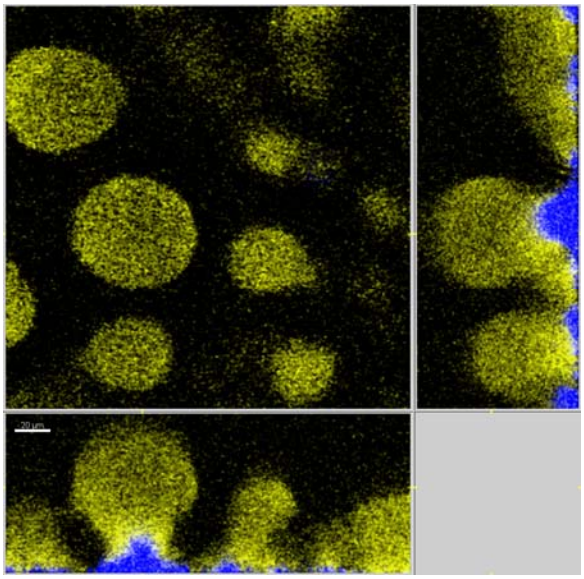
S1. Growth curves of *P. aeruginosa* strains in shake flasks containing biofilm medium at 37 degree.



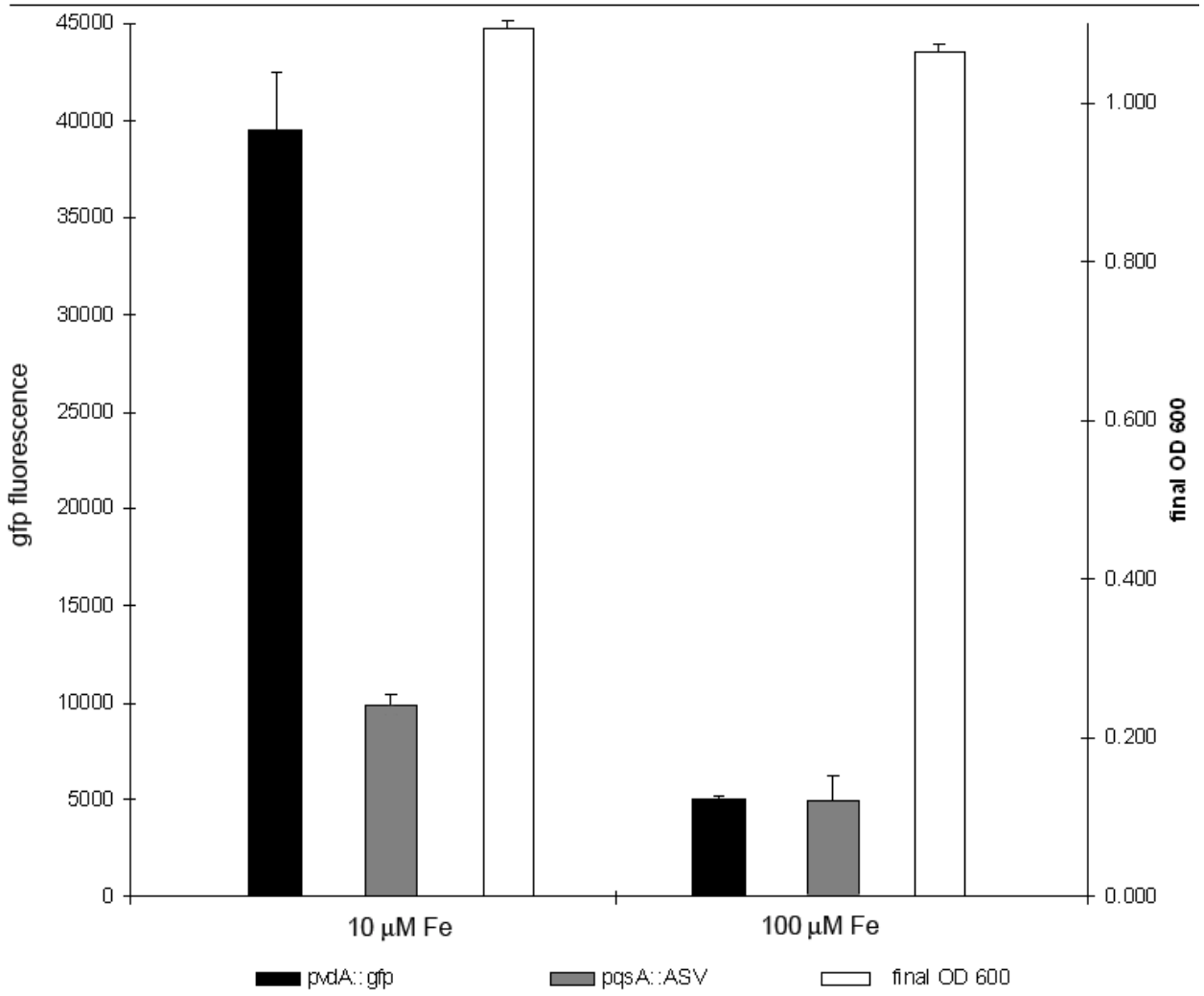
S2. 4-day-old *pilApvdA(pME6031::pvdA)*/wild-type biofilms. The *pilApvdA(pME6031::pvdA)* mutant was tagged with cfp (blue) and the wild-type was tagged with Yfp (yellow). The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. The bars are 20 µm.



S3. 4-day-old *pilA*/*fpvAB* biofilm (A) and *pilA*/*fpvB* (B) biofilm. The *pilA* mutant was tagged with cfp (blue), whereas the *fpvAB* mutant and *fpvB* mutant were tagged with yfp (yellow). The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. The bars are 20 µm.



S4. 4-day-old *pilApqsA(pUCP22::pqsA)*/wild-type biofilms. The *pilApqsA(pUCP22::pqsA)* mutant was tagged with cfp (blue) and the wild-type was tagged with Yfp (yellow). The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. The bars are 20 µm.



S5. Expression of *pvdA::gfp* and *pqsA::gfp (ASV)* fusion reporters in minimal glucose medium containing 10 µM iron and 100 µM iron. Final OD 600 was also reordered as an indication of growth.

Paper IV

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Computer-Aided Identification of Recognized Drugs as *Pseudomonas aeruginosa* Quorum-Sensing Inhibitors▽†

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Attenuation of *Pseudomonas aeruginosa* virulence by the use of small-molecule quorum-sensing inhibitors (referred to as the antipathogenic drug principle) is likely to play a role in future treatment strategies for chronic infections. In this study, structure-based virtual screening was used in a search for putative quorum-sensing inhibitors from a database comprising approved drugs and natural compounds. The database was built from compounds which showed structural similarities to previously reported quorum-sensing inhibitors, the ligand of the *P. aeruginosa* quorum-sensing receptor LasR, and a quorum-sensing receptor agonist. Six top-ranking compounds, all recognized drugs, were identified and tested for quorum-sensing-inhibitory activity. Three compounds, salicylic acid, nifuroxazide, and chlorzoxazone, showed significant inhibition of quorum-sensing-regulated gene expression and related phenotypes in a dose-dependent manner. These results suggest that the identified compounds have the potential to be used as antipathogenic drugs. Furthermore, the results indicate that structure-based virtual screening is an efficient tool in the search for novel compounds to combat bacterial infections.

One of the major problems in the treatment of infectious diseases is the occurrence of antibiotic resistance, which is prevalent among many bacterial species, in particular, strains of the opportunistic pathogen *Pseudomonas aeruginosa* (39). This bacterium is a common gram-negative species found in nosocomial infections such as urinary tract infections, respiratory system infections, dermatitis, chronic wounds, soft-tissue infections, and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunocompromised (43, 66). Furthermore, people who are suffering from the genetic disease cystic fibrosis are highly susceptible to chronic lung infection with this organism (16).

