Lehrstuhl für Mikrobiologie
der Technischen Universität München

A proteomics view of quorum-sensing regulated and surface induced genes in representative *Pseudomonas* and *Burkholderia* species

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. W. Höll
Prüfer der Dissertation:	1. Univ.-Prof. Dr. K.-H. Schleifer
	2. apl.-Prof. Dr. A. Görg

Die Dissertation wurde am 19. 04. 2004 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 28. 05. 2004 angenommen.

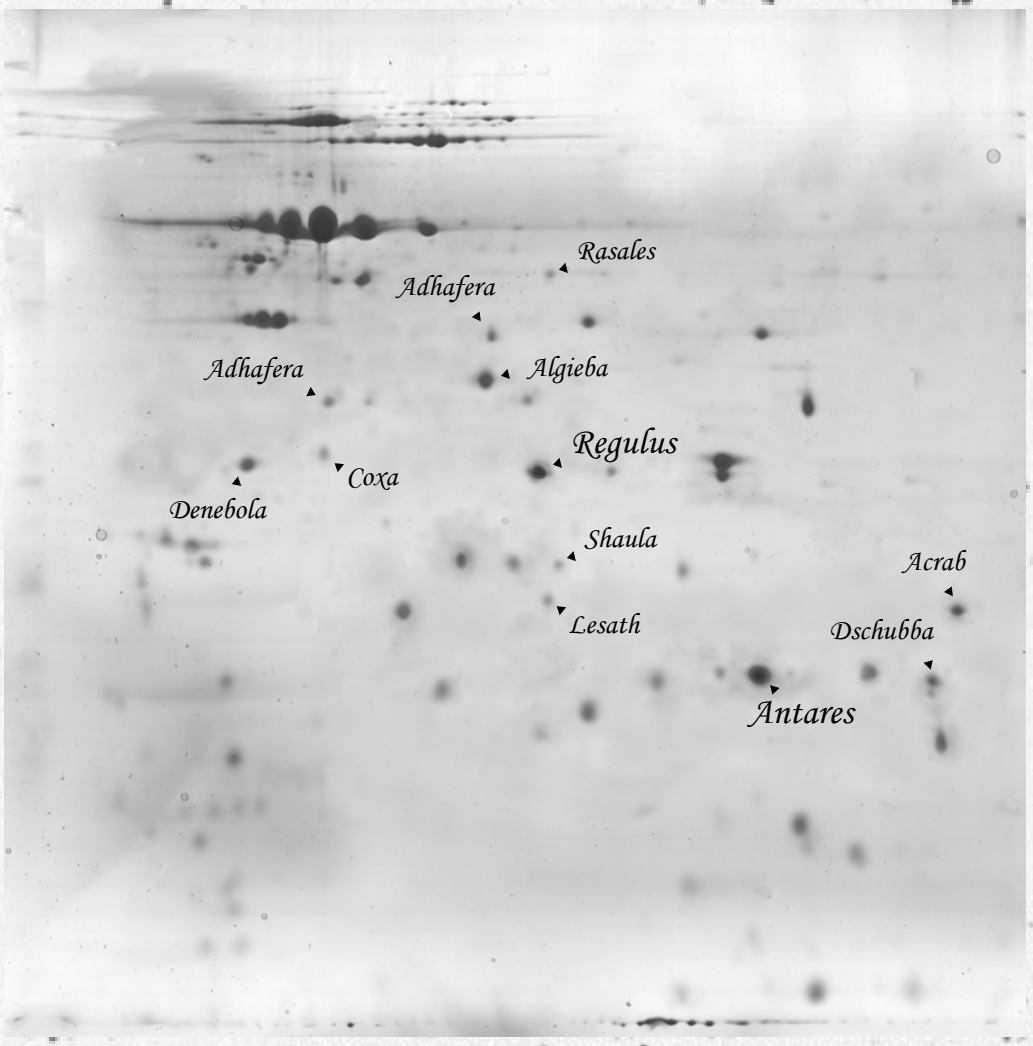
Table of contents

List of Tables

List of Figures

Abbreviations

2-DE	two dimensional gel electrophoresis
3-oxo-C12-HSL	N-3oxo-dodecanoyl homoserine lactone
AHL	N-Acyl-L-homoserine lactone
AIDS	acquired immune deficiency syndrome
C4-HSL	butanoyl-homoserine lactone
C8-HSL	octanoyl-homoserine lactone
CF	Cystic fibrosis
CMR	Comprehensive Microbial Resource
DTT	dithiothreitol
EPS	extracellular polymeric substances
H_2O_{MQ}	Milli-Q water
IAA	iodoacetamide
IPG	Immobilized pH Gradients
kDa	kilo Dalton
MW	molecular weight
NCBI	National Centre for Biotechnology Information
NL	non-linear
ORF	open reading frame
p*I*	isoelectric point
PVDF	Polyvinylidene fluoride
QS	Quorum-sensing
SDS	Sodium dodecyl sulphate
TIGR	The Institute for Genomic Research



Leo y la esquiva constelación de Escorpión

1 Introduction

1.1 Biofilms and quorum-sensing: rather a social than an isolated life.

Until recently, bacteria were considered as individual organisms unqualified to communicate or to coordinate group activities. These attributes were thought to be restricted to higher organisms. Nowadays it is recognised that bacteria communicate within the same species, between different species and organise coordinated activities like the formation of biofilms (Federle and Bassler, 2003; Greenberg, 2003b).

In nature, bacteria are usually present as co-operative consortia (also referred to as biofilms) rather than as free-floating (planktonic) cells. Bacterial biofilms are defined as complex communities attached to a surface surrounded by a thick matrix of extracellular polymeric substances (EPS). These structures are crossed by fluid-filled channels that enable nutrient transport to the interior parts of the biofilm and the elimination of waste products (O'Toole *et al.*, 2000; Hall-Stoodley *et al.*, 2004). In natural environments biofilms of mixed bacterial communities and of individual species exist. Structural analyses have demonstrated that thick biofilms have a complex architecture with differentiated three-dimensional structures like pillars or mushrooms. Several steps are required for the biofilm formation (1) initial transient association with the surface, (2) irreversible attachment and production of EPS matrix, (3) stable association as a member of a microcolony, the early development of biofilm architecture, (4) building of a three-dimensional biofilm (maturation), and (5) detachment and dispersion of single cells from the matrix (Watnick and Kolter 2000; Stoodley *et al.*, 2002). Biofilm formation permits bacteria to survive in harsh conditions. The association with a surface increases the stability of the consortium and facilitates the attachment of additional cells in close proximity. The mature biofilm protects the community from environmental disorders such as UV exposure, metal toxicity, acids and others (Hall-Stoodley *et al.*, 2004). Bacteria growing in biofilms differ in their physiology from that of planktonic bacteria. One of the most obvious differences is the increased resistance of biofilm forming bacteria towards antimicrobial agents and general host defences such as phagocytic clearance (Stewart and Costerton 2001; Lewis, 2001). In many cases, gene and protein expression patterns are altered in planktonic versus biofilm-grown cells, including genes involved in motility and attachment, metabolism, or bacteriophage genes (Whiteley *et al.*, 2001; Sauer *et al.*, 2002).

The formation of biofilms is based on bacterial interactions to coordinate concerted activities. The behaviour of entire bacterial populations is often coordinated by cell-to-cell communication. A bacterial consortium can therefore be described as a

"multicellular unit" (Shapiro, 1998). Nealson *et al.* (1970) described light production in the marine microorganism *Vibrio fischeri* in response to secreted signal molecules when the cell density was higher than 10^{10} cells/ml. The signal molecules were designated as "autoinducers" and identified as *N*-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL) (Eberhard *et al.*, 1981). Using these chemical signals bacteria can respond as groups and sense when the population has reached the "quorum" to coordinate a certain activity. This phenomenon has been designated as "quorum-sensing" (QS) (Fuqua *et al.*, 1994).

V. fischeri lives in symbiotic association with different eukaryotic hosts in the deep sea. The hosts e.g. certain anglerfishes have developed a specialised light organ where *V. fischeri* can reach a high cell density and accumulate *N*-acyl homoserine lactones (AHLs). The molecules are trapped inside the light organ with the bacteria and cannot diffuse into the seawater. This specialised eukaryotic light organ seems to be the only niche where *V. fischeri* is highly bioluminescent. In this symbiotic relationship the eukaryotic host affords a protected habitat with a higher nutrient concentration and uses the light from the bacteria for anti-predation strategies, mating or prey attraction (Nealson and Hastings, 1979; Engebrecht *et al.*, 1983).

Light production is coordinated by the *lux* regulon that comprises (1) the *luxICDABE* operon consisting of the *luxI* gene, encoding the 3-oxo-C6-HSL synthase, followed by the *luxCDABE* structural genes necessary for the bioluminescence, and (2) the *luxR* gene encoding the transcriptional activator protein LuxR. At low cell densities *luxI* is transcribed at a basal level leading to a low concentration of 3-Oxo-C6-HSL. AHLs are freely diffusible through the cell membrane and accumulate in the medium with increasing cell density. On reaching a critical threshold concentration 3-Oxo-C6-HSL binds to the LuxR-receptor protein. This complex in turn binds to a 20 bp palindromic DNA promoter element, the *lux*-box, and activates the transcription of *luxICDABE* resulting in the bioluminescent phenotype and in a positive autoregulation of *luxI* (a positive feedback loop) (Engebrecht and Silverman, 1984; Kaplan and Greenberg, 1985).

At the beginning quorum-sensing was considered a peculiarity of few Gram-negative bacteria; to date it is known that a wide variety of microorganisms are capable to coordinate the expression of certain phenotypes in a cell-density dependent manner (Whitehead *et al.*, 2001). Gram-positive bacteria, for instance, are using oligopeptides to communicate with each other. These autoinducing peptides (AIPs) are secreted by peptide transporters because the cell membrane is not permeable to them. Two-component transduction systems are used for the detection of the AIPs and the activation of the QS system (for reviews see de Kievit and Iglewski, 2000; Miller and Bassler, 2001).

Many AHLs and AIPs are highly specific: only the bacterial species that produce the respective molecule can sense and respond to the signal. The species specificity permits

bacteria to detect their own cell density even when other species are present. But it is also known that cross-species communication occurs using signal molecules as the AI-2 (furanosyl borate diester) recognised by a broad range of bacteria (Bassler *et al.*, 1997) or the unidirectional communication between *P. aeruginosa* and *B. cenocepacia* in mixed biofilms (Riedel *et al.*, 2001; for review see: Federle and Bassler, 2003).

Although the connection between quorum-sensing and biofilm formation has only been demonstrated in some species, it remains in discussion for other species that are not known to communicate but can form biofilms (Greenberg, 2003a). Davies *et al.* (1998) showed that quorum-sensing plays a role in the development of *Pseudomonas aeruginosa* biofilms under certain conditions. A mutation that blocks the synthesis of a signal molecule (*lasI* mutant; see description below 1.2.1) leads to a flat, undifferentiated biofilm that is highly sensitive to the biocide sodium dodecyl sulphate. When the mutant was grown in a medium supplemented with synthetic signal molecules, however, it was able to form wild-type biofilms. The same behaviour was seen in *Burkholderia cenocepacia*: Huber *et al.* (2001) demonstrated that a functional *cep* quorum-sensing system (see description below 1.2.2) is necessary for the effective surface colonisation of *B. cenocepacia* H111. Quorum-sensing was also found to be involved in biofilm formation of *S. liquefaciens* (Labbate *et al.*, 2004).

In medicine the ability of bacteria to function as a cooperative consortium is crucial for the development of numerous infectious diseases. Biofilms cause a variety of persistent infections (from dental caries to lethal cystic fibrosis) with different implications and, besides biofilm formation; quorum-sensing regulates the expression of many virulence factors for example in *P. aeruginosa* proteases and exotoxin A are shown to be regulated by QS (Eberl, 1999; Costerton *et al.*, 1999).

1.2 Quorum-sensing regulated phenotypes: two sides of one coin

In many bacteria quorum-sensing regulates a wide variety of phenotypes for example bioluminescence of *V. fischeri* and *V. harveyi* (Nealson and Hastings, 1979), transfer of tumor-inducing plasmids (Ti plasmids) of *Agrobacterium tumefaciens* (Zhang *et al.*, 1993), carbapenem antibiotic production of *Erwinia carotovora* (McGowan *et al.*, 1995), swarming motility of *Serratia liquefaciens* (Eberl *et al.*, 1996), biofilm formation and production of virulence factors of *P. aeruginosa* and *B. cenocepacia* (de Kievit and Iglewski, 2000; Huber *et al.*, 2001). It has been demonstrated that many bacteria do not express virulence factors until the population density is high enough to overwhelm host defences and to establish the infection (Greenberg, 2003b).

The fact that bacteria, sensing their population density, decide whether a particular phenotypic trait is expressed or not, affects the environment in positive and negative ways. On one hand, QS plays an important role in the synthesis of virulence factors and

biofilm formation of human pathogens such as *P. aeruginosa* and *B. cenocepacia,* which consequently allows the bacteria to co-colonise the host tissues (Eberl, 1999; Riedel *et al.*, 2001). Another example are plant pathogenic bacteria of the genus *Erwinia* that cause soft-rot disease due to the QS regulated production of a battery of plant cell wall-degrading enzymes (Pirhonen *et al.*, 1993). On the other hand, there are bacteria like *Pseudomonas aureofaciens,* which synthesise phenazine antibiotics that protect wheat from an ascomycete fungus. The synthesis is controlled by a PhzR/PhzI 3-Oxo-C6-HSL-dependent QS system (Wood *et al.*, 1997). It is known that production of AHLs is common among plant-associated bacteria (Cha *et al.*, 1998). The production of signal molecules has also been found in the plant growth-promoting *Pseudomonas putida* that has not only antagonistic activity against plant pathogens, but also degrades toxic organic compounds (Berg *et al.*, 2002; Weller, 1988).

1.2.1 Pseudomonas aeruginosa

P. aeruginosa, an ubiquitous environmental bacterium, is capable to infect a wide variety of animals and plants. It is classified as an opportunistic human pathogen and the leading source of Gram-negative nosocomial infections in patients with burn wounds, immunocompromised individuals suffering from cancer or AIDS, or affected by medical devices (de Kievit and Iglewski, 2000). *P. aeruginosa* is also associated with chronic lung infections in approximately 90% of the individuals suffering from cystic fibrosis (CF) (Høiby and Frederiksen, 2000; Lyczak *et al.*, 2000). CF, the most common lethal inherited disease among the Caucasian population, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (cftr) gene, encoding a chloride channel. Impaired CFTR function leads to high salt concentrations in epithelial secretions and the production of thick dehydrated mucus in the ducts of exocrine glands, e.g. the airways of the lungs. The increased mucus viscosity impairs mucociliary and alveolar clearing, promoting colonization of the lung epithelium by opportunistic pathogenic bacteria (Tümmler and Kiewitz, 1999; Boucher, 2004)

Recently, the genome of *P. aeruginosa* PAO1 has been fully sequenced and 5570 open reading frames (ORF) have been annotated (Stover *et al.*, 2000). Approximately 5% of the genes encode known or putative virulence factors that contribute to the pathogenicity of this bacterium (Wolfgang *et al.*, 2003), including exoenzymes (elastase, alkaline protease), secondary metabolites (pyocyanin, hydrogen cyanide, pyoverdin) and toxins (exotoxin A) (Passador *et al.*, 1993; Winson *et al.*, 1995). Most of these pathogenic traits as well as the maturation of biofilms have been reported to be regulated by quorum-sensing (Davies *et al.*, 1998; Yoon *et al.*, 2002; Van Delden and Iglewski, 1998)

Two quorum-sensing systems have been identified in *P. aeruginosa*: the *las* system consisting of the transcriptional activator LasR and the AHL synthase LasI which directs the synthesis of N-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) and

the *rhl* system consisting of RhlR and RhlI which directs the synthesis of N-butanoyl-homoserine lactone (C4-HSL). The two systems do not operate independently as the *las* system positively regulates the expression of both *rhlR* and *rhlI*. Thus, the two quorum-sensing systems of *P. aeruginosa* are hierarchically arranged with the *las* system being on top of the signalling cascade (Figure 1a) (for reviews see Pesci *et al.*, 1997; de Kievit and Iglewski, 2000).

Additional regulators including RsaL, QscR, GacA, Vfr, MvaT and RpoS, have been shown to be an integral part of the *P. aeruginosa* QS circuits (Figure 1a). The *rsaL* gene encodes a repressor (RsaL) of virulence genes, overexpression of *rsaL* results in reduced *lasB* expression and decreased elastase activity (de Kievit *et al.*, 1999). It has been described that the principal role of the quorum-sensing control repressor (QscR), a LuxR-homolog, is the repression of *lasI*. Consequently, the quorum-sensing controlled genes are only transcribed in habitats where they are useful. A *qscR* mutant produces the LasI-generated signal earlier than the wild type resulting in a premature transcription of a number of QS regulated genes (Chugani *et al.*, 2001). The global activator GacA, the response regulator of the GacA/GacS two-component system, has also been reported as a positive regulator of *P. aeruginosa* QS controlled virulence factors (Reimann *et al.*, 1997). It is well documented that *las*R expression is positively regulated by the virulence factor regulator (Vfr). Vfr is a cyclic AMP receptor protein (CRP), which binds to a CRP-binding consensus sequence (CCS) upstream of *las*R (Albus *et al.*, 1997). MvaT is a global regulator of virulence gene expression that acts as a repressor of quorum-sensing in *P. aeruginosa*. A mutation in *mvaT* showed altered swarming ability and enhanced production of the LasB and LasA proteases, 3-oxo-C12-HSL, and C4-HSL (Diggle *et al.*, 2002). RpoS negatively affects *rhlI* transcription; a *rpoS* mutant showed increased *rhlI* transcription and consequently higher C4-HSL levels and enhanced RhlR-RhlI-controlled virulence factor production (Whiteley *et al.*, 2000).

The importance of the cell-cell communication systems in the pathogenicity of *P. aeruginosa* has been investigated in various animal models: in all cases mutants defective in quorum-sensing (*lasI*, *rhlI*, or *lasI rhlI* double mutant) were substantially less virulent than the parent strains (Tang *et al.*, 1996; Tan *et al.*, 1999; Wu *et al.*, 2001).