The resistance of *P. aeruginosa* to multiple antibiotics is the result of a variety of specific mechanisms that includes an inherent β-lactamase and a broad-spectrum efflux pump system. Furthermore, impermeability of the membrane, adaptive mutations, and horizontal transfer of resistance genes also contribute to its resistance (38). Besides these specific resistance mechanisms, the ability of *P. aeruginosa* to grow as structured communities of cells enclosed in a self-produced polymeric matrix, known as a biofilm (11), significantly adds to its tolerance to antimicrobial agents (12). Current antibiotics are susceptible to resistance development, as there will inevitably be selection pressure for bacteria able to grow in the presence of these growth-inhibiting compounds (39). Therefore, new approaches to combat microbes without selecting for resistance would hold great promise for the treatment of infectious diseases. An approach that does not target bacterial growth is the use of antipathogenic drugs that work by decreasing bacterial virulence and rendering bacteria incapable of establishing infection. An area where the use of antipathogenic drugs has received much attention is virulence attenuation by blocking bacterial intercellular communication, i.e., quorum sensing (QS) (recently reviewed in reference 52). In gram-negative bacteria, most QS systems are members of the LuxR-LuxI homologous system that use acyl homoserine lactone (AHL) signal molecules. These systems function by means of a LuxR homolog, the transcriptional activator, and a LuxI homolog, the AHL synthase. LuxI produces the required AHL molecule, which diffuses out into the local environment, and upon reaching the required concentration, the signal molecule binds to and activates LuxR, which in turn activates the transcription of the target genes (20, 59, 68). In *P. aeruginosa*, QS is mediated through the LuxRI-type systems LasRI and RhlRI, which sense 3-oxo-C12-homoserine lactone (HSL) and C4-HSL, respectively, and via the Pqs system, which senses Pqs (2-heptyl-3-hydroxy-4-quinolone). The systems are hierarchically arranged, with LasR regulating the Rhl and Pqs systems (35, 60). AHL-mediated QS coordinates the production of virulence factors and plays a role in biofilm formation (4, 15, 45). Furthermore, QS-controlled activities, as well as QS signals, evidently also affect the activities of host immune systems (34, 37, 53, 64). By using various QS inhibition assays and high-throughput screening, small antagonistic molecules displaying some structural similarities to AHL signals have been identified as QS inhibitors (QSIs) and shown to greatly reduce the virulence of *P. aeruginosa* both in vitro and in vivo (29, 42, 50).

Recently, computer-aided drug design, especially structure-

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based virtual screening (SB-VS), has emerged as a new tool in pharmaceutical chemistry (40, 54). The increasing availability of structural data and the affordability of high-performance computing platforms have broadened the applicability of this method (54). SB-VS has been adopted as an effective paradigm for lead discovery that fits in well alongside high-throughput screening programs. Screening has been successfully used to find inhibitors of various enzymes and proteins, including human carbonic anhydrase II, human protein tyrosine phosphatase 1B, and the omnipresent bacterial enzyme DNA gyrase, which in the latter case resulted in novel classes of inhibitors with potential for use as antibiotics (6, 17, 27). In addition to the discovery of novel antibiotics by virtual screening, the approach also holds great promise for the discovery of antipathogenic drugs and especially for the discovery of new QSIs. The structures of TraR from *Agrobacterium tumefaciens* and the ligand binding domain of LasR from *P. aeruginosa* bound to their natural ligands have become available recently and can be exploited in SB-VS (7, 67, 71). The availability of the LasR structure is particularly important, as it can be used to complement the traditional QSI discovery strategies previously mentioned.

In the present study, 147 recognized drugs and natural compounds were selected from the SuperNatural and SuperDrug databases (18, 26) on the basis of their two-dimensional (2D) structural similarity to the *P. aeruginosa* LasR natural ligand, the identified QSIs furanone C30 and patulin, or the QS agonist TP-1. The automated docking program Molegro Virtual Docker (MVD) was used to screen these selected compounds for QSI candidates. Six top-ranking drugs were acquired and tested for biological activity. Three compounds, salicylic acid, nifuroxazide, and chlorzoxazone, showed significant, dose-dependent inhibition of QS-regulated gene expression and related phenotypes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. *P. aeruginosa* PAO1 (33), an isogenic *P. aeruginosa lasR* mutant (29), and an isogenic *P. aeruginosa lasI rhlI* mutant (29) were used in this study. PAO1 with a mini-Tn*5* insert containing a translational fusion of the LasR-regulated *lasB* promoter and a gene coding for an unstable version of green fluorescent protein (GFP), *gfp*(*ASV*), and an extra copy of *lasR* under the influence of the *lac* promoter (constitutively expressed in *P. aeruginosa*) (28) was used for the LasR inhibition assay. For the RhlR inhibition assays, the plasmid pMHRA was introduced by electroporation into *P. aeruginosa* PAO1 and *P. aeruginosa lasR*. pMHRA contains an RlhR-regulated *rhlA*::*gfp*(*ASV*) translational fusion inserted into the vector pMH391 (28). The *P. aeruginosa* PAO1 strain and the *lasR* mutant used for the Pqs inhibition assay carried plasmid pAC37 containing a transcriptional fusion of the Pqs-regulated *pqsA* promoter and *gfp*(*ASV*) (70). Measurement of virulence factor production was carried out with the wild-type PAO1 strain and a QS-deficient PAO1 *lasI rhlI* mutant strain (29). GFP-tagged variants of these two strains were used for flow cell biofilm experiments. Tagging of the strains was done with mini-Tn*7*–enhanced GFP as previously described (36). *Serratia liquefaciens* MG1 (wild type) and an *swrI* (swarming-deficient) mutant (19) were used to assess the effect of the QSIs on the swarming motility of the bacterium.

For the QS inhibition and LasR specificity assays, ABT minimal medium (BT plus 10% A10) supplemented with 0.5% glucose (wt/vol) and 0.5% Casamino Acids (wt/vol) (28) was used to grow the cells. This medium supplemented with 0.6% Bacto agar (BD, NJ) was used in the *S. liquefaciens* swarming assay. Modified Luria-Bertani (3) medium containing 4 g NaCl liter^{-1} was used as a basic medium to grow cells overnight (ON) prior to carrying out the swarming assay and the virulence factor assays and prior to inoculating the flow cell biofilm system. Virulence factor assays were carried out with cultures grown in modified BM2 medium (24) containing 0.5 mM Mg^{2+}, 0.01 mM $FeCl_3$, 0.06 M glucose, and 0.2% (wt/vol) Casamino Acids. Flow cell biofilms were cultivated in FAB minimal medium (30) supplemented with 0.3 mM glucose. Selective media were supplemented with ampicillin (100 mg liter^{-1}), carbenicillin (500 mg liter^{-1}), gentamicin (60 mg liter^{-1}), or streptomycin (100 mg liter^{-1}) where appropriate.

Protein structure file and drug/natural ligand database. The X-ray crystallographic structure of the *P. aeruginosa* LasR ligand binding domain bound to its natural ligand *n*-3-oxododecanoyl-L-homoserine lactone (OdDHL; Protein Data Bank [PDB] identification [ID]: 2UV0) (7) was used in the SB-VS of small molecules. Recognized drugs and natural compounds were selected from the SuperDrug and SuperNatural databases (18, 26) on the basis of their 2D structural similarity to the *P. aeruginosa* LasR ligand OdDHL (45), the identified QSIs furanone C30 and patulin (29, 51), or the LasR agonist TP-1 (42). The structures of the similarity templates are displayed in Fig. 1A. The 2D structures of the selected compounds were downloaded from Chembank (http://chembank.broad.harvard.edu/) (56) or drawn manually in MarvinSketch ver. 4.1.10 (ChemAxon Ltd., Hungary) and then saved as 2D coordinate structure-data file (SDF) files. The SDF files were merged into a single SDF file database by OpenBabel ver. 2.1.1 (OpenEye Scientific Software) and then converted to a three-dimensional (3D) structure database in CORINA ver. 3.4 (Molecular Networks GmbH, Germany).

Molecular docking. The automated docking software MVD ver. 2007.2.2.0 (65) (Molegro Aps., Denmark) was used in the SB-VS to dock the compounds from the created database. The LasR structure file contained four monomers of the ligand binding domain each in a complex with one OdDHL ligand, but only the E monomer in a complex with the ligand named OHN 1169 [E] was chosen for importation into the MVD workspace. Water was excluded from the workspace, and standard preparation of the molecules was used in the importation of the database.

Docking of the database was carried out in the following way. Initially, a template based on the present ligand was created to award poses of the docked compounds that showed similarity to the molecule. The template consisted of four individual criteria: steric, hydrogen donor, hydrogen acceptor, and ring. Subsequently, the actual docking procedure was set up by choosing template docking, the Ligand Evaluator scoring function, and the default search algorithm MolDock Optimizer. The binding site restraining the search space was sphere shaped and centered on the OdDHL ligand with a radius of 15 Å. Docking of each compound was repeated five times to ensure conformation to the lowest-energy state due to the iterative nature of the process. Five poses of each molecule in a complex with the protein were returned, one for each run, and these were finally ranked according to their rerank scores. For the specific settings for the docking process, see Fig. S1 and Table S1 in the supplemental material. In addition to the database of selected compounds, the docking process was supplemented with the LasR natural ligand structure for reference together with the structures of known LasR agonists and antagonists for comparison (Fig. 1A and Table 1). Scenes of molecules in complex with the LasR ligand binding domain were generated with PyMOL ver. 1.00 (DeLano Scientific LLC, San Carlos, CA; http://www.pymol.org) by importing the 3D poses of the docked molecules from MVD together with the structure of the LasR ligand binding domain (PDB ID: 2uV0).

QSI candidates selected for assessment of QS-inhibitory activity. The drugs selected on the basis of the docking results were tested for the ability to inhibit LasR-controlled *lasB*::*gfp* gene expression from *P. aeruginosa*. Salicylic acid, nifuroxazide, and chlorzoxazone were purchased from Sigma-Aldrich in their pure form, while indoramine (Wydora; Riemser Arzneimittel AG), tiaprofenic acid (Surgamyl; Patheon Ltd., United Kingdom), and donepezil (Aricept; Pfizer SA) were acquired as pills. Stock solutions or suspensions of the compounds were made by dissolving or suspending the compounds and pills in 96% ethanol. The pill solutions were filtered to remove the binding material, and sufficient content and purity of the active compounds were verified by high-performance liquid chromatography (HPLC)-mass spectrometry (MS).

QS inhibition assays. The LasR inhibition assay was carried out by growing the *P. aeruginosa lasB*::*gfp* strain in a 96-well microtiter tray (black polystyrene; Nunc) together with serial twofold dilutions of the putative inhibitors as previously described with modifications (50). Briefly, growth medium and inhibitor stock solution were initially added to the first column of wells to give a final inhibitor concentration of 1,600 μg/ml in a volume of 300 μl. A volume of 150 μl of medium was added to each of the rest of the wells. Next, serial twofold dilutions of the inhibitors were made by transferring 150 μl of medium between the wells in a sequential manner. The final two columns of wells were kept without added inhibitor as a reference. Finally, 150 μl of cell culture with an optical density at 450 nm (OD_{450}) of 0.2 was added to the wells to give a final OD_{450} of 0.1 and inhibitor concentrations ranging from 800 μg/ml to 1.5625 μg/ml in a volume of 300 μl. The added culture was prepared from an ON

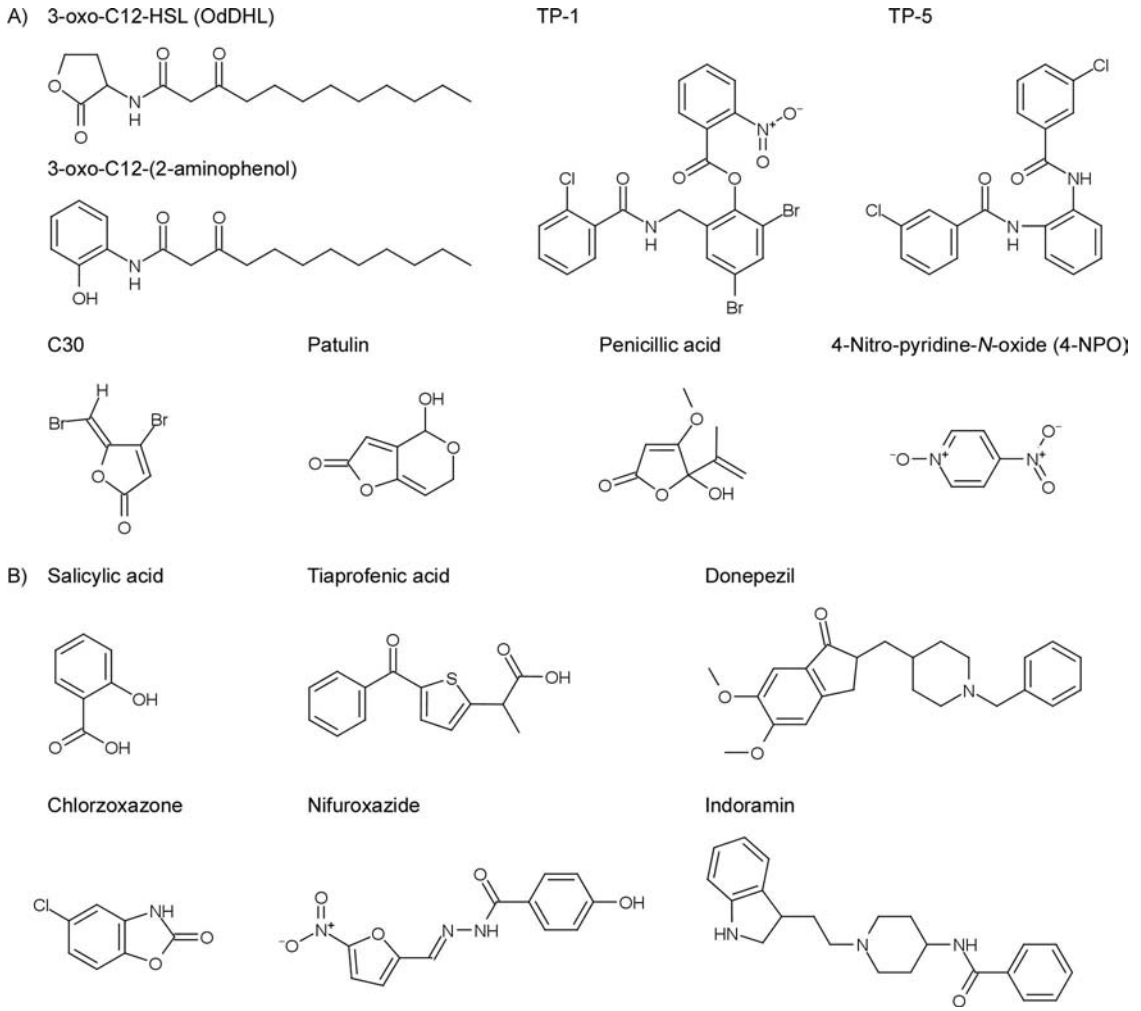

FIG. 1. 2D structures of the compounds tested in this study. (A) Structures of the known LasR binding compounds used in the 2D similarity search against the SuperNatural and SuperDrug databases (OdDHL, TP-1, furanone C30, and patulin) and included in the SB-VS for reference (OdDHL) and comparison (all). (B) Structures of the six compounds tested for the ability to inhibit LasR in *P. aeruginosa*.

culture grown at 30°C by appropriate dilution with growth medium based on the measurement of OD_{450}. The OD of the ON culture was measured with a Thermo Scientific GENESYS 10 UV spectrophotometer. The microtiter tray was incubated at 34°C in a Wallac 1420 VICTOR2 plate reader (Perkin-Elmer, MA), and the instrument was set to measure GFP(ASV) expression by means of the protein's fluorescence at 535 nm upon excitation at 485 nm and the growth in the wells as the OD_{450} every 15 min for at least 14 h. The Rhl and Pqs inhibition assays were carried out in essentially the same way as the LasR inhibition assay. However, instead of using serial twofold dilutions, salicylic acid, nifuroxazide, and chlorzoxazone were added to final concentrations of 200 μg/ml, 200 μg/ml, and 100 μg/ml, respectively.

TABLE 1. Names and types of LasR binding compounds used as references and for comparison in the SB-VS

Compound	Type	Reference
3-Oxo-C12-HSL	Natural ligand	45
3-Oxo-C12-(2-aminophenol)	Inhibitor	62
4-Nitropyridine-*N*-oxide	Inhibitor	50
Furanone C30	Inhibitor	29
Patulin	Inhibitor	51
Penicillic acid	Inhibitor	51
TP-1	Activator	42
TP-5	Inhibitor	42

LasR specificity assay. The LasR specificity assay was carried out in the same way as the Rhl and Pqs inhibition assays. *P. aeruginosa lasR* harboring reporter plasmid pMHRA or pAC37 was grown ON with the required antibiotics to maintain the plasmids, while the assay was carried out without added antibiotics to avoid secondary effects from the possible interaction between the antibiotics and the added inhibitors.