Lately, several studies intended the global characterization of the *P. aeruginosa*-QS regulon. Whiteley *et al.* (1999) constructed a library of random insertions using a transposon containing a promoterless *lacZ*, the library was screened for AHL induction of *lacZ*. They reported that approximately 4% of the genes annotated in the *P. aeruginosa* PAO1 genome might be QS regulated. More recently, three independent studies comparing *P. aeruginosa* wild type and QS mutant using DNA-microarray analysis showed that a remarkably high number of genes are QS regulated: Schuster *et*

al. (2003) identified 315 positively and 38 negatively regulated genes, Wagner *et al.* (2003) identified 394 positively and 222 negatively regulated genes and Hentzer *et al.* (2003) identified 163 positively regulated genes. When the results of the three studies were compared only 77 genes were invariably QS regulated and this set of genes was tentatively designated the "general quorum-sensing regulon" (Hentzer *et al.*, 2003). Although these studies generated an impressive amount of novel information on quorum-sensing in *P. aeruginosa,* a recent proteome analysis, comparing extracellular proteins of *P. aeruginosa* wild type and QS mutant by two-dimensional gel electrophoresis, identified several previously unknown QS regulated proteins (Nouwens *et al.*, 2003b). Therefore only the combination of different experimental approaches such as transcriptomics and proteomics will improve the understanding of the *P. aeruginosa* cell-cell communication system.

1.2.2 *Burkholderia cepacia* complex

The *Burkholderia cepacia* complex (Bcc) was first described as a phytopathogen, associated with the soft-rot of onion bulbs (Burkholder, 1950). *B. cepacia* genomovar III is a member of the Bcc; Vandamme *et al.* (2003) proposed to formally classify this genomovar as *Burkholderia cenocepacia*. Members of Bcc occur ubiquitous in nature and can be found in soil, water and plant rhizosphere. Representatives belonging to genomovar II (*B. multivorans*) and III (*B. cenocepacia*), have more recently emerged as opportunistic pathogens of humans that primarily infect immunocompromised patients with cystic fibrosis (CF) (Govan and Deretic 1996; Isles *et al.*, 1984). However, *B. cenocepacia* is also abundant in the rhizosphere of several economically important crops and can be isolated from various habitats including soil, water and plant surfaces (Balandreau *et al.*, 2001). In most cases lung infection by *B. cenocepacia* occurs in patients who are already colonised with *P. aeruginosa*. It has been suggested that *P. aeruginosa* produces an extracellular factor, which modifies the epithelial cell surface of the lung thereby facilitating the attachment of *B. cenocepacia*. Co-colonization can result in three clinical outcomes: asymptomatic carriage, slow and continuous decline in lung function or, for approximately 20 percent of the patients, fulminate and fatal pneumonia, the so-called "cepacia syndrome" (Isles *et al.*, 1984; Saiman *et al.*, 1990; Jones *et al.*, 2001). *B. cenocepacia* produces at least four types of siderophores: salicylic acid, pyochelin, cepabactin, and ornibactin. Ornibactin-mediated iron acquisition has been shown to be important in respiratory infections. Pyochelin production has been associated with 62% of clinical isolates, and related to severe pulmonary disease in CF patients. Cepabactin is produced by few clinical isolates (11%) (Lewenza and Sokol, 2001; Darling *et al.*, 1998). Some strains produce different extracellular factors such as protease, chitinase and lipase; some of which have been reported to be regulated by quorum-sensing. The *cep* quorum-sensing system of *B. cenocepacia*

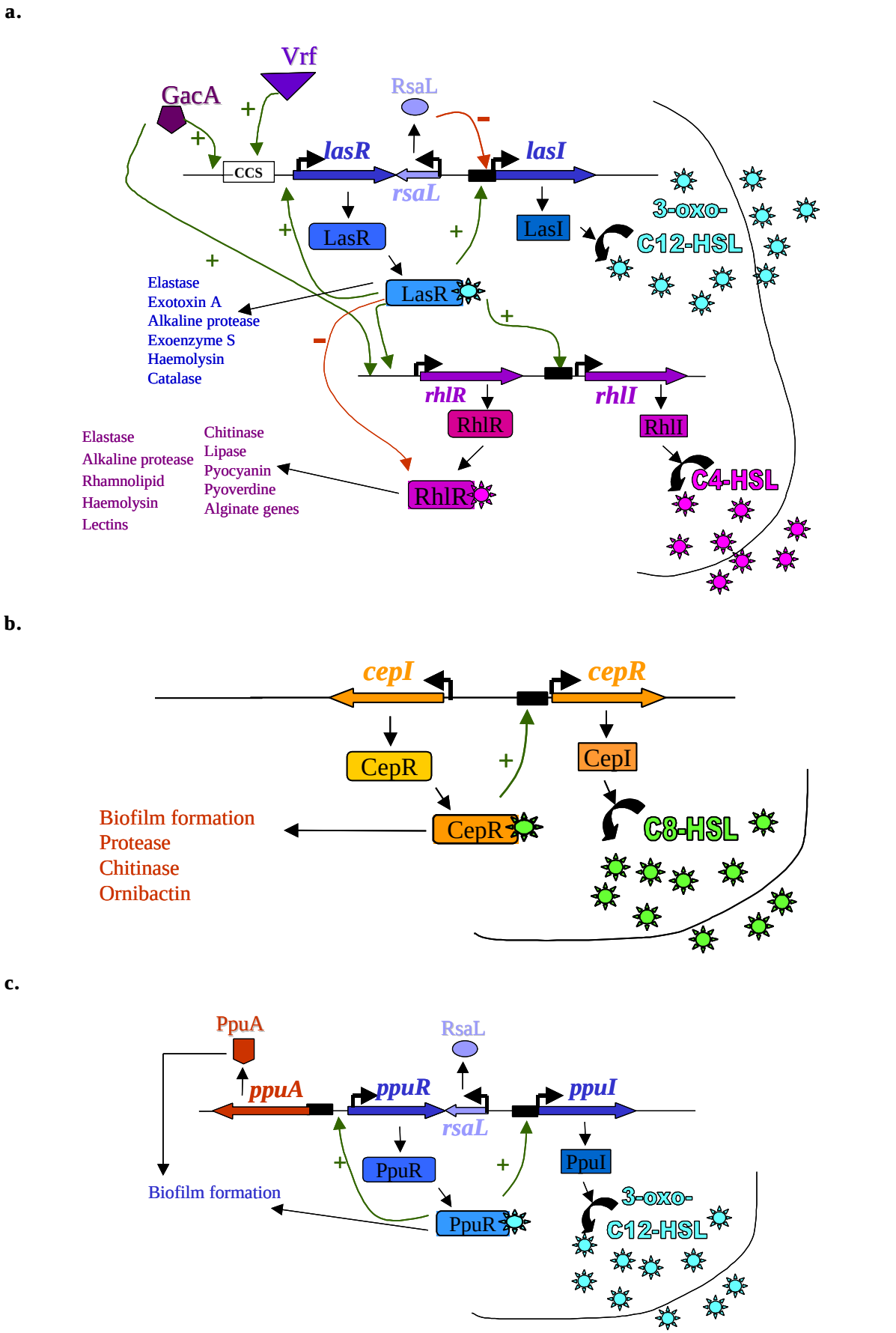


Figure 1 Schematic drawings of the quorum-sensing circuits of a) *P. aeruginosa* (*las/rhl* system), b) *B. cenocepacia* (*cep* system), c) *P. putida* (*ppu* system).
Black boxes represent the respective *lux* box.

consists of the AHL synthase CepI, which directs the synthesis of *N*-octanoylhomoserine lactone (C8-HSL) and, as a minor product, *N*-hexanoylhomoserine lactone (C6-HSL) and the transcriptional activator CepR, which binds to C8-HSL (Figure 1b) (Lewenza *et al.*, 1999).

Negative quorum-sensing regulation has been reported for the siderophore ornibactin, *cepR* or *cepI* mutations resulted in hyperproduction of ornibactin but not salicylic acid. Positive quorum-sensing regulation has been described for proteolytic and chitinolytic activity, biofilm formation and swarming motility, as well as for the expression of AidA (autoinducer dependent protein A) (Huber *et al.*, 2001; Lewenza and Sokol, 2001).

The importance of the quorum-sensing system of *B. cenocepacia* in pathogenicity has been investigated in the nematode *Caenorhabditis elegans*. It was demonstrated that *cepI* and *cepR* mutants were attenuated in their virulence against *Caenorhabditis elegans*. Therefore, it was concluded that the *cep* system of *B. cenocepacia* H111 is required for efficient killing of the nematode (Köthe *et al.*, 2003). Studies in rats and mice showed consistently that a functional *cep* system contributes to the severity of *B. cenocepacia* infections (Sokol *et al.*, 2003).

It has been demonstrated that the *cep* system regulates a variety of phenotypes, but currently very little is known about the global impact of the quorum-sensing system on gene expression in *B. cenocepacia*. Therefore, functional genomics (transcriptomics and proteomics) should be employed to map the entire QS regulon and to identify further AHL-controlled genes in *B. cenocepacia*.

1.2.3 *Pseudomonas putida*

P. putida is considered as highly attractive candidate for agricultural and environmental applications. It can be found in terrestrial and aquatic environments and can use a vast range of substrates for growing (Espinosa-Urgel *et al.*, 2002). It has been demonstrated that *P. putida* improves the growth of potatoes, sugar beets, and radish when it is applied to the seeds. Antagonistic activities have been described where *P. putida* produces antibiotics such as 2,4-diacetyl-phloroglucinol against *E. carotovora*, the pathogen *Verticillium dahliae* and other fungal pathogens (Weller, 1988; Berg *et al.*, 2002).

Recently, the genome of *P. putida* KT2440 has been fully sequenced and 5420 open reading frames (ORFs) have been annotated (Nelson *et al.*, 2002). Analysis of the genome of *P. putida* points to agricultural applications, biocatalysis, bioremediation and production of biopolymers. A high number of enzymes and transporters for secondary metabolites were found, that supports the ability of *P. putida* to degrade toxic compounds and to produce epoxides, catechols, amides and heterocyclic compounds. It has also been shown that *P. putida* KT2440 lacks a variety of virulence factors of pathogenic Pseudomonads, including exotoxin A, specific hydrolytic enzymes, and type

III secretion systems. On the other hand, it was found that surface adhesion proteins, polymer biosynthesis, type IV pili, stress-related proteins and global regulators such as GacA are present in this strain. Those genes are usually considered to be important for pathogenesis but in this case they might play an important role for colonization of and survival on surfaces (Nelson *et al.*, 2002; Cao *et al.*, 2001).

It has been described that some *P. putida* strains produce AHL signal molecules (Elarsi *et al.*, 2001). The plant growth-promoting *P. putida* IsoF isolated from tomato roots produces a wide spectrum of AHLs including 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL, and 3-oxo-C12-HSL (Steidle *et al.*, 2001). The *ppu* quorum-sensing system of this strain was recently described and four genes were found to be involved in this circuit: *ppuI*, *rsaL*, *ppuR*, and *ppuA* (Figure 1c). None of these genes were found in the *P. putida* K2440 genome (Steidle *et al.*, 2002). *PpuI* encodes the AHL synthase PpuI, which directs the synthesis of mainly N-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) and N-3-oxo-decanoyl-homoserine lactone (3-oxo-C10-HSL). *PpuR* encodes the transcriptional activator PpuR that binds to the AHLs and positively regulates the *ppuI* expression (feedback loop), influences biofilm structural development and regulates the expression of *ppuA* (Steidle *et al.*, 2002). *RsaL* encodes a protein similar to RsaL of *P. aeruginosa*, a negative regulator of the quorum-sensing system (the function of this gene in *P. putida* has not been described yet). The expression of *ppuA* has been found to be AHL dependent, PpuA is similar to long-chain fatty acid CoA ligases, and it has been speculated that it could be involved in the fatty acid composition of the cell membrane. *P. putida* IsoF formed very homogenous unstructured biofilms that uniformly cover the surface, while the *ppuI* mutant F117 formed structured biofilms with characteristic microcolonies. When the medium was supplemented with AHL signal molecules, the mutant biofilm lost its structure changing into an unstructured biofilm similar to the one formed by the wild type IsoF. Data also suggested that *ppuA* is involved in biofilm maturation, because the *ppuA* mutant formed biofilms with the same structure as the ones formed by the *ppuI* mutant (Steidle *et al.*, 2002).

By employing proteomics, Sauer and Camper (2001) showed that 45 proteins from an intracellular-enriched protein extract (obtained by sonication) were differentially expressed between planktonic and sessile cells of *P. putida* (ATCC 39168) following 6 h of attachment, indicating that this strain undergoes a global change in gene expression after the attachment to a surface. Additionally, it was shown in the same strain that 16 proteins were differentially expressed when planktonic cells were grown in medium supplemented and not supplemented with 3-oxo-C12-HSL, respectively. Only one protein, PotF (periplasmatic putrescine binding protein), was found to be down-regulated in both, surface-associated growth during the early biofilm formation and

AHL addition. Therefore, it was suggested that quorum-sensing does not play an important role in the initial attachment process.

Steidle *et al.* (2002) provided strong evidences that QS plays a crucial role in the late stages of biofilm development of *P. putida* IsoF. Furthermore, the *P. aeruginosa* and *P. putida* genomes are highly similar (85%) (Nelson *et al.*, 2002) and PpuI is functionally interchangeable with LasI (Steidle *et al.*, 2002). It can be speculated that several *las* system regulated genes present in *P. aeruginosa* might be also present in *P. putida*. Most probably additional QS regulated phenotypes exist which can be identified by protein pattern comparison of wild type versus AHL-deficient mutant.

1.3 Quorum-sensing: a bacterial double-edged sword

The impact of quorum-sensing on biofilm development and in the expression of many virulence factors in bacteria makes it an attractive target for development of novel therapeutics against bacterial infections (Hentzer and Givskov, 2003).

Conventional treatment of infectious diseases is based on antibiotics that kill or inhibit growth of bacteria (bactericides or bacteriostatic compounds). The development of resistance against these therapeutics is one of the major concerns in medical therapies. Today, it has been recognised that bacteria can adapt to the selective pressure imposed by antibiotics. The bacterial population that remains after therapies focused on killing or inhibiting growth exhibit (1) increased abilities to degrade antibacterial compounds, (2) decreased permeability, (3) decreased affinity for antibiotics, or (4) increased efflux of antibiotics. For this reason it has been proposed that the alternative to antibiotics is the development of antipathogenic drugs that attenuate bacterial virulence without inhibiting growth. In this way the organism is not able to establish a successful infection and as consequence it is cleared by the host immune response (Hentzer and Givskov, 2003).

Quorum-sensing provides an exceptional target for novel antipathogenic drugs. It has been shown that virulent organisms such as *P. aeruginosa* and *B. cenocepacia* are attenuated by the inhibition of their QS systems (Hentzer *et al.*, 2002; Wu *et al.*, 2001; Köthe *et al.*, 2003).

Quorum-sensing can be blocked in different ways: (1) the inhibition of AHL signal generation, (2) the inhibition of AHL signal dissemination, and (3) the inhibition of AHL signal reception. Many higher organisms have developed strategies to disrupt bacterial signalling pathways. It has been demonstrated that exudates from pea (*Pisum sativum*) affect QS regulated phenotypes in bacteria (Teplitski *et al.*, 2000). Polyphenolic compounds commonly produced by plants, for example epigallocatechin gallate (EGCG), ellagic acid, and tannic acid specifically block AHL-mediated communication between bacteria (Huber *et al.*, 2003). The Australian macroalga *Delisea pulchra* is the best-

investigated example of an organism, which produces metabolites (halogenated furanones) with biological activities including antimicrobial properties (de Nys *et al.*, 1993). It has been shown that some of these furanones specifically interfere with bacterial signalling processes. In presence of halogenated furanones, designated as quorum-sensing inhibitors (QSI), the half-life of the LuxR protein is reduced up to 100-fold suggesting that these compounds destabilise the AHL-dependent transcriptional activator (Givskov *et al.*, 1996; Manefield *et al.*, 2002). Artificial derivates of the *D. pulchra* furanones were shown to repress quorum-sensing in *P. aeruginosa* (Hentzer *et al.*, 2002); the application of the synthetic derivates (the C30 compound) to *P. aeruginosa* biofilms resulted furthermore in an increased bacterial susceptibility to tobramycin and SDS. In a mouse pulmonary infection model, the drug inhibited quorum-sensing of the infecting bacteria and promoted their clearance by the mouse immune response. In a recent microarray analysis it was reported that 1.7% of the *P. aeruginosa* genome was affected by the addition of the furanone compound C-30. 80% of the genes repressed by the furanone were already recognised as quorum-sensing regulated genes, including numerous *P. aeruginosa* virulence factor such as elastase, LasA protease, rhamnolipid production, phenazine biosynthesis, and chitinase (Hentzer *et al.*, 2003).

The application of halogenated furanones as human therapeutics has limitations due to their cytotoxic potential at higher concentrations but they are useful model compounds to investigate QS inhibition *in vitro*. The successful control of infections in animal models using QSIs demonstrated that quorum-sensing is indeed a promising target for antibacterial drugs development (Smith and Iglewski, 2003; Hentzer and Givskov, 2003).

1.4 Comparative proteome analysis: an essential tool to study biological systems

During the last decade the analysis of the regulation and function of individual genes has changed into global studies that analyse the entirety of genes (genome or transcriptome) and proteins (proteome) of one organism (Tyers and Mann, 2003). After the release of complete genome sequences for several microorganisms, it has been recognised that the DNA sequence information is not sufficient to elucidate biological functions. “Genomics”, the large-scale analyses of genes, provide a lot of information about changes in genetic constitution associated with a given phenotype especially when the presence or absence of a gene is correlated with a biological function. However, the existence of an open reading frame (ORF) in genomic data does not necessarily represent the existence of a change in the physiological state of the cell. During stress, drug administration, disease or health the genome remains invariable but the protein content of a cell will change. Questions about altered biological

conditions should be answered downstream of the DNA level, which means the analysis of the gene products (mRNA and proteins) known as “functional genomics” (Pandey and Mann, 2000). The analysis of mRNA levels, so called “transcriptomics”, is a valuable tool to determine the alteration in gene expression due to a given environmental or genetic stimulus. Transcriptome analyses are less time consuming than proteome studies and can be easily automated; this facilitates sample handling and reduces methodology-inhabited errors. Furthermore, it can be up scaled to cover the analysis of complete genomes by DNA-microchips (Harrintong *et al.*, 2000). However, the active product of a gene is a protein and proteins are responsible for biological functions. The evaluation and prediction of biochemical functions of proteins delivers the basis to understand biological mechanisms. Therefore, mRNA information must be complemented with data generated at the protein level also regarding other biological influences including the stability, post-transcriptional, co-translational and degradative modification of proteins cannot be predicted by mRNA expression analysis (Anderson and Anderson, 1998; Williams, 1999). Furthermore, it has been demonstrated in yeast that the correlation between mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data (Gygi *et al.*, 1999; Turner and Varshavsky, 2000).