Measurement of virulence factors. *P. aeruginosa* PAO1 and the QS-deficient *lasI rhlI* strain were inoculated in modified BM2 medium to an OD_{600} of 0.01 from ON cultures of the two strains. Inhibitors were added from stock solutions to a final concentration of 200 μg/ml for salicylic acid and nifuroxazide and to a final concentration of 100 μg/ml for chlorzoxazone. After 18 h of growth at 37°C, the OD_{600} of the cultures was measured and the assays were carried out. The OD was measured with a Thermo Scientific GENESYS 10 UV spectrophotometer. Regular BM2 medium was used as a blank sample. Estimation of exogenous proteolytic activity was carried out as described by Hentzer et al. (28), with some modifications. Briefly, 150 μl of culture supernatant, centrifuged at 15,000 × *g* and 4°C for 15 min, was mixed with 250 μl of an azocasein solution (2% [wt/vol] in 2 mM $CaCl_2$ and 40 mM Tris-HCl [pH 7.8]; Sigma-Aldrich) and allowed to react for 45 min at 37°C. Undigested substrate was precipitated with 1.2 ml of trichloroacetic acid (10% [wt/vol]) for 15 min at room temperature and subsequently centrifuged at 15,000 × *g* for 10 min. Nine hundred microliters of the supernatant was transferred to new Eppendorf tubes and mixed with 750 μl of 1 M NaOH. Proteolytic activity was measured as the OD_{440} against a blank sample

run in parallel with the other samples and divided by the OD_{600} of the culture to estimate the relative protease production. Pyoverdine production was measured according to a spectrophotometric method previously described by Höfte et al. (32), with modifications. Briefly, the concentration of pyoverdine in the culture supernatant was measured directly at 400 nm. Because the compound nifuroxazide also absorbed light at 400 nm, a sample containing medium and nifuroxazide was used as a control for measurement of the nifuroxazide-treated replicates. Relative pyoverdine production was estimated as the OD_{400} divided by the OD_{600} of the cultures. Rhamnolipid production was estimated by two modified biosurfactant production assays: a drop collapse assay (5) and an emulsification activity assay (10). For the drop collapse assay, 2-μl spots of mineral oil were added to the lid of a 96-well microtiter plate (Nunc). The lid was equilibrated for 1 h at room temperature, after which 5-μl drops of culture supernatant were added to the surface of the oil spots. The shape of the drops was inspected after 1 min. For the emulsification activity assay, 2 ml of *n*-hexadecane was added to 2 ml of culture supernatant. The mixture was vortexed at high speed for 2 min and allowed to stand for 6 h before inspection. Emulsification activity was defined as the height of the emulsion layer divided by the total height and expressed as a percentage.

Cultivation of biofilms in flow chambers. Biofilms were grown for 4 days in flow chambers with individual channel dimensions of 1 by 4 by 40 mm. The flow system was assembled and prepared as described previously (63). Inoculation of the system was carried out by injecting 300 μl of an ON culture diluted to an OD_{600} of 0.001 into each flow channel with a small syringe. After inoculation, the flow chambers were left upside down without flow for 1 h to allow bacterial attachment to the glass cover, after which medium flow was started with a Watson Marlow 205S peristaltic pump. The flow chambers were irrigated with medium with or without QSIs (salicylic acid at 20 μg ml^{-1}, nifuroxazide at 20 μg ml^{-1}, and chlorzoxazone at 10 μg ml^{-1}). The concentrations of the QSIs were reduced by a factor of 10 compared to those in the batch assays due to the continuous flowthrough of medium. The mean flow velocity in the flow chambers was 0.2 mm s^{-1}, corresponding to laminar flow with a Reynolds number of 0.02. The biofilms were grown at 30°C.

Microscopy and image acquisition. All microscopy observations and image acquisitions were done with a Zeiss LSM510 confocal laser scanning microscope (CLSM) equipped with detectors and filter sets for monitoring of GFP. Images were obtained with a 40×/1.3 objective. Simulated 3D images and sections were generated with the Imaris software package (Bitplane AG).

COMSTAT image analysis. CLSM images were analyzed by use of the computer program COMSTAT (30). Thresholds for the different image stacks were determined automatically, and connected volume filtration was used in the analysis.

***S. liquefaciens* swarming assay.** Swarming plates consisted of ABT minimal medium supplemented with glucose, Casamino Acids, and 0.6% Bacto agar. QSIs were mixed into the medium immediately before casting at concentrations of 200 μg/ml for salicylic acid and nifuroxazide and 100 μg/ml for chlorzoxazone. The plates were left for drying without lids in a fume hood for 1 h at room temperature. Five-microliter drops of 10-times-diluted ON cultures of *S. liquefaciens* MG1 and an *swrI* mutant were placed on the appropriate plates and incubated for 18 h at room temperature before pictures of each plate were taken. The areas of the swarming zones were calculated by use of the free ImageJ software. Measurements were done in triplicate. The viability of *S. liquefaciens* in the presence of the QSI compounds was assessed by monitoring growth at 30°C in a 96-well microtiter tray via OD_{600} measurement. The concentrations of the QSI compounds were equal to those used in the swarming assay.

RESULTS

Virtual screening for LasR QSI candidates. The initial 2D similarity search for drugs/natural compounds similar to the *P. aeruginosa* LasR natural ligand 3-oxo-C12-HSL, the proven QSIs furanone C30 and patulin, and the tri-phenyl LasR agonist TP-1 (Fig. 1A) in the publicly available (http://bioinformatics.charite.de/content/index.php) SuperNatural and SuperDrug databases (18, 26) resulted in 149 similar compounds. A 3D structural database of the compounds was subsequently docked against the ligand binding domain of LasR (PDB ID: 2UV0) in the docking program MVD. Furthermore, the screening included docking of the natural ligand itself and structures of known LasR antagonists and agonists (Fig. 1A and Table 1) for comparison. Six top-ranking drug/natural ligands (Fig. 1B) displaying docking scores better than that of the recognized QSI 4-nitropyridine-*N*-oxide (4-NPO), which was set as the cutoff for selection, were acquired to test their LasR-inhibitory potential. The docking scores of the six acquired compounds, together with those of the OdDHL reference and the known LasR binding compounds, are displayed in Table 2.

TABLE 2. Rerank scores, similarity scores, and molecular weights of the acquired compounds, the reference ligand, and the known LasR binding compounds included for comparison[a]

Compound	Mol wt	Similarity score	Rerank score
Acquired compounds			
Indoramine	340.4	−491.29	−121.281
Nifuroxazide	274.2	−447.124	−86.3983
Tiaprofenic acid	258.3	−396.031	−71.4197
Donepezil	370.4	−499.284	−70.7016
Chlorzoxazone	169.6	−344.493	−69.6008
Salicylic acid	138.1	−336.595	−66.156
Reference, 3-oxo-C12-HSL	297.4		−129.739
Known LasR binders			
3-Oxo-C12-(2-aminophenol)	305.4	−470.817	−93.8255
Patulin	154.1	−354.068	−70.1662
4-NPO	140.1	−305.698	−63.1366
Furanone C30	257.9	−265.816	−47.9107
Penicillic acid	170.2	−315.516	75.4073
TP-1	568.6	−497.462	220.864
TP-5	385.2	−398.532	431.434

[a] The compounds were ranked on the basis of their rerank scores. The scores of the individual compounds are the best of five scores derived from independent docking rounds.

Determination of LasR inhibition. In order to determine the QS-inhibitory potential of the six acquired drugs, they were initially screened by means of a LasR inhibition assay based on a *lasB*::*gfp*(ASV) translational fusion in *P. aeruginosa* PAO1. *lasB* codes for the virulence factor elastase and has been shown to be under the transcriptional control of LasR (22). As shown in Fig. 2, three of the six compounds, salicylic acid, nifuroxazide, and chlorzoxazone, showed significant reductions in the number of relative fluorescence units (RFU) over time in a dose-dependent manner. This suggests that the compounds possess inhibitory activity against LasR and function as QSIs. Furthermore, the compounds did not inhibit growth at the concentrations used. These results are in accordance with the finding that QS deficiency in *P. aeruginosa* exerts no effect on the growth of the bacterium (25).

Inhibition of the Rhl and Pqs QS systems. The activity of the three compounds salicylic acid, nifuroxazide, and chlorzoxazone against LasR-controlled *lasB*::*gfp* expression prompted us to investigate their inhibitory effects on the other major parts of the *P. aeruginosa* QS circuit, the Rhl and Pqs systems, which are both under LasR regulation (35, 60). Inhibition of the systems was investigated by assays similar to the LasR assay, with Rhl system inhibition being assessed by an *rhlA*::*gfp*(*ASV*) translational fusion and the Pqs inhibition being assessed by a *pqsA*::*gfp*(*ASV*) transcriptional fusion as described in Materials and Methods. *rhlA* is the first gene of the *rhlAB* operon that

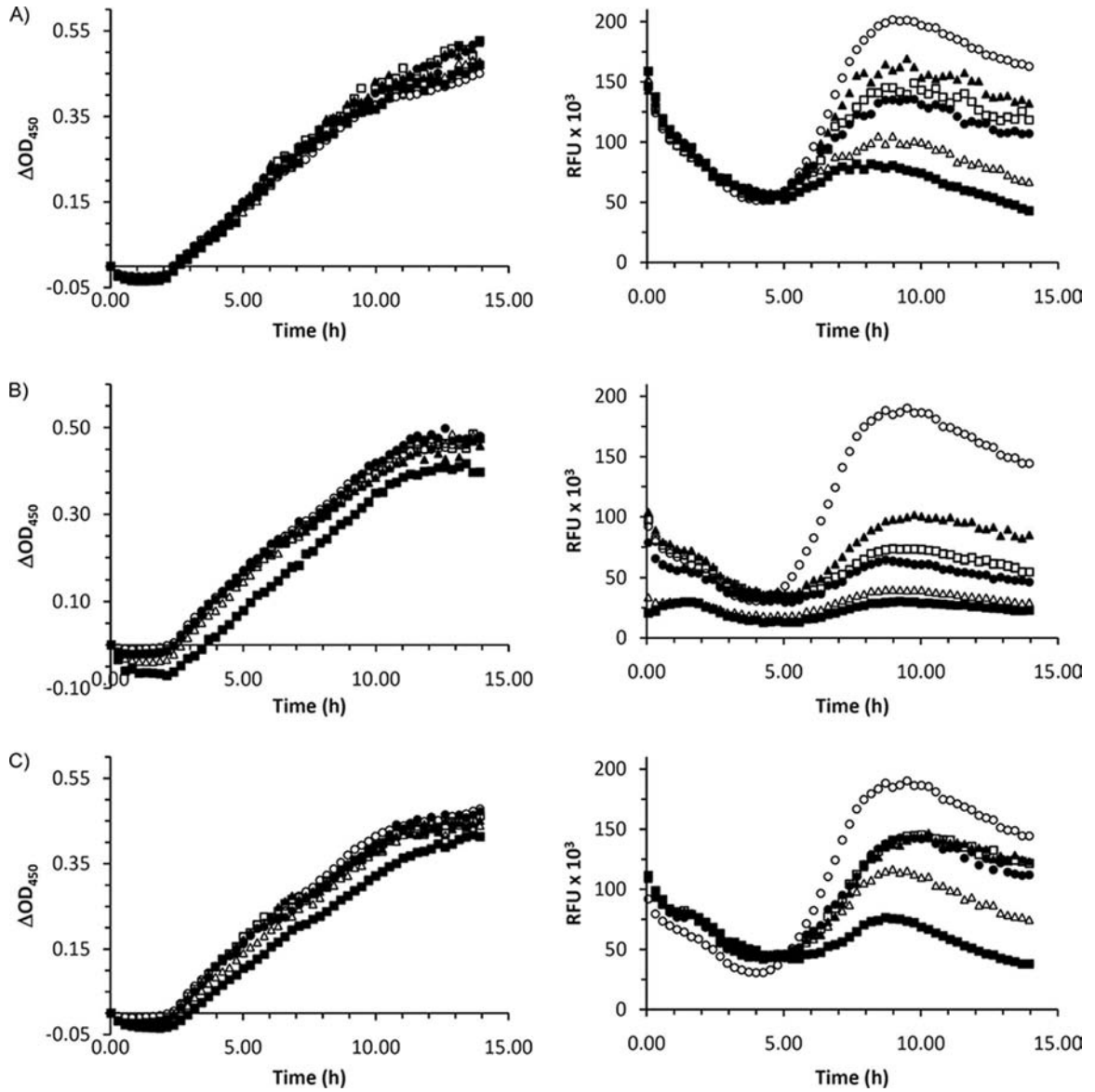

FIG. 2. Dose-response curves of the three drugs salicylic acid (A), nifuroxazide (B), and chlorzoxazone (C) when incubated together with PAO1 *lasB*::*gfp*(*ASV*). The three compounds reduce the RFU level (GFP fluorescence units divided by OD_{450}) at various concentrations (right), indicating inhibition of LasR, while displaying no effect on growth (left). Growth is displayed as the increase in OD_{450} compared to the initial level. Salicylic acid and nifuroxazide were tested at concentrations of 400 (■), 200 (Δ), 50 (●), 12.5 (□), 3.125 (▲), and 0 (○) μg/ml, while chlorzoxazone was tested at 200 (■), 100 (△), 20 (●), 6.25 (□), 1.5625 (▲), and 0 (○) μg/ml. Results are representative of three independent experiments.

codes for a rhamnosyltransferase essential for the production of rhamnolipid, the major product controlled by the Rhl QS system (44), and *pqsA* is the first gene of the *pqsABCDE* operon that is required for production of the Pqs signal (21). The two assays should thus give a relevant indication of the levels of Rhl and Pqs QS system inhibition by the three identified active compounds. The compounds were tested at concentrations of 200 μg/ml for salicylic acid and nifuroxazide and 100 μg/ml for chlorzoxazone. These concentrations displayed the largest degree of LasR inhibition without inhibiting growth (Fig. 2). As Fig. 3A shows, treatment of the two reporter strains with the three compounds resulted in a reduction of RFU levels in all cases. The reductions were statistically significant at the $\alpha = 0.01$ level (single-factor analysis of variance [ANOVA]). This added further evidence to the finding that the three identified compounds induce conditions of reduced QS activity in *P. aeruginosa*.

The results, however, were inconclusive regarding the specificity of the compounds. It could not be inferred if inhibition of the Rhl and Pqs systems occurred solely as a result of LasR inhibition or if the two systems were also directly inhibited by the compounds. To investigate this further, the GFP reporter plasmids for the two systems were introduced into a *P. aeruginosa lasR* mutant. The fluorescence levels from these reporter strains should only relate to the activity of the Rhl and Pqs systems abolishing fluorescence occurring as a result of system

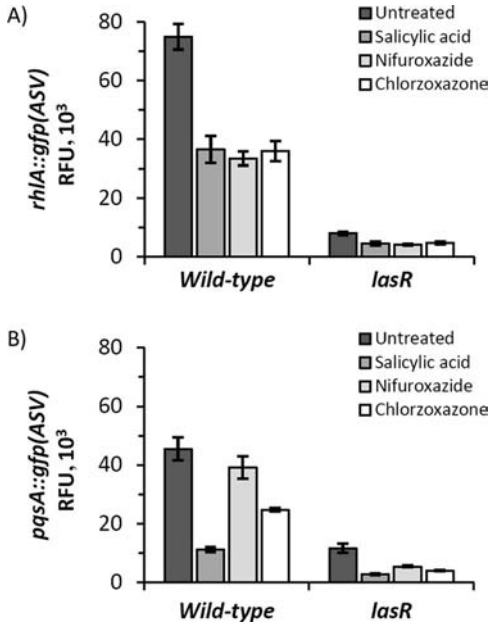

FIG. 3. Expression of *rhlA*::*gfp*(*ASV*) (A) and *pqsA*::*gfp*(*ASV*) (B) in wild-type and *lasR* mutant *P. aeruginosa* PAO1 treated with the three identified LasR inhibitors. Results are average RFU values taken from a single time point measurement corresponding to maximal induction of the reporters in the late log phase of growth. Inhibitors were added at concentrations of 200 μg/ml for salicylic acid and nifuroxazide and 100 μg/ml for chlorzoxazone. Averages and SDs of eight replicates are shown.

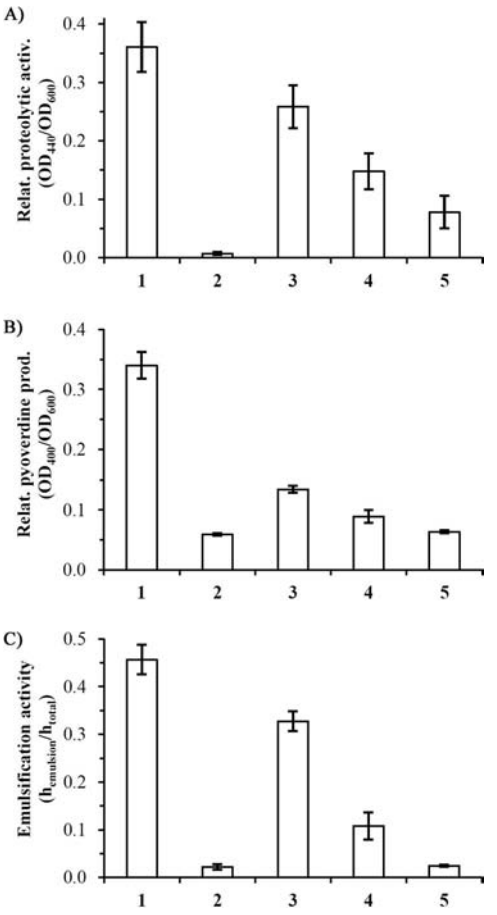

FIG. 4. Inhibition of exogenous protease production (A), pyoverdine production (B), and rhamnolipid production (C) in *P. aeruginosa* treated with the three identified LasR inhibitors. 1, untreated; 2, *lasI rhlI* mutant (QS deficient); 3, salicylic acid treated; 4, nifuroxazide treated; 5, chlorzoxazone treated. Results were taken after 18 h of growth at 37°C. Averages and SDs of five replicates are shown. Relat., relative; activ., activity; prod., production.

activation by LasR. As shown in Fig. 3B, expression of the *rhlA*::*gfp*(*ASV*) and *pqsA*::*gfp*(*ASV*) reporters was significantly lower in the *P. aeruginosa lasR* mutant than in the wild type, in accordance with LasR acting as a positive regulator of both the Rhl and Pqs systems. Interestingly, the experiment also showed that the three compounds reduced the expression of the *rhlA*::*gfp*(*ASV*) and *pqsA*::*gfp*(*ASV*) reporters in the *lasR* mutant compared to that in the untreated *lasR* mutant, indicating that the compounds, to some degree, exert an effect on the Rhl and Pqs systems independently of the effects seen from LasR inhibition.