The expression “proteome” refers to all the proteins encoded by the genome including protein isoforms and modifications, protein-protein interactions, protein activity patterns. Proteomics, the study of proteomes, links proteins with phenotypes associated with special strain characteristics and provides important information how genomes function (Wasinger *et al.*, 1995; Pandey and Mann, 2000; Humphery-Smith and Blackstok 1997). While nucleic acids are chemically homogeneous, proteins show widely different chemical properties. This is one of the major problems of proteomics. Protein analysis has always to struggle with variable sample material, sample degradation, post-translational modifications and dynamic range of abundance. So far there is no unique methodology that is able to resolve all the variables (Anderson and Anderson, 1998; Rabilloud, 1996). Proteomics has traditionally employed two-dimensional electrophoresis (2-DE) for the analysis of complex protein mixtures (O’Farrell, 1975; for review: Görg, 2000). In the beginning, the identification of proteins was accomplished by Edman sequencing. Later on, 2-DE has been combined with mass spectrometry (MS) which is more sensitive that Edman and can also identify posttranslational modifications. Various MS technologies have been developed such as time-of-flight (TOF) MS, or soft ionisation methods including matrix-assisted laser desorption ionization (MALDI) and electro-spray ionisation (ESI) (for reviews see: Graves, 2002; Zhu, 2003; Görg, 2000). The limitations of 2-DE in terms of automation, analysable protein range, protein solubility etc., have prompted the development of other proteomics

approaches including multi-dimensional protein identification technology (MUDPIT), isotope coded affinity tags (ICAT) or protein-arrays. The major advantage of these technologies is the higher detection sensibility, but quantitative reproducibility in comparative studies with valid statistical data has still to be evaluated. The ability to quantitatively analyse changes in the protein expression profile and to characterise protein modifications makes the 2-DE approach the method of choice for proteomics studies (Rabilloud, 2002).

The major limitations of the 2-DE include (1) the dynamic protein expression range that complicates the visualisation of low abundant proteins, (2) the poor solubility of hydrophobic proteins, and (3) the analysis of proteins with extreme basic or acidic pH, extreme low or high molecular weight or extremely hydrophobic proteins (Görg, 2000; Rabilloud, 1998; Görg *et al*., 1999). Problems concerning pH gradients instability and reproducibility problem were overwhelmed by the introduction of the "immobilines" for the isoelectric focussing (IEF) (Bjellqvist *et al*., 1982; Görg *et al*., 1988). The separation of complex protein samples has experienced an improvement of at least one order of magnitude using a combination of narrow pH gradients and sample pre-fractionation. It has been demonstrated that the sub-cellular fractionation of proteins prior to 2-DE increases the number of visualisable proteins of one organism. In addition, the pre-fractionation provides information about sub-cellular localization and biological function of the proteins (Corthals *et al*., 1997; Rabilloud, 2002).

In the case of bacterial pathogens, proteomics can be employed to compare protein expression profiles under various conditions, thereby identifying proteins that are related with antibiotic resistances, determining novel targets for drug design and the effects of these drugs on cellular physiology or mode of pathogenicity (Williams, 1999; Cordwell *et al*., 2001). Membrane and surface proteins are the interface between the organism and the surrounding medium. In pathogenic bacteria they play an important role in various mechanisms of pathogenicity (e.g. adhesins, pore-forming channels for the secretion of toxins and other virulence factors, antibiotic resistance). The study of membrane proteins is known to be difficult due to their intrinsically hydrophobic nature and to the number of transmembrane regions which makes the solubilisation of these molecules for 2-DE very challenging. However, several solubilising agents have been developed and more membrane proteins have become accessible (Rabilloud, 1998; Santoni *et al*., 2000). Secreted proteins are important for several pathogenic processes. The analysis of these proteins by 2-DE can be difficult due to the presence of contaminating proteins from undefined sources (e.g. medium components), in addition the expression of extracellular proteins is very sensitive to any change in the growth conditions and it is closely controlled by the growth phase of the culture making the extracellular protein patterns very variable. Therefore, extracellular proteins from

bacterial sources are not commonly compared despite the valuable information that this fraction contains (Cordwell *et al.*, 2001; Nouwens *et al.*, 2003a).
Despite all difficulties an enormous progress has been made to elucidate the regulation of mRNA and proteins in response to altered genetic or biological conditions. Therefore, the combination of global approaches such as proteomics and transcriptomics appears to be most promising to improve our understanding of the microbial universe.

1.5 Aims of this work

The main objective of the presented work was to identify quorum-sensing regulated and surface-induced genes of *P. aeruginosa*, *P. putida* and *B. cenocepacia* by means of comparative proteome analysis. These strains were chosen because of their intensively studied QS systems and the availability of defined AHL-negative mutants. Further on, down stream experiments were planned to verify the biological function of QS regulated or biofilm-specific proteins.
An initial extended proteome analysis of the *P. aeruginosa* PAO1 quorum-sensing regulon aimed on the identification of QS regulated genes and served as reference map to determine (1) proteins regulated directly by the QS superregulators GacA and Vrf by comparing PAO1 wild type and respective mutant strains, (2) QS regulated proteins involved in the pathogenicity of *P. aeruginosa* in cystic fibrosis patients by comparing clinical isolates with the type strain PAO1 and to (3) perform a target validation of the quorum-sensing inhibitor compound furanone C30 by a comparison of treated and untreated PAO1 wild type.
In *B. cenocepacia* H111 the *cep*-system controls various phenotypes. Another goal of this work was to compare the protein pattern of QS negative mutant and wild type strain to identify additional QS regulated proteins in *B. cenocepacia*.
In *P. putida* IsoF biofilm development is influenced by QS. In order to find surface-induced genes an appropriate biofilm growth model should be established and 2-DE patterns of planktonic and biofilm cells should be compared. A comparative proteome analysis of an AHL-deficient mutant with *P. putida* IsoF wild type should consequently elucidate which of the surface induced proteins are in addition QS regulated.

2 Materials and methods

2.1 Bacterial strains

Table 1 Bacterial strains

Organism	Relevant genotype and characteristics	Growth Medium	Reference
Pseudomonas aeruginosa			
PAO1	Wild type, *Pseudomonas*	ABC+ CASA	Holloway, 1955
MH710	PAO1 *lasI rhlI* double mutant	ABC+ CASA	Hentzer *et al.*, 2003a
TB10839	Clinical isolate	LB	Kiewitz and Tümmler 2000
TB121838	Clinical isolate	LB	Kiewitz and Tümmler 2000
PAO1-GAC	PAO1 *gacA* mutant	ABC+ CASA	M. Givskov, Laboratory collection
PAO1-MVFR	PAO1 *mvfR* mutant	ABC+ CASA	M., Givskov, Laboratory collection
Pseudomonas putida			
IsoF	Wild type isolate from tomato rhizosphere	LB/ABG	Steidle *et al.*, 2001
F117	IsoF *ppuI* mutant	LB	Steidle *et al.*, 2001
Burkholderia cepacia			
H111	Clinical isolate	ABC/LB	Römling *et al.*, 1994
H111-I	H111 *cepI* mutant	ABC/LB	Huber *et al.*, 2001
Escherichia coli			
C600		LB	L. Eberl, Laboratory collection.

2.2 Cultivation of organisms: Planktonically and biofilm

ABt minimal medium (Clark and Maaløe, 1967) was prepared with H_2O_{MQ}. Modified Luria Bertani (LB) medium (Andersen *et al.*, 1998) was either prepared with tap water (for *P. aeruginosa* TB clone isolates) or with distilled water (for *P. putida*). All media were autoclaved for 20 min at 121°C and a pressure of 1.2 bar. For agar plates the media were supplemented with 1.5% agar.

2.2.1 *Pseudomonas aeruginosa*

PAO1 wild type and *lasI rhlI* double mutant strains were grown in 1.5 l ABt minimal medium supplemented with 20 ml 1 M citrate (ABC) and 5ml/l of a 20% Casamino-acids solution(Becton, Dickinson and Company) (ABC+CASA). The *lasI rhlI* double mutant was complemented by addition of 2 µM N-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) and 2 µM N-butanoyl-homoserine lactone (C4-HSL) to the growth medium ABC+CASA. The quorum-sensing inhibitor furanone C30 was added to the growth medium in a final concentration of 10µM. Reproducible growth experiments were performed in the following way:
15 ml of an overnight pre-inoculum (OD_{600} 2.0) in ABC+CASA of PAO1 and *lasI rhlI* double mutant respectively were added to 600 ml of sterilised ABC+CASA
At OD_{600} 0.3 the cultures were divided, 1200 ml ABC+CASA and the respective additives (AHL molecules and furanone) were supplied resulting in 1500 ml of the following cultures:

PAO1 wild type
PAO1 wild type + 10 µM furanone C30 (added each hour)
lasI rhlI double mutant
lasI rhlI double mutant + 2 µM 3-oxo-C12-HSL + 2 µM C4-HSL (added once)

The strains were grown until the end of the exponential growth phase (OD_{600} 1.2) under vigorous agitation (250 rpm) at 37°C (appendix 1).
TB clone isolates TB121838 and TB10839 were grown in 1.5 l LB medium at 250 rpm and 37°C until the late exponential growth phase (OD_{600} 2.5) (Buschmann, 2004; appendix 2).
Strains PAO1-GAC and PAO1-MVFR were grown in 1.5 l ABC+CASA until the end of the exponential growth phase (OD_{600} 1.5) under vigorous agitation (250 rpm) at 37°C. Additionally, the control strains PAO1 wild type and *lasI rhlI* double mutant were grown in parallel under the same conditions until OD_{600} 1.2.

2.2.2 Burkholderia cenocepacia

In order to study the quorum-sensing regulon of *B cenocepacia* the wild type H111 and H111-I *cepI* mutant strains were grown in 1 l ABC medium. Complementation of the *cepI* mutant was accomplished by the addition of 1 µM octanoyl-homoserin lactone (C8-HSL). The strains were grown until OD_{600} 2.0 (Hoboth, 2002; appendix 3).

2.2.3 Pseudomonas putida

P. putida IsoF wild type and F117 *ppuI* mutant strains were grown in 1.5 l LB until the end of the exponential growth phase (OD_{600} 2.0) under vigorous agitation (250 rpm) at 30°C. Additionally, F117 was grown in LB supplemented with 2 µM 3-oxo-C12-HSL (Steidle, 2002). The planktonic growth was compared with the silicone tubes model for biofilm growth (see 2.2.4.2).

For comparison of planktonically growth and glass wool biofilm grown cells (see 2.2.4.1), *P. putida* IsoF wild type was grown in 500 ml of ABt minimal medium supplemented with 10 ml of 1 M glucose (ABG) under vigorous agitation (250 rpm) at 30°C.

2.2.4 Biofilm models

To investigate the biofilm growth of *P. putida* two models systems were employed, the glass wool model with ABG minimal and the silicone tubes model using LB medium. Microtitre plates (Nunc, Roskilde, Dk) were used to investigate anaerobic biofilm growth.

2.2.4.1 Biofilm growth in glass wool

Glass wool	2.5 g of sterilised glass wool Ø 15µm (Merck*****) per 100 ml ABG medium in an 500ml Erlenmeyer flask

***** Diameter and total surface area of the glass wool should be constant.

Glass wool was cleaned with boiling water from any fat or chemicals that can inhibit the attachment of the cells and sterilised at 180°C for 2-3 h as described by Steyn *et al.* (2001). 0.1 ml of a 24 h pre-inoculum of *P. putida* IsoF in ABG medium was added to 100 ml of ABG medium with 2.5 g of dried glass wool (final cell number = $4x10^6$ CFU/ml). The cultures were incubated 18 h at 30°C without agitation.

2.2.4.2 Biofilm growth in silicone tubes

Reagents and equipment

		Diameter Internal x External	
Silicone tubes Novodirect	**1**	1x3 mm	Bubble trap tubes
	2	2x6 mm	Medium and waste tubes
	3	3x6 mm	Biofilm tubes
Connectors Novodirect	**A**	2-1 mm	
	B	3-1 mm	
	C	3-2 mm	
	T	2-2-2 mm	
Peristaltic pump	205U/CA12 Watson Marlow		
Marprene tubes	Marprene tubes made for 205UCA pump		
Hypodermic needles	Ø 0.6 x 30mm		
Silicone	Silicone rubber transparent		
Sterilised filters	Membrane filters 0.2µm		
Sodium Hypochlorite	0.5% in sterilised H_2O_{MQ}		

Biofilm system assembly and procedure (Figure 2) (Christensen *et al.*, 1999, modified).
For filling up the system with liquid a high flow rate was used (40 rpm). When the bubble traps were overflowing the high flow was kept for 5min and then reduced to 20 rpm (7 ml/100 sec), the traps were closed with the stoppers and the tubes were consequently filled up. The flow was kept for 5-10 min.
The sterilisation was done with 2 l 0.5% hypochlorite pumped through the system during 4h, first for 3h at 5rpm (1 ml/ min) and then for 1 h at 10 rpm (1,75 ml/ min).
The hypochlorite was washed out of the system using 8 l sterile H_2O_{MQ}. The water flow was kept over night at 5rpm (1 ml/ min). Then the system was connected to the medium bottle and filled up, the medium flow was kept at 5rpm (1 ml/ min) for 30 min before inoculation.
8 channels of the pump were connected to a bottle with 10 l LB medium and 4 channels were connected to a bottle with 5 l LB medium supplemented with 2 µM 3-oxo-C12-HSL to complement the mutant. Before the inoculation, the flow was stopped. An O/N pre-inoculum of *P. putida* IsoF and F117 was used to inoculate each tube (4 tubes per strain) with 20 ml of culture.

After 3h (cell attachment to the tube surface) the flow was set to 0,5rpm (0,1 ml/ min) for 1h. In order to keep the flow faster than the cells can divide, the dilution rate (D) should exceed the instantaneous growth rate constant (**µ**) thus, the planktonic cells are washed out and only surface-attached cells remain (Heersink, 2002; White, 1995). The biofilm was grown at 1rpm (0,2 ml/ min) at 30°C for approx. 60 h. **µ** and D were calculated as described by White (1995).

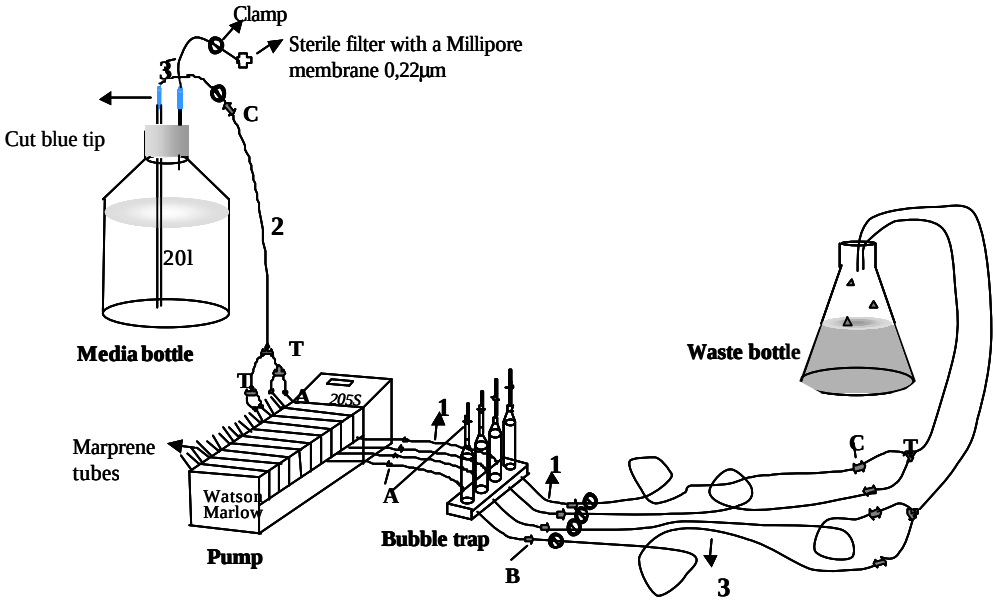


Figure 2 Schematic drawing of the silicone tube biofilm growth model system.
The components of the system are: (1) 15 l bottle with 10 l LB medium connected with two silicone tubes N°2 (Ø2 mm), one to dispense the medium and the second connected to a filter that provides the bottle with sterile air, (2) a peristaltic pump with marprene tubes, 3) bubble traps connected using silicone tubes N°1 (Ø1 mm), 4) biofilm tubes N°3(Ø3 mm, 100 cm) and (5) waste bottle.

2.2.4.3 Microtitre plates

P. aeruginosa PAO1 wild type and *lasI rhlI* double mutant in absence and presence of 2 µM 3-oxo-C12-HSL and 2 µM C4-HSL were grown in 30 ml ABC medium. 1 ml of these cultures was used to inoculate 30ml ABA medium (ABt + 10 mM arginine). The mutant was grown in absence and presence of 2µM 3-oxo-C12-HSL and 2µM C4-HSL. The medium was supplemented with 300 µl 20% KNO_3 and 12 µl Resazurin 0,5% to enable and monitor anaerobic growth in the microtitre plates. The cultures were incubated at 37°C with vigorous agitation. When the OD was 1.0 (after approx. 5 h) 100 µl of each strain were pipetted into the wells (32 wells per strain) of a microtitre plate. The microtitre plates were sealed with microseal® film and incubated at 30°C for 40 h. The medium was then removed and 100 µl of 0,1% crystal violet were added. After 30 min the colorant was removed and the wells were washed. For quantification the crystal violet was solubilized in DMSO and the absorbance of a mixture with 750 µl ethanol was measured at 570nm.

2.2.5 Haem acquisition

Reagents

Iron chelator 2,2' dipyridyl Sigma Aldrich	1 M (20% ETOH /H_2O_{MQ})

Utilization of haemoglobin was tested growing *P. aeruginosa* PAO1 wild type and *lasI rhlI* double mutant (in absence and presence of 2 µM 3-oxo-C12-HSL and 2 µM C4-HSL) using: (a) LB medium supplemented with 4 mM of free iron chelator 2,2' dipyridyl and 0.5 µM of bovine haemoglobin (Sigma) (LBH). Growth was monitored spectrophotometrically as described in appendix 1 and (b) LBH agar plates with 4 mM of free iron chelator 2,2' dipyridyl and 0.4 µM of bovine haemoglobin incubated 36 h at 37ºC.

2.3 Fractionation of cellular proteins

Sample fractionation prior to two-dimensional gel electrophoresis reduces the complexity of protein extracts, improves the visualisation of the protein spots and simplifies the analyses (Corthals *et al.*, 1997). Three different protocols were used to obtain intracellular, extracellular and surface-bound protein fractions (appendix 1).

Reagents

Protease inhibitor	Protease inhibitor cocktail®, Roche
Glycine buffer	0.2 M glycine pH: 2.2 with HCl
Rehydration buffer* ?	8 M urea 2% CHAPS 0.5% (v/v) IPG-buffer corresponding pH
Resolving buffer*?	30mM Tris 7M Urea 2M Thiourea 4% CHAPS
Phenol	Equilibrated phenol pH 8.0 ultrapure USB

*These solutions should not be warmed up.

? 0.5g Serdolit were added to 50ml of rehydration buffer without IPG buffer/resolving buffer, stirred for 10min and filtered.

2.3.1 Sample preparation from planktonically grown cells.

1.5 l culture was divided to obtain different protein fractions. First approx. 200–400ml (1-2 g of cells) were separated and used for the intracellular fraction. The remaining culture was used for the preparation of cell surface proteins. Cells were harvested by centrifugation at 5000 rpm for 30 min and the supernatant of the 1.5 l was collected and used for the extracellular fraction (appendix 1).

2.3.1.1 Intracellular proteins

The pellet was washed twice with 0.9% NaCl, resuspended in 7-10 ml 50 mM Tris HCl pH 7.5 supplemented with 1/3 tablet of protease inhibitor. Cells lysis was performed in a

sonicator (UP 200S Dr. Hielscher) with approximately 40% amplitude for 2 min using the 1.5 cm tip. Cell debris was removed by centrifugation at 4°C and 12.000 rpm for 1 h and the supernatant was divided in aliquots of 1ml.

2.3.1.2 Cells surface proteins[1]

Surface-bound proteins were extracted as described by Nilsson *et al.* (2000). The protocol was modified as follows:
4g of cells were resuspended in 100ml of glycine buffer and stirred for 15 min at 20°C. Cell debris was removed by centrifugation at 7000rpm for 15 min, the pH of the supernatant was adjusted > 7.5 with 10 N NaOH. 3 volumes of acetone were added to precipitate the proteins O/N. The pellet was recovered by centrifugation at 12000 rpm during for 15 min and washed twice with ethanol and once with acetone.

2.3.1.3 Precipitation of extracellular proteins[1]

The supernatant proteins were precipitated using 15% of trichloroacetic acid (TCA) and stirred O/N at 4°C. The pellet was recovered by centrifugation at 4°C 5000 rpm for 1.5 h and washed twice with ethanol and once with acetone.

2.3.2 Phenol extraction

1ml aliquots from the protein extracts were mixed by vortexing with 1ml phenol and incubated for 10 min at 70°C. The sample was cooled on ice for 5 min and the phases were separated by centrifugation at 4°C 5000 rpm for 10 min. The aqueous phase was discarded and 1ml of H_2O_{MQ} was added. This procedure was repeated once and proteins were precipitated with 1 ml of ice-cold acetone. The pellet was recovered by centrifugation at 4°C 15000 rpm for 20 min and washed with 1 ml of acetone centrifuging for 10 min at 4°C 15000 rpm. After the pellet was dried and pulverised the proteins were resuspended in the appropriated buffer (appendix 1).

Intracellular proteins	→	Rehydration buffer pH 4-7
Extracellular and surface-bound proteins	→	Rehydration buffer pH 3-10
Proteins for DIGE labelling	→	Resolving buffer
Biofilm surface proteins	→	Resolving buffer

[1] For the surface and extracellular proteins the pellet was dried, pulverised and resuspended in 3-7ml of 50mM Tris/HCl pH 7.5.

2.3.3 Preparation of biofilm cells

2.3.3.1 Glass wool model[2]

Glass pearls	45 g sterilised glass pearls (5mm diameter approx.) per 2.5 g glass wool in a 1l Schott bottle

The glass wool was removed from the ABG medium (see: 2.2.4.1) and soaked in 0.9% NaCl. This washing step was repeated twice and the glass wool was dried smoothly with filter paper. The washing step was repeated once more and finally the glass wool was dried for 5-10 min on filter paper.
3 times 2.5 g of glass wool were collected in a 1 l bottle containing 135 g of glass pearls. 300 ml of 50 mM Tris/HCl pH 7.5 were added and the flask was strongly shaken by hand for 30 min. The liquid was transferred to a tube and the broken pieces of glass wool were left to sink for 5 min. The supernatant was transferred with a pipette into a centrifugation tube.

2.3.3.2 Silicone tubes model[2]

After 60 h of biofilm growth, the medium flow was increased to 5rpm (1 ml/min) for 10 min to remove the planktonic cells. Afterwards the medium was replaced by 2 l 0.9% NaCl. When the tubes were filled with NaCl the flow was stopped for 10 min, during this time the cells detach from the tube surface. The flow (2 rpm, approx. 0.5 ml/min) was started again while the tubes were nipped one by one. In-between air was allowed to come into the system to drag the cells that still remained attached to the tubes. The biofilm was collected in centrifugation tubes containing distilled water and ½ tablet of protease inhibitor (Protease inhibitor cocktail®, Roche).

2.4 Protein biochemical methods

2.4.1 SDS- Polyacrylamide gel electrophoresis (SDS-PAGE)

To evaluate quality (purity and integrity) concentration of the protein extracts and for the further western blot studies, SDS-PAGE was used (Laemmli, 1970). Proteins were separated in 12% SDS-polyacrylamide gels and stained by Colloidale Coomassie (see 2.5.6.2).

2.4.2 Western Blot

Proteins were separated by SDS–PAGE and blotted to a PVDF Immobilon-P transefer membran (Millipore) in a semi-dry transfer chamber Multiphore II (Amersham Biosciences).

[2] For the fractionation of the samples the same procedure described for the planktonic cells was used.

Immune reaction was made as described by Blake *et al.* (1984). *S. marcescens* HasA antibodies (Létoffé *et al.*, 1994) were cleaned using concentrated of *E. coli* C600 cell extract. After cleaning the HasA antibodies were diluted 2000-fold for the cross-reaction. FliD antibodies (Arora *et al.*, 2000) were diluted 6000-fold.

2.4.3 Quantification of protein concentration

Coomassie-Brilliant Blue	Coomassie Plus protein assay reagent Pierce, Rockford, IL, USA

The fixed-complex formed between the Coomassie-Brilliant Blue and the protein amino groups render the proteins visible and the concentration can be measured at the reagent's maximum absorption (595nm) (Bradford, 1976). The protein concentration is proportional to the absorption and can be calculated using to compare a Bovine Serum Albumin (BSA) standard curve.

A spectral photometer (LKB Ultrospec plus, Pharmacia biotech) was used for the quantification, which was performed as described in appendix 1.

2.4.4 Quantification of enzymatic activities and exoproducts.

2.4.4.1 Chitinase activity

The chitinolytic activity can be determined using carboxymethyl-chitin-remazol brilliant violet (CM-Chitin-RBV). Once the chitin is degraded the violet colour is released from the complex and it can be measured spectrophotometrically at 550nm. 150 µl of culture supernatant[3] was incubated 3h at 37°C with 0.2% CM-Chitin-RBV and 300 µl $NaHPO_4$-buffer 100 mM (pH 6.0). The intensity of the violet colour in the supernatant is proportional to the chitinase activity.

2.4.4.2 Production of pyoverdin

Pyoverdin is a fluorescent siderophore secreted by *P. aeruginosa*. Differences in pyoverdin production were monitored spectrophotometrically at 380 nm measuring the green-/yellow colouring of the culture supernatant[3].

2.4.4.3 Total protease activity

Proteolytic activity was determined incubating the culture supernatant[3] of *P. aeruginosa* strains with 2% Azoalbumin. Production of yellow colour after the liberation of the azo-group is proportional to the protease activity and can be measured spectrophotometrically at 440nm (Ayora and Götz, 1994)

[3] Culture supernatants were rescued after cell harvesting and filter-sterilized.

2.4.4.4 LasA activity

LasA is a *P. aeruginosa* metalloprotease, its activity can be determined monitoring the lysis of *Staphylococcus aureus*. The culture supernatant[3] of *P. aeruginosa* strains was incubated with *S. aureus* cells resuspended in 10mM sodium-phosphate buffer pH 7.5. Decrease of the cell density indicates protease activity and can be measured spectrophotometrically at 600nm.

2.4.4.5 Elastase activity

P. aeruginosa elastase (LasB) is able to degrade biological tissues and cell components including collagen, fibrin and elastin. The culture supernatant[3] of *P. aeruginosa* strains was mixed with Elastin-Congored (Sigma) (10mg /ml in 50mM Tris HCl, pH 7.0). The liberation of Congored after the elastin degradation can be measured spectrophotometrically at 440nm.

2.4.4.6 Lactoferrin degradation by PrpL

PrpL endoprotease is able to cleave lactoferrin, transferrin, elastin and casein. PprL activity was measured incubating the culture supernatant[3] of the *P. aeruginosa* strains with lactoferrin (Sigma) (Wilderman *et al.*, 2001). 1mM EDTA was used to inihibit metalloproteases (LasA, LasB and AprA) and Pefablock (Merck) to inhibit the serine protease PrpL (control). Analysis of the PrpL activity was performed as described in appendix 1.

2.5 Two-dimensional electrophoresis (2-DE)

2-DE resolves complex mixtures of proteins according to their isoelectric point (pI) by isoelectric focusing (IEF, first dimension) and according to their molecular mass (second dimension) by SDS-PAGE (O'Farrell, 1975).

There are three basic steps for the sample preparation used for 2-DE analyses, 1. obtaining of the desired protein fraction (see 2.3), 2. inactivation or removal of interfering substances. In this case protease inhibitor was used and DNA, salts and some polysaccharides were removed using a phenol/water solvent extraction, and 3. protein solubilisation. The proteins were solubilized in rehydration buffer. In the case of surface proteins from biofilm a buffer with thiourea was necessary. The solubility of all the proteins from one fraction was not guaranteed but the protocols render good reproducibility in the 2-DE gels and a good sample concentration (3-9µg/µl in approx. 200 µl). (Görg *et. al.*, 2000).

2.5.1 First dimension: isoelectric focussing (IEF)

Reagents and equipment

IPG buffer Amersham Biosciences	pH 3-10 NL, pH 4-7, pH6-11
Rehydration buffer*? ?	8 M Urea 2% CHAPS 0.5% (v/v) IPG-buffer with corresponding pH. pH 3-10 NL, 4-7, 6-11
IPG stripes (pH 3-10 NL, 4-7, 6-11)	Immobiline DryStripes Amersham Biosciences
Ceramic strip holders	IPGphor strip holders
Isoelectric focussing unit	IPGphor Amersham Biosciences
DDT	Dithiothreitol plusone Amersham Biosciences

*This solution should not be warmed up
? DTT-rehydration buffer: 5,6 g DTT /2 ml rehydration buffer
? 0.5 g Serdolit was added to 50ml of rehydration buffer without IPG buffer, stirred for 10 min and filtered.

Isoelectric focussing was performed with the IPGphor (Amersham Biosciences) and IPG stripes (Amersham Biosciences). 250 µg of protein were mixed with DTT-rehydration buffer to obtain a final volume of 350 µl. DTT is a reducing agent which solves disulfide bonds and unfolds the proteins. Sample preparation and IPGphor running were performed as described by Görg *et al*. (2000). IPG strips can be stored at-20°C if they are not directly used after focussing (Table 2).

Table 2 Isoelectric focusing running conditions

Temperature Current max. Sample volume	20°C 0.05 mA per IPG-Strip 350 µl (for 180mm x 3mm strips)	
Current	Time	
30 V	12 hours	Rehydration and sample
200 V 500 V 1000 V 1000 to 8000 V	1 hour 1 hour 1 hour 1 hour (Gradient)	Initial IEF
8000 V	3 hours IPG 3-10NL 4 hours IPG 4-7 IPG 6-11	IEF to the steady-state

2.5.2 Equilibration of the IPG stripes

The focused proteins should be load with SDS to improve the protein transfer from the IPG stripe to the SDS-PAGE. Two equilibration steps are necessary to achieve the sufficient loading with SDS. Urea and glycerol are included into the buffer to diminish the electroendosmotic effect caused by positive charged molecules that can disturb the transfer of SDS-loaded proteins. Iodoacetamide (IAA) is an alkylating agent used to remove the excess of DTT (Görg *et al*., 1987a; Görg *et al*., 1987b).

Reagents and equipment

500 ml equilibration buffer	6 M urea 30% glycerin 2% SDS Applichem 16,7 ml 4x gel buffer MM5.2
IAA	Iodoacetamide Sigma
DTT	Dithiothreitol plusone Amersham Biosciences
Glass tubes with closure	Glass tubes Schott

Two equilibration solutions were used. (1) 1 g DTT/100 ml equilibration buffer and (2) 4 g IAA/100ml equilibration buffer. The stripes were incubated for 15min in each solution with gentle shaking (Görg, *et. al.*, 2000). After equilibration the strips were directly transferred to the second dimension.

2.5.3 Preparation and casting of SDS gels for second dimension.

Reagents and equipment

Polyacrylamid (PAA) solution 30% (T) Applichem*	30% Acryl amide 0,8 % Methyl-bisacrylamide
Ion exchanger resin	1 g Serdolit Serva
4x Gel buffer	1 l 1.5 M Tris (Base) Amersham Biosciences pH 8.8
Overlying buffer	70% EtOH
SDS	10% SDS Appliquem
TEMED	Tetramethylethylendiamine Serva
Ammoniumpersulfat	10 % APS
500 ml Displacing solution	250 ml 4x gel buffer 250 ml Glycerin Bromophenol blue
1 l Storage buffer	250 ml 4x gel buffer 10 ml 10% SDS
Glass plates	Cassette, 200 x 250 mm and 1.5 mm spacer Amersham Biosciences
Gel casting box	DALT Amersham Biosciences

*1 g of Serdolit was added to 1 l PAA, stirred for 10 min and filtered.

A vertical system (DALT, Amersham Biosciences) was used for the casting and running of 2-DE gels; gel thickness was 1.5 mm. The gels were poured as described by Görg *et al.* (2003).

Table 3 Standard volumes for casting 12 2-DE gels

Component	13 % (T)	11 % (T)
PAA	563 ml	477 ml
4x gelbuffer	325 ml	325 ml
10% SDS-solution	13 ml	13 ml
H_2O_{MQ}	393 ml	479 ml
10% APS	6,5 ml	6,5 ml
TEMED	429 µl	429 µl

2.5.4 Second dimension: SDS-PAGE

Reagents and equipment

Electrophoresis buffer	60.4 g Tris (Base) Amersham Biosciences 288.4 g glycine Amersham Biosciences 20 g SDS Applichem
Agarose Ultrapure	
Bromophenol blue	
MWM	Molecular weight marker Biorad
Glass plates holder	
Electrophoresis chamber	DALT vertical electrophoresis system Amersham Biosciences

The strips were rinsed with electrophoresis buffer and inserted between the glass plates in close contact with the SDS gel. 15 µl of MW was applied to a 0,5 x 0,5 cm filter paper and placed to one end of the IPG strip. Strips and marker were sealed with agarose (0,5% in electrophoresis buffer, trace of brompenol blue). The gels were run at 10ºC for 2 h at 60mA followed by 24 h at 18 W.

2.5.5 Fixing

Reagents

2l Fixing solution (Silver staining, Colloidal Coomassie)	800 ml 96% EtOH 200 ml acetic acid (glacial) 1 l H_2O_{MQ}

For every staining protocol (silver staining, Colloidal Coomassie) the gels were fixed for 1h in order to immobilise the proteins in the gel and to remove any compound that can interfere with the subsequent staining.

2.5.6 Visualization

2.5.6.1 Silver staining

This staining technique is more sensitive than some Coomassie techniques. Therefore it was chosen only when very low concentrations of protein were obtained.

washing solution	600 ml EtOH 2 l H_2O_{MQ}	Two times for 20 min
	H_2O_{MQ}	One time for 20 min
sensitizer solution	200 mg Sodium thiosulfate 2 l H_2O_{MQ}	1 min
washing	H_2O_{MQ}	Three times for 20 sec
silver staining solution	2 g Silver nitrate 0.2 ml (37%) Formaldehyde 1 l H_2O_{MQ}	20 min
washing	H_2O_{MQ}	Three times for 20 sec
development solution	5 mg Sodium thiosulfate 30 g Sodium carbonate 0.5 ml (37%) Formaldehyde 1 l H_2O_{MQ}	3-5 min
washing	H_2O_{MQ}	One time for 20 sec

stopping-solution	5 g Glycine 1 l H_2O_{MQ}	5 min
washing	H_2O_{MQ}	Three times for 10 min

2.5.6.2 Colloidal Coomassie staining

As described by Neuhoff *et al.* (1988)

Washing	Distilled water	Two times for 10 sec
Coomassie staining solution	100 g $(NH_4)_2SO_4$ 250 ml Methanol 20 g 85% *o* -phosphoric acid 0.625 g Coomassie-Brilliant Blue G250 Serva 1 l H_2O_{MQ}	O/N
Destaining	H_2O_{MQ}	O/N

2.5.7 Image capturing and gel analysis.

Gels were scanned with a densitometric Image scanner (Amersham Biosciences) and the raw images were analysed using the ImageMaster Elite ® 2-D gel analysis software version 4.0 (Amersham Biosciences) (appendix 1).

2.5.8 Protein identification

2.5.8.1 Mass spectrometry (MALDI-TOF)

The protein identification by mass spectrometry was done by Dr. Gerold Reil (Department of Chemical-technical Analysis and Chemical Food Technology, Technical University of Munich) in cooperation with Prof. Dr. Angelika Görg (Proteomics Department, Technical University of Munich) (appendix 1).

2.5.8.2 N-terminal sequencing

N-terminal sequencing of protein samples was done at the Max Plank Institute of Biochemistry in cooperation with Dr. Dr. habil. Friedrich Lottspeich (appendix 3).

3 Results and discussion

The main aim of this work was to identify quorum-sensing regulated and surface-induced genes of, *Pseudomonas aeruginosa, Pseudomonas putida* and *Burkholderia cenocepacia* by means of comparative proteome analysis.