Influence of QSIs on virulence factor production. We subsequently tested the effects of our identified QSIs on the production of three QS-controlled virulence factors: exogenous proteases, pyoverdine, and rhamnolipid. The production of exogenous proteases was assessed indirectly by measuring the ability of culture supernatant to degrade the colored protein substrate azocasein. Pyoverdine production was estimated directly through spectrophotometric measurements of the pyoverdine concentrations in culture supernatants. Rhamnolipid production was estimated indirectly by an emulsification activity assay and a drop collapse assay that estimates the presence of biosurfactants.

As shown in Fig. 4A, the three compounds significantly inhibited the production of exogenous proteases ($\alpha = 0.01$; single-factor ANOVA). However, it was evident that the reduction in protease production observed for the three inhibitors was less than that observed in the QS-deficient *lasI rhlI* mutant strain, suggesting that some QS activity remains in *P. aeruginosa* when it is treated with the inhibitors at the concentrations used. Treatment of *P. aeruginosa* with the three identified QSIs resulted in a reduction in the supernatant levels of pyoverdine (Fig. 4B), suggesting that the three compounds reduce the production of the siderophore. The results were statistically significant at the $\alpha = 0.01$ level in a single-factor ANOVA. Interestingly, inhibition of pyoverdine production occurred to a greater extent than the inhibition of protease production and the chlorzoxazone treatment resulted in pyoverdine levels comparable to that of the *P. aeruginosa lasI rhlI* mutant. This indicates that chlorzoxazone, at the concentration tested, is able to reduce pyoverdine production to a level resembling the level observed in QS deficiency. Finally, evidence was obtained that rhamnolipid production was inhibited by the three identified QSIs. An emulsification activity assay resulted in activities that were significantly reduced in cultures treated with the three compounds ($\alpha = 0.01$; single-factor ANOVA) compared to the activity in untreated wild-type cultures (Fig. 4C). As for the pyoverdine results, the emulsification results indicate that chlorzoxazone has the ability to reduce rhamno-

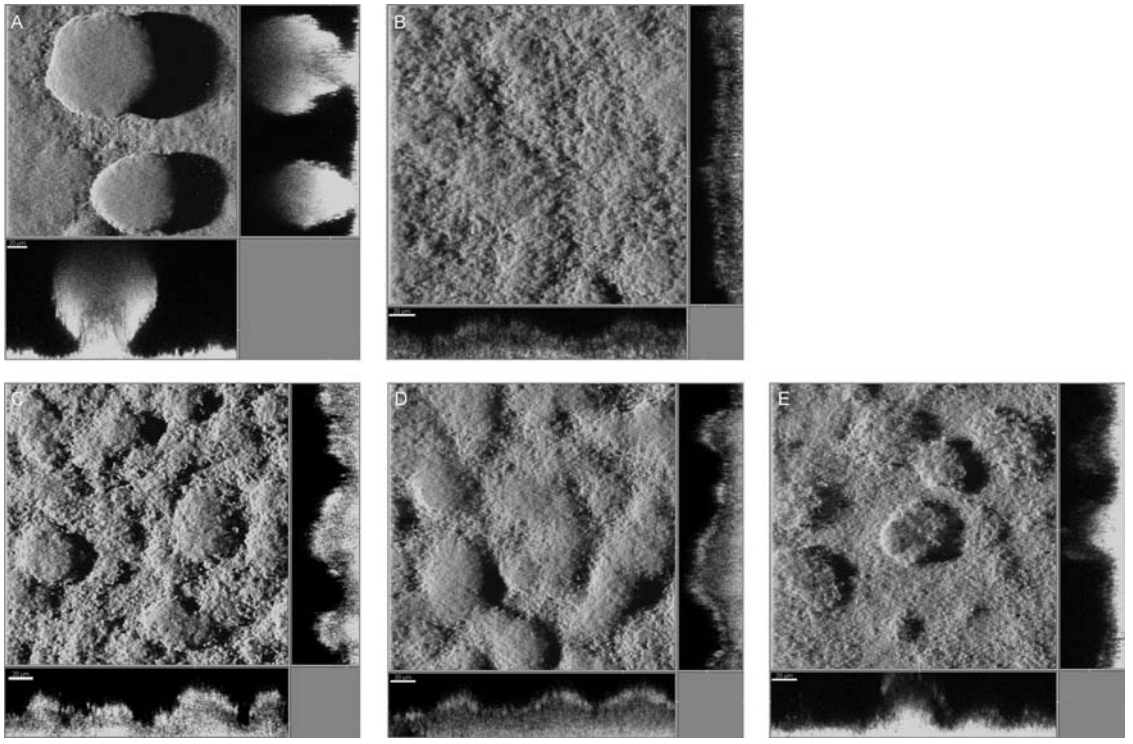

FIG. 5. CLSM pictures of 4-day-old *P. aeruginosa* biofilms. A, untreated; B, *lasI rhlI* mutant (QS deficient); C, salicylic acid treated; D, nifuroxazide treated; E, chlorzoxazone treated. The strains were GFP tagged for visualization. The main pictures are top-down 3D projections, while the flanking pictures are vertical sections. Bars, 20 μm.

lipid production to a level resembling that seen in a QS-deficient *P. aeruginosa lasI rhlI* mutant. The results were corroborated by a qualitative estimation of rhamnolipid production based on a drop collapse biosurfactant assay (5), which indicated that treatment with the three inhibitors affected the production of rhamnolipid by *P. aeruginosa* as follows: wild type, 100% production; *lasI rhlI*, 0%; salicylic acid treatment, 50%; nifuroxazide treatment, 25%; chlorzoxazone treatment, 0% (see Fig. S2 in the supplemental material).

Effects of the QSIs on flow chamber biofilm development. QS has been reported to play an important role in *P. aeruginosa* biofilm development and biofilm-related tolerance to antibiotics (4, 15, 45). Evidence has previously been presented that biofilms grown under conditions that repress QS are subject to sloughing (28), which may, at least in part, be caused by a lack of extracellular DNA matrix material (1). This prompted us to test the influence of the identified QSIs on *P. aeruginosa* biofilm formation. Biofilms were grown on glucose minimal medium in flow chambers in the presence or absence of non-growth-inhibitory concentrations of the QSI compounds, and as shown in Fig. 5, the characteristic mushroom-shaped structures normally observed in such *P. aeruginosa* biofilms (36) were not present in the inhibitor-treated biofilms. Instead, the inhibitor-treated biofilms resembled the QS-deficient *P. aeruginosa lasI rhlI* biofilm both in structure (flat and relatively unstructured) and in thickness. The finding that the inhibitor-treated biofilms were flatter and less structured than the wild-type biofilm was supported by objective image analysis. COMSTAT analysis was used for estimation of the total biomass present in biofilms formed by untreated, QSI treated, and QS-deficient cells. As shown in Fig. 6, inhibitor-treated biofilms contained less biomass than untreated biofilms, indicating impairment of the ability of *P. aeruginosa* to develop or maintain biofilms when treated with the selected QSIs. In support of these results, the QSI-treated biofilms had a total biomass comparable to that of biofilms formed by the QS-deficient *lasI rhlI* mutant strain.

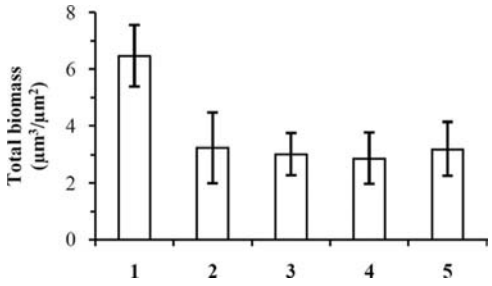

FIG. 6. Results of COMSTAT analysis for total biomass calculation of the individual biofilms. 1, untreated; 2, *lasI rhlI* mutant (QS deficient); 3, salicylic acid treated; 4, nifuroxazide treated; 5, chlorzoxazone treated. Averages and SDs from analysis of 12 images taken at random positions in three different biofilms are shown.