3.1 Part I- Global analyses of quorum-sensing regulated genes of Pseudomonas aeruginosa

3.1.1 General mapping of quorum-sensing regulated proteins

The quorum-sensing system of *P. aeruginosa* has been extensively analysed over the past few years using different approaches. Impressive amounts of novel information have been generated with transcriptome studies and the comparison of extracellular protein patterns of the *P. aeruginosa* wild type PAO1 with those of an isogenic *lasI rhlI* double mutant by means of 2-D gel electrophoresis (Schuster *et al.*, 2003; Wagner *et al.*, 2003; Hentzer *et al.*, 2003; Nowens *et al.*, 2003). One of the aims of this work was the identification of quorum-sensing regulated genes in an extended proteome analysis. The 2-DE protein patterns of the intracellular, extracellular, and surface protein fractions of the *P. aeruginosa* wild-type PAO1 and the *lasI rhlI* double mutant were compared. Our global analysis showed in total that 723 out of 1971 detected protein spots were differentially expressed (more than 2.5 fold up or down-regulated). When the double mutant was grown in a medium containing AHL signal molecules 23% of all detected spots were rescued to the wild type situation and therefore considered as QS regulated proteins (appendix 1). This is one of the major differences between this proteome analysis and the transcriptomic studies, where the number of regulated genes was significantly lower (2.9% in the study of Hentzer *et al.*, 2003, 6.3% in the study of Schuster *et al.*, 2003, and 11.1% in the study of Wagner *et al.*, 2003). This discrepancy cannot simply be explained by the differences in the interpretation of microarray and proteome data (e.g. differences in the threshold levels used for the definition of QS regulated genes or proteins). Our results clearly show that inactivation of the *P. aeruginosa* PAO1 quorum-sensing system affects the cell's protein composition more strongly than the cell's transcriptome, indicating that a major part of AHL-dependent regulation occurs at the post-transcriptional level. It was shown in two transcriptome analysis that seven of all AHL-induced genes (e.g Clp-protease, LasA, LasB) code for proteins involved in proteolysis, phosphorylation or glycosylation of proteins and that two encode known or putative chaperones (Wagner *et al.*, 2003; Schuster *et al.*, 2003). This indicates that the QS circuitry controls transcription of several genes encoding proteins that in turn will affect expression of various proteins at the post-

transcriptional level. Examples of QS regulated proteases are the ATP-dependent Clp-protease PA3326 that was shown to be AHL-controlled in all three transcriptome analyses as well as in the present proteome analysis. Members of the Clp-family protease are involved in post-translational protein modifications and often modulate the levels of various proteins (Porankiewicz *et al.*, 1999). QS regulated elastase LasB (PA3724) and alkaline protease LasA (PA1871) are involved in the post-transcriptional modification of proteins such as exotoxin ExoS and ExoT (Kamath *et al.*, 1998; Cowell *et al.*, 2003). These proteases cleave at well-defined sequence motifs. Therefore, the known cleavage sites were searched in the translated genome sequence of *P. aeruginosa* PAO1. This analysis revealed that approximately 63% of all proteins are potential targets for LasB or/and LasA, supporting the idea that the two proteases might play important roles in the processing and degradation of proteins in *P. aeruginosa* (appendix 1). Another QS regulated protease found in all studies is the PprL aminopeptidase PA2939, which cleaves amino acids from small peptides providing a source of both carbon and nitrogen to the bacteria (Cahan *et al.*, 2001). PrpL cleaves casein, lactoferrin, transferrin and elastin. It has been reported that strains producing PrpL degrade iron-binding proteins. The ability to scavenge iron from proteins such as lactoferrin contributes to the tissue damage and therefore to the infection (Wilderman *et al.*, 2001). It was clearly shown in this work that the expression of PrpL is dependent on a functional quorum-sensing system (appendix 1: Fig. 4A) supporting the influence of QS in virulence processes.

For the first time twenty of 48 identified proteins were reported as QS regulated (Table 4). 23 were in agreement with the transcriptome analyses (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003) and 5 were also found in a proteomics study (Nowens *et al.*, 2003b).

Table 4 Novel QS regulated proteins

PA No.[a]	Protein Identification[a]	PA No.[a]	Protein Identification[a]
PA0455	RNA helicase DbpA	PA1777	outer membrane protein OprF
PA2659	hypothetical protein	PA2398	ferripyoverdin receptor FpvA
PA3003	hypothetical protein	PA3704	Haeme acquisition HasAp
PA3227	peptidyl-prolyl cis-trans isomerase A PpiA	PA3785	conserved hypothetical protein
PA3250	hypothetical protein	PA4221	Fe(III)-pyochelin receptor FptA
PA4195	binding protein component of ABC transporter	PA4710	outer membrane hemin receptor PhuR
PA4345	hypothetical protein	PA5505	TonB-dependent receptor
PA5494	hypothetical protein	PA0745	enoyl-CoA hydratase/isomerase
PA1087	flagellar hook-associated protein type 3 FlgL	PA4356	xenobiotic reductase XenB
PA1094	flagellar capping protein FliD	PA5078	conserved hypothetical protein

[a]Data generated from peptide mass maps were compared to the complete translated ORFs for *P. aeruginosa* PAO1 (http://www.pseudomonas.com)

Nine of these proteins are products of genes that were identified in all three transcriptome experiments (appendix 1: table 2). The percentage of QS regulated proteins of the "secretome" was significantly higher (36%) than those of the intracellular (17%) or surface (25%) protein fractions (Figure 3 A, B, C). These data are congruent with previous studies, which have shown that inactivation of the QS system results in the down regulation of the type II Xcp secretion system (Chapon-Herve et al., 1997) and the reduced expression of many extracellular virulence factors including proteases, chitinase and lipase (Passador et al., 1993; Pearson et al., 1997).

On the other hand there are some notable disagreements between the present "secretome" analysis and the one performed by Nouwens *et al*. (2003). While the latter study reported that the most abundant protein in the supernatant of PAO1 is the LasB protease, the major protein spots in this study were identified as HasAp, which was not at all detected by Nouwens *et al*. (2003). Out of 27 proteins identified as AHL regulated by Nouwens et al. (2003), only 10 were also identified in the current study. Most strikingly, three of these 10 proteins were found to be oppositely regulated. These were identified as structural components of the *P. aeruginosa* flagellar apparatus FliC and FlgK and the arginine/ornithine binding protein AotJ. Although further work will be required to address this issue in better detail it is conceivable that some of the observed discrepancies are probably caused by differences in the experimental protocols [e.g. complex Luria–Bertani medium used by Nouwens et al. (2003) in contrast to minimal medium supplemented with citrate and casamino acids in this study]. In fact, the expression of several extracellular enzymes in *P. aeruginosa*, e.g. LasB elastase (Ohman *et al*., 1980) or lipase (Gilbert *et al*., 1991), has previously been demonstrated to be strongly dependent on medium composition. The flagellar capping protein FliD was found to be positively QS regulated in this study. FliD is responsible for mucin adhesion, thus playing an important role in the initial colonization of *P. aeruginosa* in the airways of cystic fibrosis patients (Scharfman *et al*., 2001).

A western blot analysis using anti-FliD antibodies was performed to evaluate the expression of this flagella component and two QS-regulated effects can be observed: one directly on the transcription or translation of FliD and another on the proteolytic degradation of the protein (Figure 4). FliD is a 49,4 kDa protein, and it has been reported that two regulatory mechanisms influence the *fliD* expression (Arora *et al*., 1998). These results suggest that one of the described regulatory systems is AHL-dependent since the expression in the QS mutant is reduced. In the surface fraction a strong band of about 50 kDa is present in PAO1 wild type as well as in the mutant when it is grown in a medium complemented with AHLs. In the *lasI rhlI* mutant a weaker 50 kDa band appears, indicating that the expression of FliD is reduced in this strain.

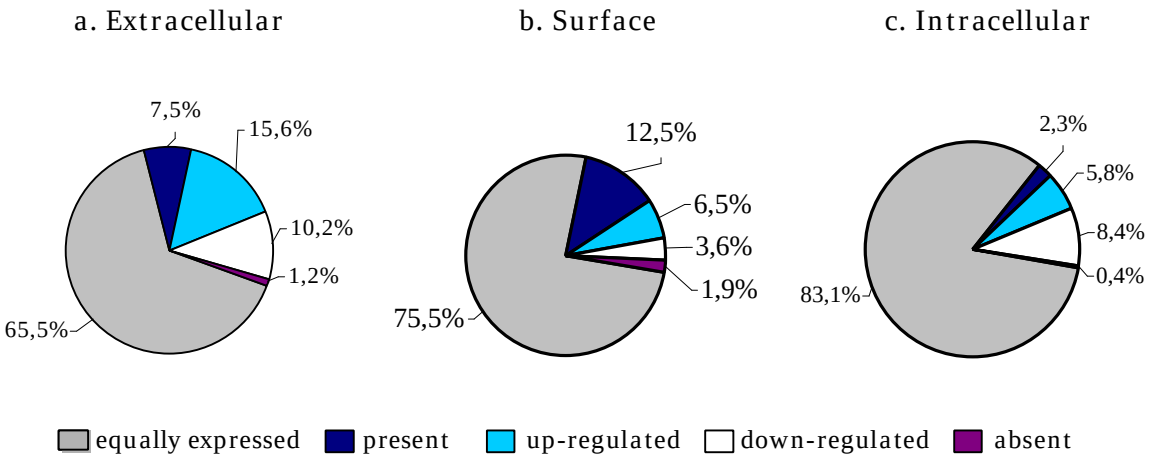


Figure 3 Percentage of quorum-sensing regulated proteins in the *P. aeruginosa* PAO1 wild type. The patterns of three different protein fractions of the wild type and *lasI rhlI* mutant were compared by means of 2-DE. The percentages refer to differentially expressed proteins in the wild type, which expression was restored in the *lasI rhlI* mutant grown in medium complemented with 2 µM 3-oxoC12-HSL and C4HSL.

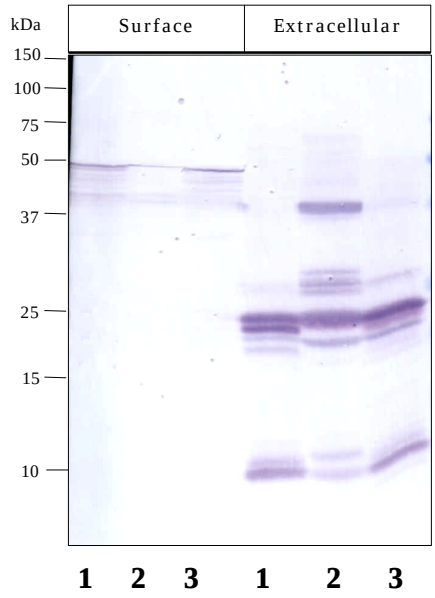


Figure 4 Western blot analysis of FliD expression. Proteins from the surface or extracellular fraction were resolved by SDS-PAGE and probed with anti-FliD-antibodies. 1. PAO1 wild type, 2. *lasI rhlI* mutant 3. *lasI rhlI* mutant + 2 µM 3-oxo-C12

In the western blot with proteins from the extracellular fraction only smaller protein fragments were found, it could be observed that the digestion pattern found in the wild type is restored in the mutant when it is grown in a medium supplemented with AHLs. This indicates that proteases responsible for this degradation process are obviously QS-regulated. The pattern of the QS mutant is different, and an additional band corresponding to a protein bigger than 37 kDa can be detected. The assembly of the cap protein is an exception in the sequential assembly of the flagellum components. It is exported before the completion of the main hook, continually displaced to the end of the growing filament and it is required as a nucleation point for the polymerisation of FliC (flagellin) (Maki *et al.*, 1998, Ikeda *et al.*,1996). In *Salmonella typhimurium* it has been

found that the chaperone FliT (14 kDa protein) binds specifically to the flagellar cap protein FliD before it is exported and self assembled at the end of the flagella (Fraser *et al.*, 1999). It can be speculated that the 37 kDa band present in the mutant is one of the digestion products of FliD still attached to one of the chaperones involved in the export of FliD before the final assembly.

The arginine/ornithine binding protein AotJ was found to be positively QS regulated in this study. The presence of more than one system for uptake of arginine in *P. aeruginosa* has been reported. Under aerobic conditions the arginine succinyltransferase pathway is used, under anaerobic conditions the arginine deaminase pathway is preferred in which arginine is converted to ornithine (Nishijyo *et a*l., 1998). The *aot* operon has been found to be involved in the transport of arginine and ornithine; the *aru* operon encodes enzymes of the arginine succinyltransferase pathway (Itoh, 1997). AotJ is the first product of the *aot* operon and it has been found to be involved in biofilm formation (Sauer *et al.*, 2002; Yoon *et al.*, 2002). An analysis of anaerobically grown biofilms in microtitreplates using AB medium supplemented with arginine as carbon source, clearly demonstrated that PAO1 wild type forms thicker biofilms than the QS deficient mutant, probably due to a lack of arginine uptake of the AHL-negative strain. The defect could be restored by adding AHLs to the medium (Figure 5). This result was in agreement with the positive QS-regulation of AotJ found in the proteome analyses.

The major protein spots in the extracellular fraction were identified as HasAp that was not at all detected by Nouwens et al. (2003) (appendix 1). HasAp is an iron regulated extracellular haem-binding protein required for the utilization of haemoglobin iron. It is synthesised as a 20.5 kDa preprotein but only the processed form is able to bind haem. It has been suggested that the expression of this haemophore is particularly important in the early stage of infection, when the haemoglobin concentration is low and thus a HasAp producing strain will out compete other colonizers by sequestering the available haem (Létoffé *et al.*, 1998). The transcriptome analyses were unable to detect differences between the wild type and the *lasI rhlI* double mutant because the total amount of HasAp isoforms produced does not differ significantly. But in the proteome analyses it was shown that in the wild type the most abundant HasAp band has a molecular mass of 18 kDa corresponding to the active haemophore form (appendix 1: fig. 2 subarea A; fig. 4B and C) while in the mutant the two dominant bands display molecular masses of 19 and 20.5 kDa (appendix 1: fig. SM2). These results suggest that the production of functional HasAp haemophore by *P. aeruginosa* is QS regulated (appendix 1). *P. aeruginosa* has a second haem-acquisition locus, *phu*, which consists of the *phuR* haemin receptor gene and the *phuSTUVW* operon encoding a typical ABC transporter. PhuR was also found to be positively QS regulated in the

proteome analyses (appendix 1). The two systems operate in parallel and thus only inactivation of both systems causes severe defects in haem or haemoglobin utilization. When the *P. aeruginosa* wild type and the *lasI rhlI* mutant were grown in a medium containing haemoglobin as the sole source of iron, it was clearly established that the ability to utilize haemoglobin as iron source is indeed QS-regulated (Figure 6 and appendix 1). The findings of this study support the hypothesis that the quorum-sensing regulatory system is networked with the iron regulatory system as it has been proposed previously by Hassett *et al.* (1999)

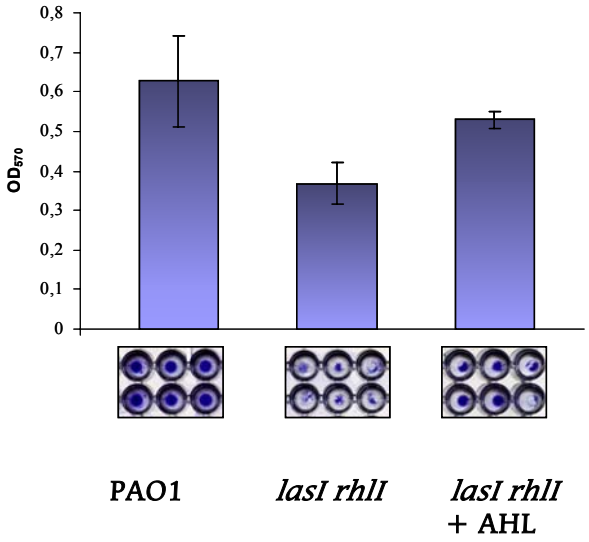


Figure 5 Biofilm formation under anaerobiosis in minimal medium with arginine as sole carbon source.
PAO1 wild type and the *lasI rhlI* double mutant (in the absence or presence of 2 μM 3oxo-C12-HSL and C4-HSL) were grown in the wells of polypropylene microtitre plates. The attached cells were stained with crystal violet. For quantification of the biofilm-assosiated dye the crystal violet was dissolved and the absorbance was determined at 570 nm. Error bars represent the standard deviation of the mean for six independent wells.

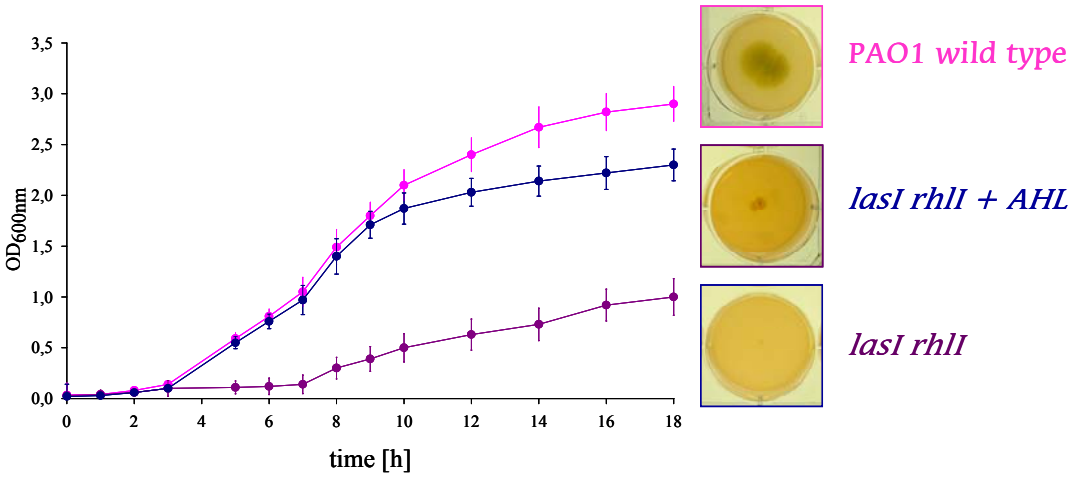


Figure 6 Growth of the PAO1 wild type and the *lasI rhlI* double mutant in medium containing haemoglobin as the sole iron source.
lasI rhlI double was grown in the absence or presence of 2 μM 3oxo-C12-HSL and C4-HSL (liquid culture and agar plates). The curves represent the average from three independent experiments. Error bars represent the standard errors of the means.

Quorum-sensing mutants of *P. aeruginosa* have been extensively analysed with respect to regulated phenotypes over the past few years. However, the recent functional genomics analyses revealed that an unexpected large number of genes are controlled by quorum-sensing, suggesting that many yet unknown functions are AHL regulated. The detailed evaluation of these new functions by phenotypic and biochemical assays is essential to validate the results of the global analyses.

Additionally, in order to study the possibility of antipathogenic drugs development, it was interesting to identify and validate additional targets of the furanone C-30, a synthetic compound that was developed on the basis of natural metabolites produced by the macroalga *Delisea pulchra* (de Nys *et al*., 1993; Givskov *et al*., 1996). Based on the general map of the QS regulated proteins of *P. aeruginosa* the influence of the furanone C-30 on the QS system was evaluated. It has been demonstrated before by transcriptomics that this compound specifically inhibits the quorum-sensing cascade of *P. aeruginosa* (Hentzer *et al*., 2002; 2003). The specificity of furanone C-30 was also demonstrated on the proteome level (appendix 1). Comparing the surface protein patterns of the wild-type PAO1 grown in the presence or absence of furanone C-30, it was observed that the protein pattern of C-30 treated cells strongly resembled the one of the *lasI rhlI* double mutant (appendix 1: Fig. SM1).