Assessment of the inhibitory activity of the QSI compounds in another microbial species. The results of the Rhl inhibition assay and the LasR specificity assays indicated that the QSIs, in addition to the effect seen through inhibition of LasR, to some degree directly inhibit the activity of RhlR. It was therefore of interest to investigate whether the three compounds could

inhibit AHL-based QS in other organisms. *S. liquefaciens* uses QS by means of C4-HSL and C6-HSL in the coordination of cellular functions such as swarming motility (19). In order to assess the activity of the QSIs on *S. liquefaciens* QS, we investigated the effects of the three compounds on *S. liquefaciens* swarming motility. A reduction of the swarming zone was observed when the bacteria were grown on agar with salicylic acid (0.347 cm^2; $n = 3$, standard deviation [SD] = 0.03) or chlorzoxazone (0.150 cm^2; $n = 3$, SD = 0.007), compared to that of bacteria grown on plates with no QSI (11.1 cm^2; $n = 3$, SD = 0.9). The sizes of the zones were similar to that of the zone observed for an *swrI* mutant (0.143 cm^2; $n = 3$, SD = 0.01) that is defective in swarming motility due to a lack of AHL synthesis (19). Nifuroxazide inhibited swarming at an intermediate, but significant, level (5.41 cm^2; $n = 3$, SD = 0.5). Growth experiments confirmed that the QSIs do not affect the growth of *S. liquefaciens* at the concentrations used in the swarmer assays (data not shown). The results thus suggest that the three QSIs also have the potential of inhibiting QS in organisms using short-to-medium-chain AHLs.

Investigation of the structural features required for LasR-inhibitory activity. It was of interest to investigate the proposed binding of salicylic acid, nifuroxazide, and chlorzoxazone to the LasR ligand binding domain as determined by the docking program MVD compared to the binding modes proposed by the program for known QSIs and the natural ligand itself. Finding a certain pattern in the proposed interactions between the active compounds and the binding site would be of interest in the identification of other QSIs, as well as for optimization of the already discovered ones. As shown in Fig. 7A to C, the three discovered QSIs were proposed to align with a ring structure in place of the homoserine lactone moiety of the natural ligand, a feature also proposed to occur for the LasR antagonists 4-NPO and furanone C30 (Fig. 7D and E). This, however, was the only pattern present since specific interactions between the compounds and LasR revealed no similarity and was therefore inadequate to improve the screening used in the present study.

DISCUSSION

The increase in antibiotic resistance seen in *P. aeruginosa* clinical isolates (8, 9) and their ability to form persistent infections through the formation of biofilms (13, 11) have drawn attention to the improvement of current treatment strategies. A great effort has been made to develop antipathogenic drugs and strategies (29, 57, 61), especially by means of reducing bacterial virulence through intercellular communication (QS). Blocking of QS in *P. aeruginosa* by the use of QSIs has been shown to be a promising strategy for the treatment of infections (29, 49), and a series of QSIs has been identified by different groups through traditional methods (29, 42, 50, 62). The traditional methods, however, have limitations that can be complemented by novel computer-aided drug design (40, 54).

Use of X-ray crystallography-derived 3D structures for structure-aided drug design is a common activity in drug discovery today (14). In this process, the structures of macromolecular targets, often proteins or protein complexes complexed with their natural ligands or identified inhibitors, are solved and the structural information is used to design inhibitors or screen molecules for putative inhibitory activity. In the present study, we have used structure-based virtual screening to identify novel *P. aeruginosa* QSIs. The database used consisted of recognized drugs/natural ligands, i.e., compounds that have a significant potential for the clinical treatment of *P. aeruginosa* infections.

Our screening identified three compounds, salicylic acid, nifuroxazide, and chlorzoxazone, that displayed QS-inhibitory activity at concentrations that did not affect bacterial growth (Fig. 2 and 3).

The results indicated a direct inhibition of LasR by the compounds, and they were also shown to inhibit the two remaining QS systems in *P. aeruginosa*, Rhl and Pqs. As LasR controls these two systems (35, 60), the observed inhibition could be an effect resulting from LasR inhibition, as opposed to direct inhibition of the two systems. To elaborate on the specificity of the compounds, Rhl and Pqs inhibition was investigated in a *P. aeruginosa lasR* mutant. The results indicated that the two systems were affected by the compounds to some degree in the absence of LasR, which suggests that the three identified QSIs directly affect the Rhl and Pqs systems as well. Salicylic acid appears to be a fairly potent inhibiter of Pqs-dependent signaling (Fig. 3), suggesting that this compound might act as a Pqs signal antagonist. Further work could usefully include microarray experiments to gain more insight into the mode of action of the three identified QSIs.

In addition, treatment of *P. aeruginosa* with the compounds resulted in a decrease in the production of QS-controlled virulence factors (Fig. 4). Interestingly, treatment with chlorzoxazone was found to result in virulence factor inhibition resembling that of a *lasI rhlI* mutant. This result suggests that chlorzoxazone has the potential to function as an effective antipathogenic drug which can be used in the treatment of *P. aeruginosa* infections. Treatment with salicylic acid and nifuroxazide resulted in virulence factor inhibition that occurred to a lesser extent than that seen in the QS-deficient *lasI rhlI* mutant strain, indicating that QS regulation is not completely switched off by these two identified inhibitors at the concentrations tested. The facts that chlorzoxazone was less inhibitory of *lasB* expression than salicylic acid and nifuroxazide (Fig. 2) but displayed more potent virulence factor inhibition (Fig. 4) support the findings that the identified QSIs affect the Rhl and Pqs systems, in addition to the Las system. Compared to other identified inhibitors, such as furanone C30, patulin, and penicillic acid, the inhibitors discovered here are not as potent (29, 51), but the fact that the compounds are already approved drugs for human use is a significant benefit in the further application and development of antipathogenic drugs. Our previously identified potent inhibitors are experimental drugs and have been important for proof of concept (29, 51), but they are not pharmaceutically relevant due to toxicity and instability.

The three compounds salicylic acid, nifuroxazide, and chlorzoxazone were also found to alter biofilm formation by wild-type *P. aeruginosa* PAO1. Treated biofilms were thinner and less structured than untreated ones and resembled the biofilms formed by the QS-deficient mutant (Fig. 5). An analysis of the total biomass present in the biofilms showed that the inhibitor-treated biofilms and the QS-deficient biofilm contained roughly half the biomass of the wild-type biofilm (Fig. 6).