Out of the 102 surface protein spots found to be QS controlled in the general mapping 67% were affected by the furanone. The changes in the protein pattern that were not related to QS regulated spots were less than 2.5% giving emphasis to the hypothesis that C-30 specifically interferes with the expression of QS controlled phenotypes (Table 5 and annexo 1: Table SM2).

Table 5 Proteins affected by the quorum-sensing inhibitor C-30

PA No.[a]	Protein Identification[a]	PA No.[a]	Protein Identification[a]
PA0315	hypothetical protein	PA2659	hypothetical protein
PA0460	hypothetical protein	PA3227	PpiA / peptidyl-prolyl cis-trans isomerase A
PA0888	AotJ / arginine/ornithine binding protein	PA3445	conserved hypothetical protein
PA5494	hypothetical protein	PA3931	conserved hypothetical protein
PA0943	hypothetical protein	PA4195	binding component of ABC-transporter
PA1250	AprI / alkaline protease inhibitor	PA4495	hypothetical protein
PA1342	binding component of ABC-transporter	PA4739	conserved hypothetical protein
PA2395	hypothetical protein	PA5481	hypothetical protein
PA244	6 glycine cleavage system protein H2		

[a]Data generated from peptide mass maps were compared to the complete translated ORFs for *P. aeruginosa* PAO1 (http://www.pseudomonas.com)

3.1.2 Impact of the superregulators Gac and Vfr on the QS regulon of *P. aeruginosa*.

The quorum-sensing circuit of *P. aeruginosa* is subject to regulation by a number of global regulators, including Vfr, GacA, RsaL, MvaT, and RpoS. *LasR* expression has been shown to be dependent on Vfr (Albus *et al.*, 1997) and it has been demonstrated that the global activator GacA activates directly or indirectly the expression of *rhlR* (Reimmann *et al.*, 1997). Recently, the global impact of these two superregulators on the gene expression in *P. aeruginosa*, was evaluated by a comparative transcriptome analysis of PAO1 wild type strain and the respective superregulator mutants (PAO1 *gacA* and PAO1 *mvfR*; M. Givskov, personal communication). Surprisingly, no differences in the expression of known quorum-sensing regulated genes could be detected at the transcriptome level. In order to validate these unexpected results a proteome analysis of wild type and mutants was performed. The 2-DE protein patterns of the intracellular, extracellular, and surface protein fractions of the *P. aeruginosa* wild type PAO1, the *mvfr* and the *gacA* mutants were compared. When *gacA* mutant was compared with the wt PAO1, 3% (40 out of 1540 protein spots) of the spots were differentially expressed, from them 27 were identified and 13 proteins were found to be QS regulated (9 identified by MALDI-TOF marked with asterisks, and 4 by comparison with the reference map of *P. aeruginosa* PAO1 QS regulon marked with **a**. in Table 6) as reported in the recent transcriptomics or proteomics studies. In the case of the *mvfr* mutant 5% were differentially expressed (74 out of 1541 protein spots) and 46 were identified, 19 proteins were found to be reported as QS regulated (13 identified by MALDI-TOF (*) and 5 by comparison with the reference map (**a)** in Table 6). Among GacA or MvfR-controlled genes were well known QS regulated virulence factors such as chitinase, flagellin, and endoprotease PrpL were found to be differentially expressed between PAO1 and PAO1 *gacA* or PAO1 *mvfR* (Figure 7 A and B, Figure 8 and appendix 4).

Clearly the proportion of QS regulated proteins affected by mutations of *gacA* or *mvfR* is much lower than the number of proteins where expression was found to be affected by the QS system when the wild type PAO1 was compared with the *lasI rhlI* mutant. Even if all the differentially expressed spots from the superregulator mutants would have been identified as QS regulated, the proportion of proteins affected by the *lasI rhlI* mutation is distinctly higher (Table 7).

Table 6 Identification of differential expressed proteins between wild type and superregulator mutants, *gacA* and *mvfr*. Regulation refers to each one of the indicated mutants. Protein were identified by MALDI-TOF MS peptide mass mapping

PA No[b]	Spot[c]	Protein description[d]	Theor. p*I*[d]	Theor. Mr[d]	Sequence coverage (%)	Average Pract.pI[d]	Average Pract.Mr (kDa)[d]	mvfr Regulation	gacA Regulation
Surface-bound proteins									
PA0122*	SF23	con. hyp. protein	4,7	14,6	37,4	3,5	13,6	absent	
PA0143*	SF24	nonspecific Nuh				7,4	36,7	down	down
PA0283	SF22	sulfate-binding protein precursor *sbp*	8,5	25,9	36,4	8,1	34,7		
PA0315*	SF18	hyp. protein				7,6	15,0	down	
PA1337	SF21	glutaminase-asparaginase *ansB*	6,7	32	38,6	7,7	36,7	down	
PA1342*	SF8	ABC-Transporter	8,5	26,8	33,0	8,1	29,9	down	
PA2575	SF5	hypothetical protein	6	22,2	62,5	7,0	20,2	up	up
PA2623	SF11	isocitrate dehydrogenase *icd*	5,1	21,1	45,5	4,9	43,6	present	absent
PA2952	SF2	electron transfer flavoprotein beta-subunit	9	26,3	32,9	8,9	25,3	up	up
PA3227[a]	SF17	PpiA				7,7	15,8	down	
PA3250[a]	SF9	hyp. protein				5,9	36,8	down	down
PA3450	SF12	probable antioxidant protein	5,3	51,9	24,1	5,5	26,6	up	up
PA3836	SF20	hypothetical protein	7,7	52,9	34,2	7,6	30,3	down	
PA4453	SF6	conserved hypothetical protein	9,1	30,2	23,7	8,7	21,7	down	
PA4468	SF14	superoxide dismutase *sodM*	5,8	30,0	22,4	6,5	20,6	present	present
PA4708	SF3	hypothetical protein	6,9	31,0	25,3	7,8	26,5	down	
PA4739[a]	SF19	con. hyp. protein				7,1	12,9	down	
PA5415	SF7	serine hydroxymethyltransferase	6,1	16,1	44,6	7,9	25,7	down	
Extracellular proteins									
PA0283	SN9 SN25	sbp sulfate-binding protein precursor	8,5 8,5	36,4 36,4	16,6 27,3	8,4 8,0	34,8 36,0	up up	
PA0888*	SN30	arginine/ornithine binding protein AotJ	6,4	27,9	41,3	5,5	26,5	down	down
PA1178*	SN3	outer membrane protein H1 precursor *oprH*	9	21,5	30	9,8	17,4	down	down
PA1777*	SN26	outer membrane protein OprF precursor	5	37,6	11,4	4,8	33,1	down	down
PA3165	SN12	histidinol-phosphate aminotransferase hisC2	5	39,4	10	4,6	43	down	
PA3785*	SN7	conserved hypothetical protein	6,3	16,9	41,1	6,4	17,5	down	

PA4067	SN4	outer membrane protein OprG precursor	4,9	25,2	26,7	4,6	23,4	down	
PA4175*	SN1	Pvds-regulated endoprotease, lysyl classPrpL	6,5	48,2	34	6,5	49,5		down
PA4175*	SN8	Pvds-regulated endoprotease, lysyl classPrpL	6,5	48,2	16,9	6,6	24,7		down
PA4175*	SN14	lysyl class PrpL	6,5	48,2	11,3	5,9	25	down	down
PA4221*	SN17 SN18	fptA Fe(III)-pyochelin receptor precursor-	5,9 5,9	79,9 79,9	30,6 15,7	6,0 4,6	76,4 54,6	down down	
PA4710[a]	SN29 SN28	outer membrane hemin receptor Phu	6,7 6,7	84,7 84,7		6,0 6,2	87,6 87,6	down down	down down
PA5199	SN2	two-component sensor EnvZ	5,9	48,8	21,9	6,0	50,2	up	down
Intracellular proteins									
PA0962	IC22	probable dna-binding stress protein	5	17,55	47,4	5,0	14,7		down
PA1587	IC2 IC19	lipoamide dehydrogenase-glc	6,5 6,5	58,5 50,1	34,5 18,8	6,5 6,6	57,8 57,2	absent absent	down down
PA2300*	IC26 IC27	Chitinase ChiC	5,2 5,2	53 53	33,6 12,3	5,2 5,3	55,6 56	absent absent	down down
PA3309	IC3	conserved hypothetical protein	5,5	16,5	68,2	5,7	17,8	absent	down
PA3326*	IC5	Clp family ATP-dependent protease	5,5	22,1	55,7	5,6	21,4	down	
PA3529	IC20 IC21	probable peroxidase	5,4 5,4	21,8 21,8	26,5 15	5,4 5,5	22,2 22,1	down down	
PA3807	IC17	nucleoside diphosphate kinase *ndk*	5,5	15,6	48,3	5,7	14,1	down	
PA4238	IC6	DNA-directed RNA polymerase alpha chain *rpoA*	4,9	36,6	35,4	5,1	43,1	down	
PA4352	IC1	conserved hypothetical protein	5,9	30,9	24,1	6,3	34,6	absent	down
PA4356[a]	IC7	xenobiotic reductase XenB				5,7	43,5		down
PA4468	IC18	superoxide dismutase *sodM*	5,8	22,5	30	6,1	21,1	down	
PA4932	IC25	rplI 50S ribosomal protein L9 rplI	5,4	15,5	44,6	5,6	14,7	down	
PA4935	IC11	30S ribosomal protein S6 *rpsF*	4,8	16,1	37,4	4,9	14,9	absent	down
PA5178	IC16	conserved hypothetical protein	5,4	15,4	60,7	5,5	13,9	down	down

* Proteins identified by MALDI TOF peptide mass mapping and reported as QS regulated in previously published studies.
[a] Proteins identified as QS regulated by comparison with the QS regulon of *P. aeruginosa* PAO1 (Appendix 1).
[b]Data generated from peptide mass maps were compared to the complete translated ORFs for *P. aeruginosa* PAO1(www.pseudomonas.org)
[c] Spot no. refers to numbers shown in Fig. 7A and B
[d] Isoelectric point and molecular mass obtained in the experiments (practical) and from the databases(theorical)

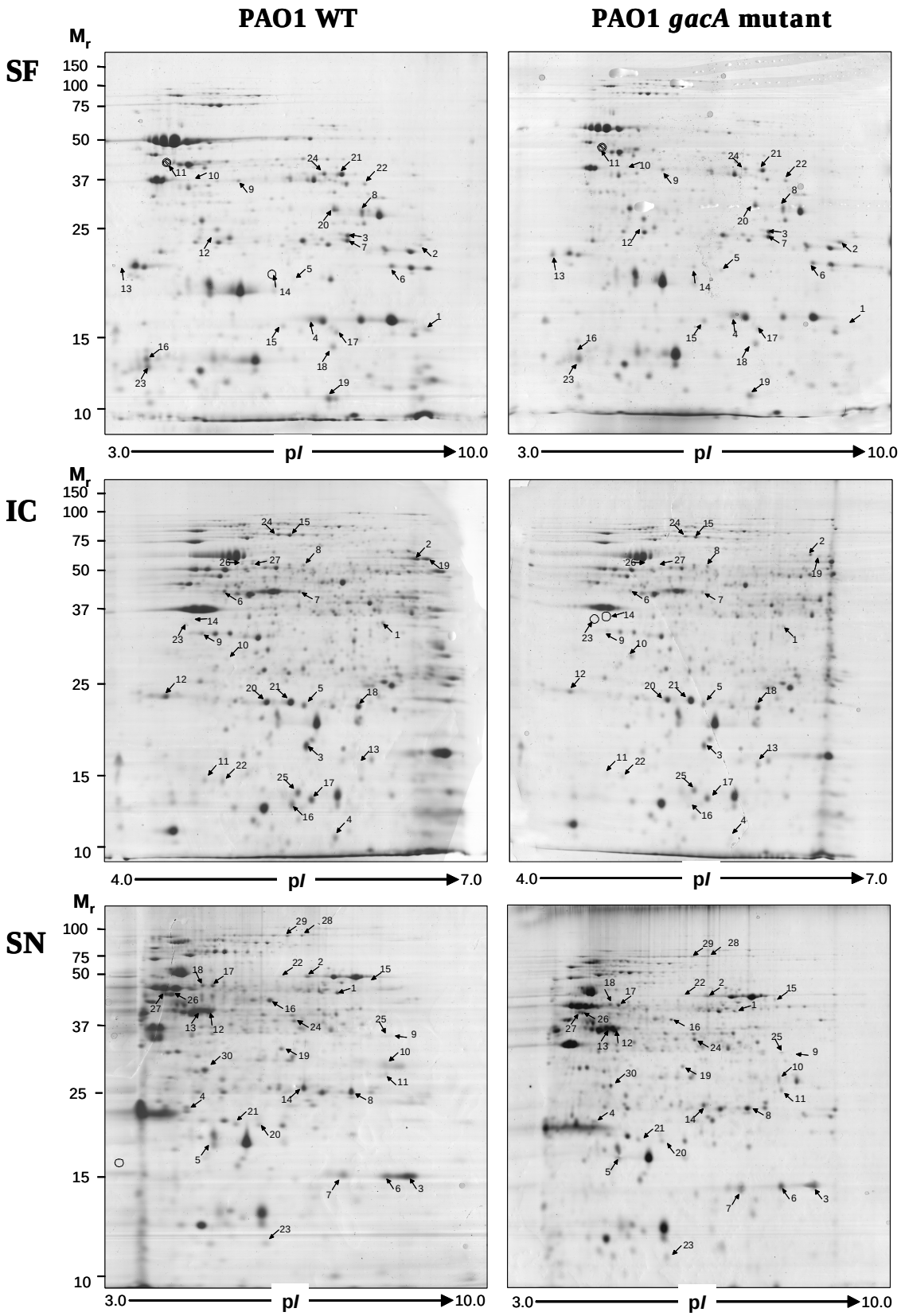


Figure 7 Comparative two-dimensional gel electrophoresis (2-DE) of *P. aeruginosa* PAO1wild type and superregulator mutants.
A: Surface-bound (SF), extracellular (SN) and intaracellular (IC) protein patterns of the *P. aeruginosa* PAO1wild type compared with PAO *gacA* mutant. Dry strips with pH gradients from 4-7 or nonlinear 3 to 10, followed by an SDS PAGE on 13% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250. ⊗ N° 11 Only present in *mvfr* mutant.

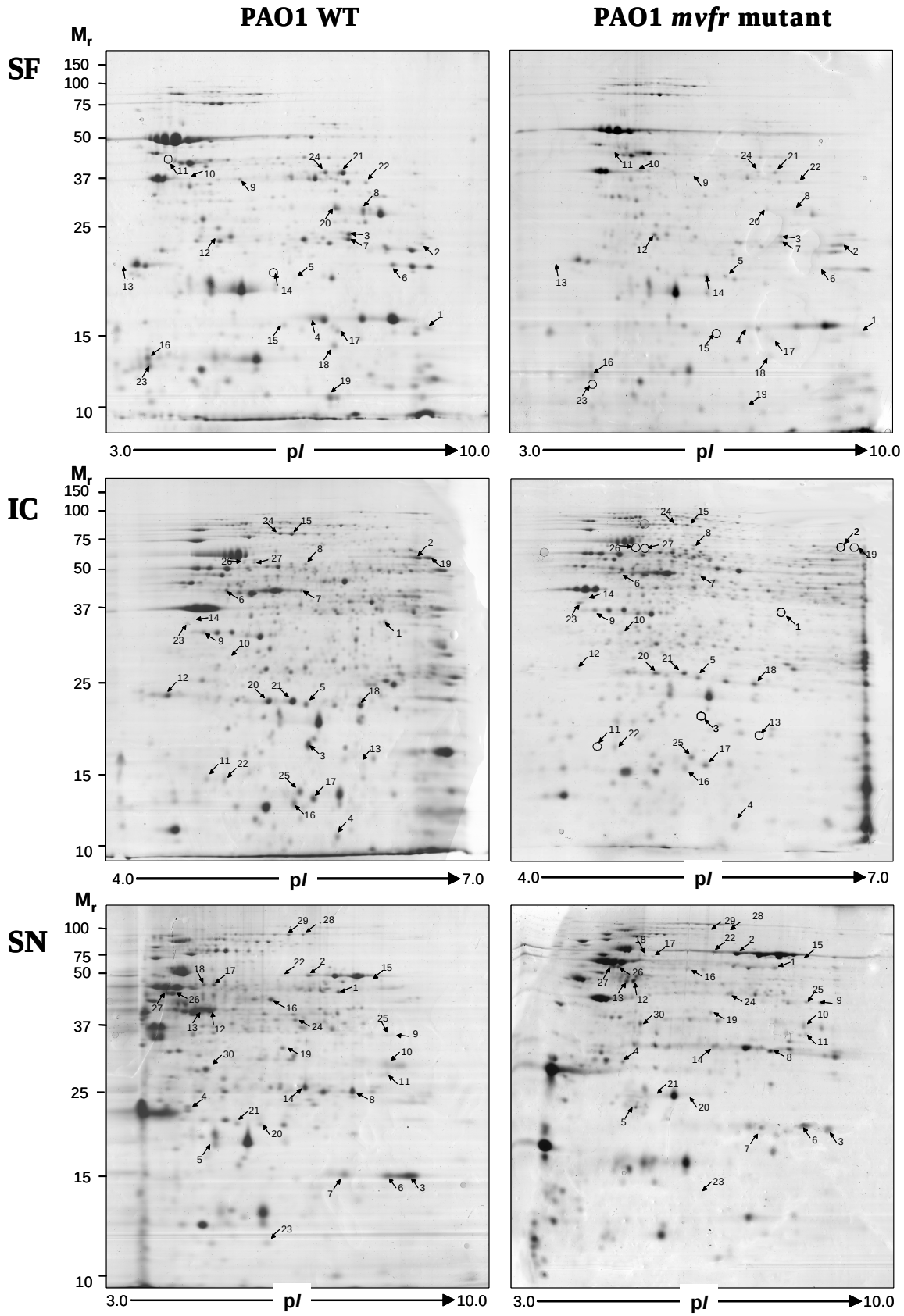


Figure 7. Comparative two-dimensional gel electrophoresis (2-DE) of *P. aeruginosa* PAO1wild type and superregulator mutants.
B: Surface-bound (SF), extracellular (SN) and intaracellular (IC) protein patterns of the *P. aeruginosa* PAO1wild type compared with PAO *mvfr* mutant. Dry strips with pH gradients from 4-7 or nonlinear 3 to 10, followed by an SDS PAGE on 13% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250.

Table 7 Global comparison of differentially expressed proteins between GacA, MvfR and *lasI rhlI* mutants.

	Differentially expressed	**Identified[a]**	**Identified as QS regulated[b]**
gacA	40	26	13
mvfR	74	46	19
lasI rhlI	467*	48	47

*****All quorum-sensing regulated protein spots (Appendix1).
[a] Identified by MALDI TOF peptide mass mapping or by comparison with the reference map of the QS regulon (Appendix 1).
[b] Reported as QS regulated in previously published studies Marked with * or [a] in the Table 6.

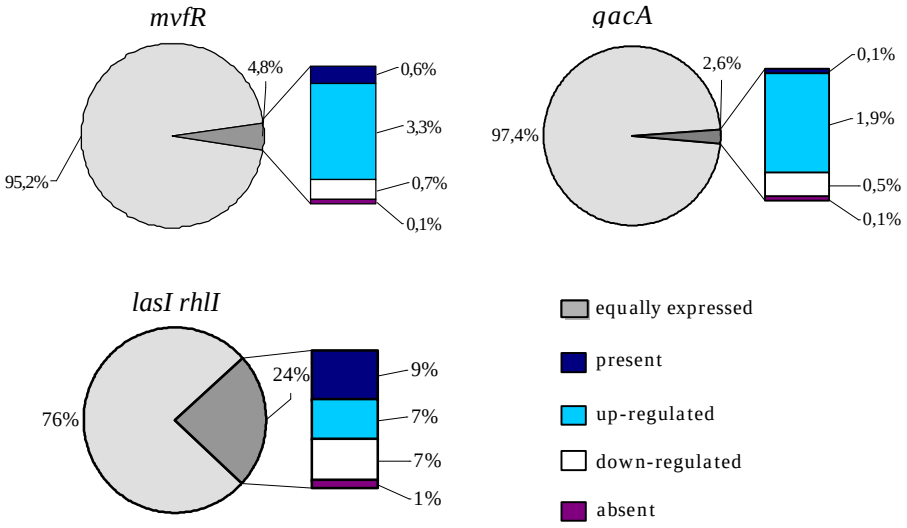


Figure 8 Percentage of differentially expressed protein spots between *P. aeruginosa* PAO1 wild type and each one of the respective mutants.

These results show that the effect of Vfr on the quorum-sensing system of *P. aeruginosa* is higher than the effect of GacA. The same can also be concluded from the phenotypic analysis of some virulence factors such as exoproteases and pyoverdine production that were more strongly reduced in the *mvrf* mutant than in the *gacA* mutant. The LasB activity was not affected at all in either *mvrf* or *gacA* mutants (Figure 9). The proteome analysis confirms the observation that GacA and MvfR affect QS regulated phenotypes in *P. aeruginosa*. However, the effect of the mutation in either *gacA* or *mvfr* does not compromise the entirety of the QS system as it was expected.

Furthermore, proteins that are not reported as quorum-sensing regulated were found to be differentially expressed between the mutants and the wild type PAO1, including the lipoamide dehydrogenase (PA1587), serine hydroxymethyltransferase (PA5415), the

two-component sensor EnvZ and probable dna-binding stress protein (PA0962) (Table 6). This indicates that the regulators have an additional not QS related impact on protein expression.

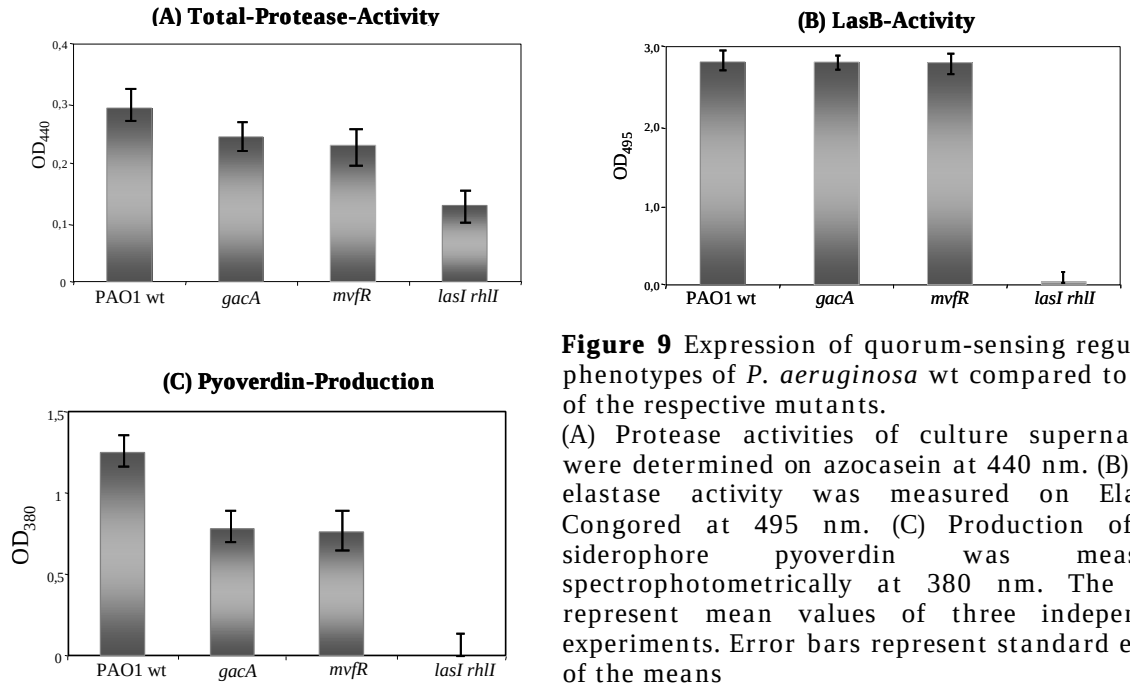


Figure 9 Expression of quorum-sensing regulated phenotypes of *P. aeruginosa* wt compared to each of the respective mutants.
(A) Protease activities of culture supernatants were determined on azocasein at 440 nm. (B) LasB elastase activity was measured on Elastin-Congored at 495 nm. (C) Production of the siderophore pyoverdin was measured spectrophotometrically at 380 nm. The data represent mean values of three independent experiments. Error bars represent standard errors of the means

3.1.3 Quorum-sensing and pathogenesis

Previous work has provided clear evidence that quorum-sensing plays a pivotal role for the pathogenicity of *P. aeruginosa*. AHL-deficient mutants were shown to be attenuated in a number of animal models including the neonatal mouse model of pneumonia (Wu *et al.*, 2001) and a *Caenorhabditis elegans* model (Tang *et al.*, 1996). Comparing the protein patterns of two *P. aeruginosa* strains from the TB clonal lineage isolated from the sputa of CF-patients (TB121838 and TB10839) that differ dramatically in their pathogenic potential, several QS regulated proteins were found differentially expressed between the two strains (appendix 2: Fig. 1, Fig. SM1 A, B). Both isolates are genetically closely related. The major difference between them is that TB10839 is highly cytotoxic for macrophages and adheres strongly to human airway epithelia and mucins (Kiewitz and Tümmler, 2000). In total the intensity of about 4% of all detected protein spots differs more than two-fold between the two strains. Remarkably, the extracellular sub-proteome showed a higher diversity (5.5%) than the intracellular and surface derived sub-proteomes (3.2% and 3.3%, respectively). These findings support the study of Wehmhöhner et al. (2003) who demonstrated that particularly the secretome expression is a sensitive measure of *P. aeruginosa* strain variation (appendix 2). 12 out of 19

identified proteins had been reported as QS regulated before and were found to be expressed at reduced levels in TB121838 compared to TB10839. One of the differentially expressed proteins, exclusively present in TB121838, did not match with any ORF of PAO1 but was identified as ORF C62 (Larbig *et al.*, 2002) of the *P. aeruginosa* clone C. The corresponding gene is located in a strain specific gene island of this clone (appendix 2: Table 1). These results were further corroborated by a phenotypic characterization of the two strains which showed that the expression of QS regulated phenotypes such as the exoprotease LasB, elastase, and chitinase were indeed reduced in strain TB121838, and the synthesis of the siderophore pyoverdin was completely abolished (Figure 10 and appendix 2). Therefore, it can be speculated that the reduced virulence of TB121838 may, at least in part, be a consequence of the down-regulation of QS regulated functions.

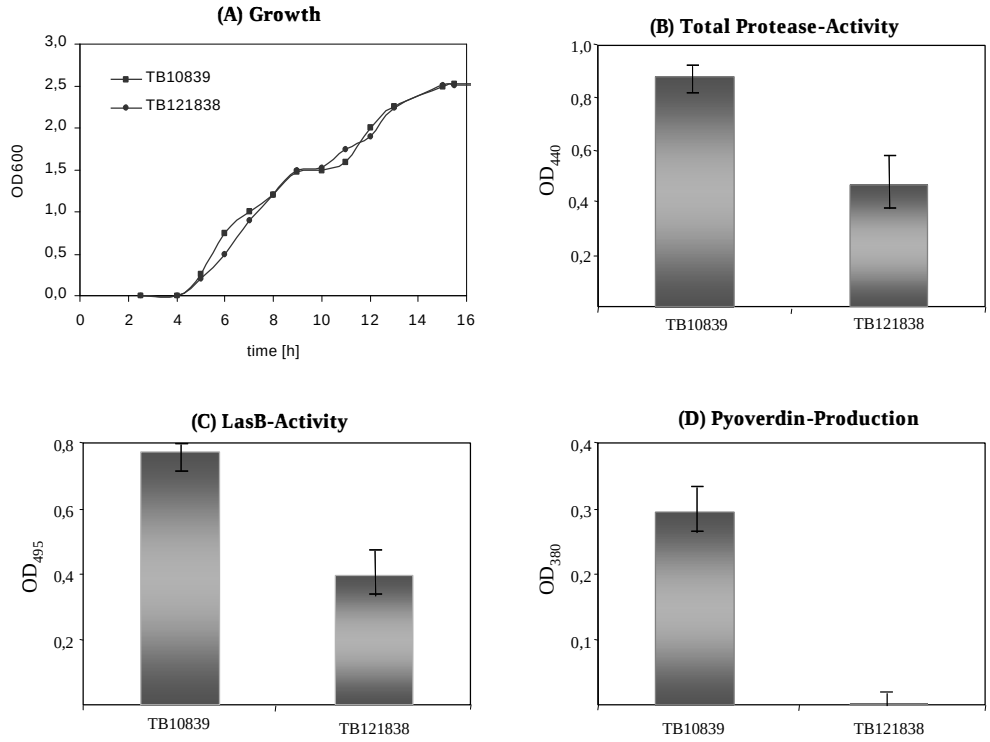


Figure 10 Comparison of growth, and expression of quorum-sensing regulated phenotypes of TB10839 and TB121838 in LB medium.
(A) Growth was measured spectrophotometrically at 600 nm. When the cultures reached an OD_{600} of 1.5 samples of culture supernatants were taken. (B) Protease activities of spent culture supernatants were determined on azocasein. (C) LasB elastase activity was measured on Elastin-Congored at 495 nm. (D) Presence of the siderophore pyoverdin in the supernatant was measured spectrophotometrically at 380 nm. The data represent mean values of three independent experiments. Error bars represent the standard errors of the means.

3.2 Part II- Quorum-sensing in Burkholderia cenocepacia H111, a global regulatory system

Employing 2-DE we have shown that the *cep* quorum-sensing system of *B. cenocepacia* H111 controls expression of at least 56 different proteins spots (6%) out of 985 detected spots (Figure 12 and appendix 3: Table 2). These protein spots were considered as QS regulated because the expression level in the wild type was fully restored in the AHL-deficient *cepI* mutant by the addition of the respective signal molecule to the growth medium. This result indicates that QS represents a global gene-regulation system in *B. cenocepacia* and is consistent with previous findings showing that a number of apparently unrelated functions including the production of extracellular hydrolytic enzymes, swarming motility, biofilm formation, and synthesis of the siderophore ornibactin are *cep* regulated (Huber *et al.*, 2001; Lewenza *et al.*, 1999).

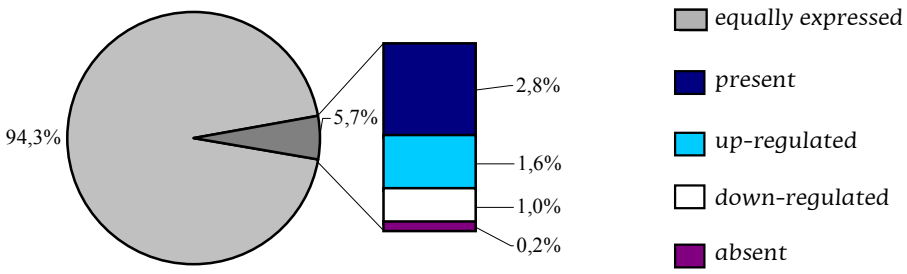


Figure 11 Percentage of QS regulated proteins *B. cenocepacia* H111 wild type.
The protein patterns of the wild type were compared with those of the isogenic *cepI* mutant. Only the spots which expression rate was fully restored to the wild type level when the mutant was grown medium supplemented with 2 μM C8-HSL were considered QS regulated.

As the sequence annotation of the *B. cenocepacia* genome was not finished the identification of *cep*-regulated proteins by MALDI-TOF was not feasible. N-terminal amino acid sequences for 19 protein spots were determined and 10 QS regulated proteins were identified (appendix 3: Table 3). Three spots identified an ORF with high similarity to AidA of the plant pathogen *Ralstonia solanacearum* AW1 (53% identity). *aidA* (autoinducer dependent) is located upstream of the *sol* quorum-sensing locus of this organism and was shown to be regulated by the LuxR homologue SolR (Flavier *et al.*, 1997). A search for sequences resembling the *cep* box unravels a putative CepR binding site upstream of the identified ORF. Until now the function of AidA is unknown but in a recent study of Huber *et al.* (submitted) it has been shown that the expression of this ORF product is growth phase-dependent. It has also been found that it is involved in infection processes because it is essential for slow killing of *C. elegans* (Huber *et al.*, submitted).

Another protein found to be QS regulated in the present work is FimA. Six spots corresponded to an ORF with strong similarity (50% identical amino acids) to FimA, the major subunit of type I pili of *Escherichia coli* (Peek *et al.*, 2001). For *E. coli* it has been demonstrated that type I pili are important virulence factors as these surface structures mediate specific adherence to mammalian host tissues and biofilms on abiotic surfaces (Mulvey *et al.*, 1998; Pratt and Kolter, 1998). As biofilm formation of *B. cenocepacia* is controlled by the *cep* system (Huber *et al.*, 2001) it appears possible that FimA is involved in biofilm formation.

The results of the present study may also indicate that the *cep* QS system is involved in the development of resistance against oxidative stress and in type I secretion system. A probable peroxidase and the superoxide dismutase SodB were found to be down-regulated in the *cepI* mutant. Aditionally, the spot QS7, 54% identical to PA0314 of *P. aeruginosa*, is an ABC transporter involved in a hypothetical type I secretion system (appendix 3)

3.3 Part III-Identification of quorum-sensing and surface induced genes in Pseudomonas putida.

3.3.1 Establishing of a biofilm growth model for 2-DE analyses

Prior to the identification of surface-induced genes by comparing protein patterns of planktonic and biofilm cells an appropriate biofilm growth model had to be established. The culturing of copious amounts of biofilm is a prerequisite for obtaining enough protein biomass for 2-DE sample preparation. Two model systems were tested in this work: glass wool and silicone tube grown biofilms.

Glass wool as substratum for cell attachment provides a large surface-to volume ratio and should in principle support the growth of a sufficient amount of biomass (see: 2.2.4.1) (Steyn *et al.*, 2001).

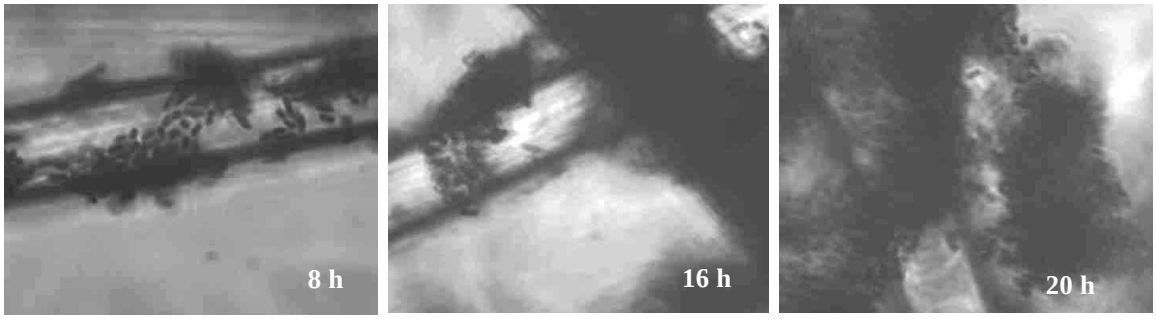


Figure 12 Biofilm formation of *P. putida* IsoF on glass wool.
Initial attachment to the substratum after 8 h. Development of dense biofilms after 16 h and 20 h. Samples of glass wool were stained with 0.01% crystal violet and inspected by bright-field microscopy.

P. putida IsoF biofilms could be grown on glass wool (Figure 14 A, B, C) but the number of cells was not high enough to perform 2-DE analyses of all subcellular fractions. Harvesting of biofilm cells was done after 18h when big microscopic cell clusters were observed on the glass wool.

After 20 h, the biofilm started to detach from the surface. Only 200 µg of protein were obtained from the intracellular fraction after 18h using 7.5 g of glass wool and it was not possible to obtain more than 50 µg of protein from the surface fraction. For this reason, only intracellular protein patterns of biofilm cells could be compared with the ones of the planktonic counterpart, although the biofilm mode of growth should exhibit the most dramatic effect on the expression of surface-associated proteins.

The patterns were visualised using silver staining and three gels (one of each growth) were used for the analyses (Figure 15).