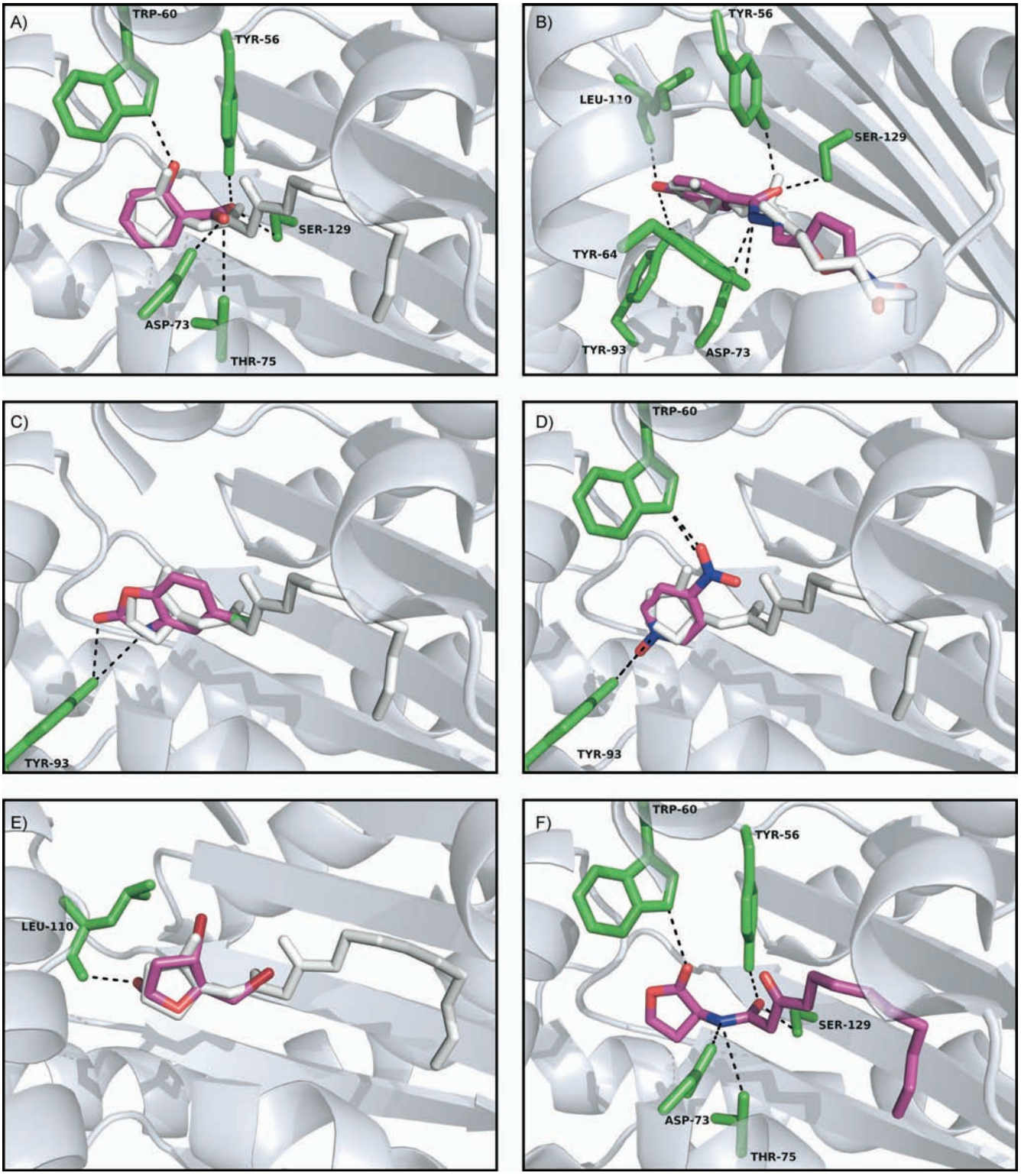

FIG. 7. Scenes displaying the conformations (magenta) of the identified LasR inhibitors and known LasR inhibitors as proposed by MVD after docking of the compounds. A, salicylic acid; B, nifuroxazide; C, chlorzoxazone; D, 4-NPO, the known LasR inhibitor used as cutoff in the docking; E, furanone C30, a potent LasR and QSI; F, OdDHL, the natural LasR ligand, as it is present in the LasR crystal structure with its hydrogen bonds (black lines) to the protein (green residues). In scenes A to E, hydrogen bonds proposed by MVD to occur upon binding are shown as black dotted lines together with the interaction residues (green). The conformation of OdDHL (white) has been included in scenes A to E for comparison, and the backbone of the LasR ligand binding domain is shown in blue-white with α-helical residues 65 to 72 omitted for clarity.

These results agree with a study by Davies et al. (15), who presented evidence that QS is involved in the maturation of *P. aeruginosa* biofilms. Furthermore, alteration of *P. aeruginosa* biofilms through the action of QSIs has been shown to result in a decrease in the tolerance of antibiotics and an increase in the effectiveness of phagocytosis by polymorphonuclear leukocytes (4, 13, 29). These observations emphasize the importance of the ability to alter biofilm formation by blocking QS, as shown by the inhibitors discovered in this study. Interestingly, the prolonged continuous exposure of *P. aeruginosa* to salicylic acid and nifuroxazide occurring in the flow chambers at decreased concentrations, compared to the virulence factor assays, apparently improved the potency of the compounds. This indicates that the activity of the compounds, and QSIs in general, against virulence factor production should also be assessed in comparable treatment schemes to fully estimate the potency of the compounds in question.

The finding that the three QSIs, to some degree, exerted inhibitory effects on the Rhl QS system independently of LasR prompted an investigation of QS inhibition in other bacterial species that use AHLs similar in length to the Rhl system autoinducer C4-HSL. The compounds were found to reduce swarming motility of *S. liquefaciens*, which uses C4- and C6-HSL for QS-regulated swarming (19). This result thus indicates that the three compounds have the potential of inhibiting QS in other bacterial species, which can have implications for the treatment of mixed-species infections with, e.g., *P. aeruginosa* and *Burkholderia cenocepacia*.

Salicylic acid is a small organic acid that is produced by plants and functions in the induction of defense responses against pathogenic attack (58). It is the major metabolite of aspirin and is responsible for the antiinflammatory properties of the drug in humans (31). In agreement with our findings, previous studies have implicated salicylic acid in the reduction of *P. aeruginosa* virulence (2, 48). Besides reducing the production of virulence factors and biofilms, the compound was shown to reduce the infectious potential of the bacterium against the plant *Arabidopsis thaliana* and the nematode *Caenorhabditis elegans* (48). Our results confirm these previous findings and directly establish salicylic acid as a QSI in *P. aeruginosa*.

Nifuroxazide is a synthetic antimicrobial agent used in the treatment of enteric infections (41). The antimicrobial effects of the compound should, at first glance, prevent it from being a useful antipathogenic drug. The results presented here (Fig. 2) showed, however, that the compound, at the concentrations used, did not affect bacterial growth. This discrepancy between the normal use of the compound and the observed effect is not unique to nifuroxazide. Recent studies showed that ceftazidime, tobramycin, and macrolide antibiotics, used at subinhibitory concentrations, displayed strong QS-inhibitory effects (23, 60, 69). Thus, nifuroxazide should still be a candidate in the development of antipathogenic drugs despite its antimicrobial activity at high concentrations.

The third drug found to be QS inhibitory, chlorzoxazone, is a centrally acting muscle relaxant when administered to humans (46). In addition to its medical effects, the compound has also been found to be hepatotoxic in some patients (47). The high QSI activity of the compound at the concentrations tested, however, makes the compound an interesting antipathogenic drug candidate, and therefore the exact hepatotoxic effects at the amounts required should be assessed before the compound is excluded from further development. Due to its small size, the compound may also be used as a lead compound from which other nontoxic QSIs for use as antipathogenic drugs can be synthesized.

Our discovery of three new QSIs prompted us to investigate the proposed binding of the three compounds to the LasR ligand binding domain in order to improve the screening procedure. Knowing specific interactions required for inhibition is of great value in the search for new inhibitors. A pattern in the interactions between the molecule and its binding site indicating inhibitory activity was, however, not present (Fig. 7). The identified feature of having a ring structure in place of the homoserine lactone moiety of the natural ligand is inadequate for screening improvement. This lack of features indicating inhibitory activity should also be seen in light of the mode of binding between LasR and its natural ligand. The structure of LasR complexed to OdDHL reveals that the ligand is deeply buried in a cavity inside the protein (7). Combined with results displaying irreversible binding of the ligand to LasR (55), this indicates that competitive binding of QSIs occurs with the protein in a more open conformation. Therefore, the available structure of the LasR ligand binding domain with bound OdDHL may not be representative of the structure to which the inhibitors bind, which is why structural features and interactions required for inhibition may be impossible, or at least difficult, to determine. The combination of SB-VS and a preliminary similarity search for molecules similar to already known LasR binding compounds presented here has, however, proven to be successful in the discovery of new QSIs.

Despite its insufficiencies for using the structure of LasR as a powerful tool in the future discovery of QSIs, the screening strategy used here has proven successful, with a hit rate of 50% (three active compounds out of six tested). Unlike the three indentified QSIs, the three compounds that did not show QSI activity were all from pills. Although HPLC-MS confirmed that the extraction procedure was adequate, we cannot exclude the possibility that some of the filler used in the pills binds the active compound under the conditions of the experiment and thereby limits its accessibility to the bacteria. (Oral delivery of the pills to humans has the benefit of extremes of pH to disrupt such binding.) The relatively small amount of compounds included for the SB-VS renders the possibility of false negatives, i.e., compounds with undiscovered activity that were discarded on the basis of the virtual screening process. Despite the desire to detect as many active compounds as possible, the occurrence of false negatives lies in the very nature of virtual screening. The methods used in the process are far from perfect, resulting in the possible exclusion of active compounds. In the screening strategy used here, the potent inhibitor furanone C30 would have been discarded on the basis of the cutoff values used. To avoid or reduce this phenomenon, an increased number of compounds should be tested in future searches.

In conclusion, we have used SB-VS for the identification of three *P. aeruginosa* QSIs. One of them, salicylic acid, was discovered previously to be an inhibitor of *P. aeruginosa* virulence, including virulence regulated by QS, which has been further established in this study. In addition, we have identified

the two new QSIs nifuroxazide and chlorzoxazone. These two compounds display a higher level of inhibition than salicylic acid, with chlorzoxazone treatment resembling conditions of QS deficiency, which is of particular interest in the further development of effective antipathogenic drugs active against *P. aeruginosa*.

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