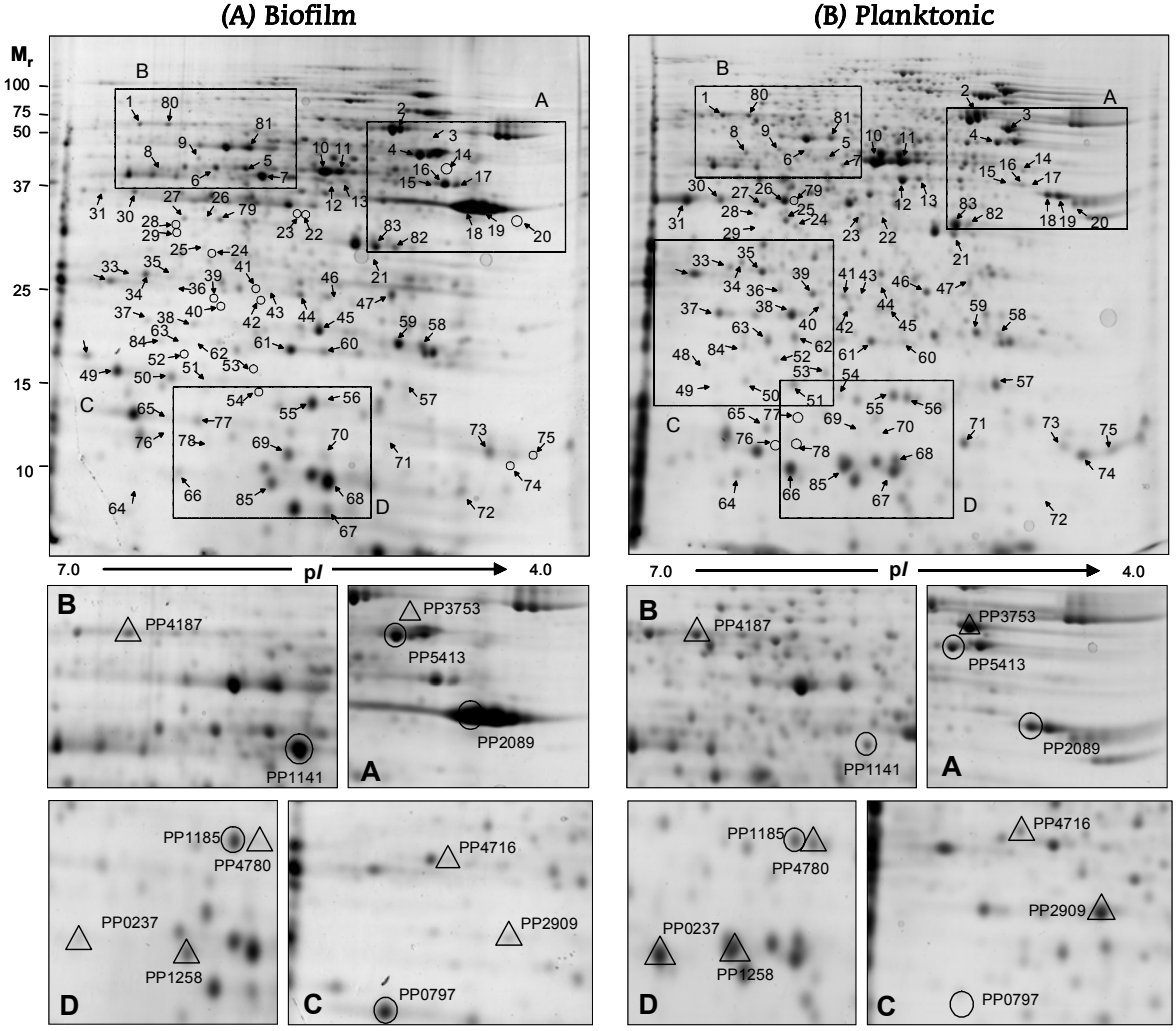


Figure 13 Comparative two-dimensional gel electrophoresis (2-DE) of intracellular proteins of *P. putida* IsoF grown as biofilm on glass wool (A), and in planktonic culture (B).
The proteins were separated on Immobiline Dry strips with pH gradients from 4 to 7, followed by an SDS PAGE on 13% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250. Regions of interest (A to D) are shown below in better detail. Circles indicate positively biofilm-induced spots; triangles indicate negatively biofilm-regulated spots.

Of 723 detected proteins spots, 12% were found to be differentially expressed more than 2.0-fold (Figure 14). Four protein spots were exclusively detected, 28 were up-regulated and 37 were down-regulated in the biofilm grown cells compared to the planktonic counterpart. 16 protein spots were exclusively expressed in the planktonic cultures.

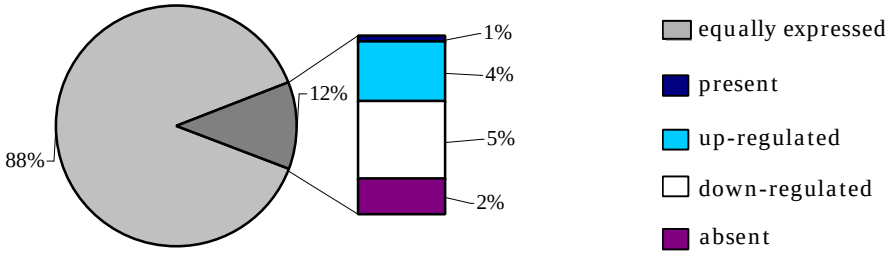


Figure 14 Proportion of differentially expressed intracellular proteins in the biofilm growth of *P. putida* IsoF on glass wool.
The protein pattern of biofilm growth was compared to the protein patterns of the planktonic cultures. The percentages refer to the biofilm mode of growth

In order to characterize these proteins by means of mass spectrometry additional gels were stained with Coomassie and 13 proteins differentially expressed between planktonic growth and biofilm growth were identified (Table 8 and appendix 5 A, B)

Table 8 Identification and functional cathegory of genes that are differentially expressed in *P. putida* after 18 h of attachment to glass wool.

Protein Identification[a]	PP No.[a]	Regulation	Spot No
Cell envelope			
phosphoglucosamine mutase	PP4716	-3,1	34
outer membrane protein H1	PP1185*	2,2	55
DNA metabolism			
phage integrase, putative	PP2501	-9,5	57
Energy metabolism			
lipoamide dehydrogenase component	PP4187	-2,5	80
ATP synthase F1, beta subunit	PP5413*	2,0	4
Fatty acid and			
acyl-CoA dehydrogenase, putative	PP4780	-7,0	56
Hypothetical proteins			
conserved hypothetical protein	PP0797*	22,7	49
conserved hypothetical protein	PP2909	-6,4	38
Regulatory functions			
transcriptional regulator, AraC family	PP3753	-35,5	3
Transport and binding			
sulfonate ABC transporter, sulfonate-binding protein SsuA	PP0237	-5,3	66
ABC transporter, periplasmic amino acid-binding protein	PP1141*	6,9	7
outer membrane protein OprF	PP2089*	4,2	18
Unknown function			
epimerase, putative	PP1258	-1,5	85

[a]Data generated from peptide mass maps were compared to the complete translated ORFs for *P. putida* KT2440 (http://www.tigr.org)
*The orthologous in *P. aeruginosa* was reported to be QS regulated in one of the global studies.

To determine which gene in *P. aeruginosa* was the corresponding orthologous gene in *P. putida* a blast search with the amino acid sequence against the translated genome of *P. aeruginosa* was done. A protein of *P. aeruginosa* was considered orthologous if it showed an identity higher than 50% and the length of the alignment was approximately the entire length of the *P. putida* protein (Durbin *et al.*, 1998) (appendix 6). Interesting, five out of 13 identified proteins have a counterpart in *P. aeruginosa* that have been reported as QS regulated (Table 8).

An interesting protein found to be more than 9-fold up-regulated in planktonic cultures was the putative phage integrase PP2501. This protein seems to be unique in *P. putida* since there is not a significant match over its entire length when compared to the complete NCBI protein database. Nevertheless, there is a region of approximately 100 amino acids that is shared with several other proteins from different bacterial species, which corresponds to a phage integrase domain. It could be hypothesized that PP2501 is involved in the acquisition of genes via horizontal transfer. This process has been already described in a number of studies (for review see: Weinel *et al.*, 2002)

Even if interesting results can be achieved using the glass wool model, too much effort is necessary to obtain enough protein for a 2–DE comparison. In contrast, with the silicon-tubes model copious amounts of biofilm and higher protein concentrations were obtained (see 2.2.4.2) (Christensen *et al.*, 1999). After 60 h of continuous medium flow, the mature biofilm was harvested and 4 x 100cm tubes (Ø 3 mm) were used to obtain 600 µg of proteins from the surface fraction. Therefore, this model was used for further study.

3.3.2 Differentially expressed proteins between the biofilm mode of growth and planktonic cultures *of P. putida*

Surface protein patterns of *P. putida* IsoF grown as biofilm and planktonically were compared to determine surface-controlled genes, it was expected that this protein fraction would be the most representative to study the difference between cells attached to a surface and the free swimming cells. The cells were grown as described in Materials and Methods (2.2.4.2). Five hundred protein spots were detected, 26% of which were differentially expressed (= 1.5-fold). Ten protein spots were exclusively detected, 73 were up-regulated and 32 were down-regulated in the biofilm mode of growth. Fourteen protein spots were exclusively expressed in planktonic cultures (Figure 15; Figure 16). Twenty nine differentially expressed proteins were identified by MALDI-TOF mass spectrometry (Table 9 and appendices 7, 8).

Bearing in mind that 26% of the proteins detected in one subproteome are differentially expressed it can be concluded that the biofilm mode of growth has a significant effect on protein expression. As shown in Table 9, proteins belonging to different functional categories are differentially expressed showing that bacteria growing in biofilms are

indeed physiologically different from free-living organisms. These results are in agreement with studies proposing that bacteria undergo a variety of metabolic changes in the biofilm growth (for review see: Hall-Stoodley *et al.*, 2004).

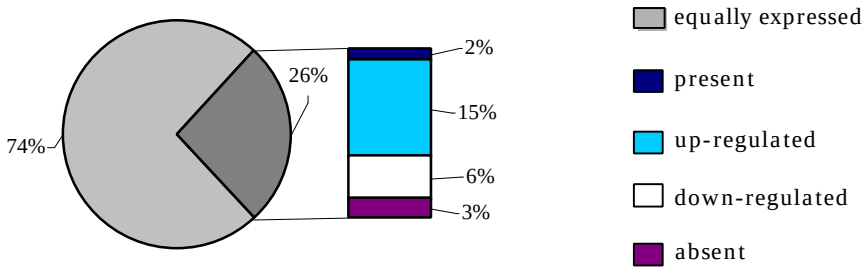


Figure 15 Percentage of surface-induced proteins in the biofilm growth on silicon tubes.
The protein patterns of *P. putida* IsoF wild type grown in the silicone tubes and as planktonic cultures were compared. The percentages refer to the biofilm mode of growth.

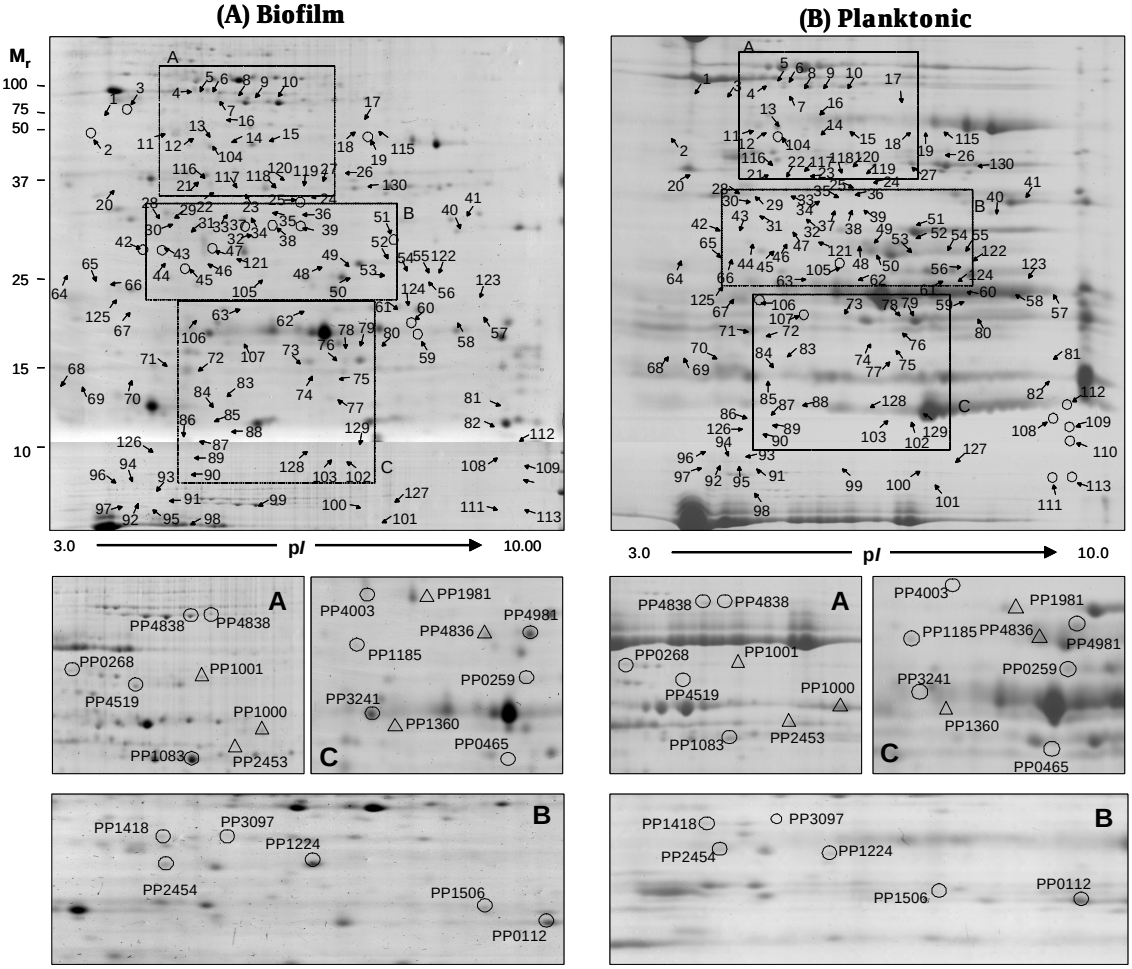


Figure 16 Comparative two-dimensional gel electrophoresis (2-DE) of surface-bound proteins of *P. putida* IsoF grown as biofilm in silicone tubes (A), and in planktonic culture (B).
The proteins were separated on Immobiline Dry strips with nonlinear pH gradients from 3 to 10, followed by an SDS PAGE on 13% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250. Regions of interest (A to C) are shown below in better detail. Circles indicate positively biofilm-induced spots; triangles indicate negatively biofilm-regulated spots.

Recently, Sauer and Camper (2001) investigated the impact of surface-associated growth on the gene expression in *P. putida* ATCC 38168. At that time, the sequencing of the genome of *P. putida* KT2440 was not finished therefore the proteins were identified by N-terminal sequencing and compared to the annotated genome of *P. aeruginosa* PAO1. In order to compare this study with the present work, the orthologous protein in *P. aeruginosa* of the respective differentially expressed *P. putida* proteins had to be identified. Since it is known that the proteome of sessile bacteria is strongly dependent on the nature of the biofilm substratum, the medium surrounding the cells, and the step of biofilm development, it is not surprising that only three proteins found in the present work were also found to be differentially expressed by Sauer and Camper (2001); the ornithine carbamoyltransferase (PP1000), the arginine deaminase (PP1001) and L-asparaginase II (PP2453). These proteins belong to the functional category "energy metabolism" defined by CMR's TIGR and were found down-regulated in cells grown as biofilms in both studies.

3.3.3 Quorum-sensing regulated genes in *P. putida* Iso F

For the identification of QS regulated proteins the protein profiles of the *P. putida* IsoF parent strain and an isogenic *ppuI* mutant were compared. In order to compare QS regulated proteins with biofilm induced proteins the growths were made under the same conditions used for the comparison between biofilm and planktonic cells (see **2.2.4.2**). More than 480 protein spots were detected and 52 were differentially expressed (= 1.5-fold). They were only considered as truly QS regulated if their expression level was fully restored to the wild type level when the mutant was grown in LB medium supplemented with 2 µM 3-oxo-C12-HSL (Figure 17).

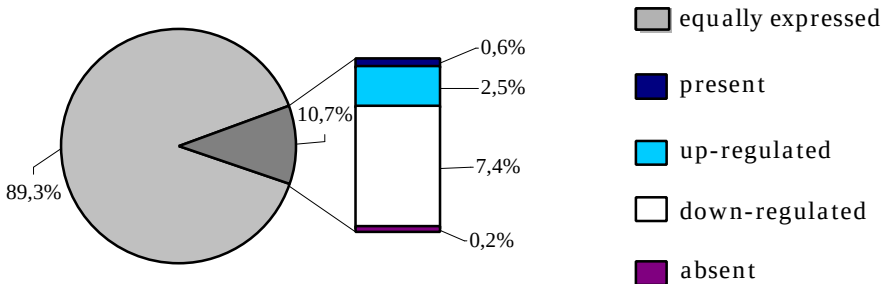


Figure 17 Percentage of quorum-sensing regulated surface-associated proteins in *P. putida* IsoF wild type.
Patterns from the surface-protein fraction of the wild type were compared with those of the isogenic *ppuI* mutant. Only the spots which expression rate was fully restored to the wild type level when the mutant was grown medium supplemented with 2 µM 3-oxo-C12-HSL were considered QS regulated.

Three protein spots were exclusively expressed, twelve were up-regulated and 36 proteins were down-regulated in the wild type strain. One protein spot was exclusively expressed in the *P. putida ppI* mutant (Figure 18).

23 new direct or indirect target genes outside the *ppu* gene cluster found by Steidle *et al.* (2002) were identified in the present work (Table 9 and appendices 9, 10). Interestingly, for six of these 23 QS regulated proteins of *P. putida* orthologous proteins with 50% identity were also found to be QS regulated in *P. aeruginosa*. Examples are outer membrane proteins and transporters (PP1185, PP2089, PP1418) and conserved hypothetical proteins (PP3241, PP4836, PP4981) (marked with stars in Table 9). Proteins involved in protein fate and synthesis were found to be quorum-sensing regulated supporting the idea of AHL-dependent regulation at the post-transcriptional level as it was also observed in the analyses of the QS regulon of *P. aeruginosa*.

In total 11% of the detected surface proteins were considered QS regulated, indicating that the *ppu* system acts as a global regulatory system in this rhizosphere isolate.

Figure 18 Comparative two-dimensional gel electrophoresis (2-DE) of surface-bound proteins of *P. putida* IsoF wild type, *ppuI* mutant and *ppuI* mutant + AHL.
NEXT PAGE: (A) *P. putida* IsoF wild type (B) *ppuI* mutant grown in the absence and (C) presence of 2 µM 3-oxo-C12-HSL. The proteins were separated on Immobiline Dry strips with nonlinear pH gradients from 3 to 10, followed by an SDS PAGE on 13% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250. Regions of interest boxed (A to D) are shown below in better detail. Circles indicate positively QS regulated spots

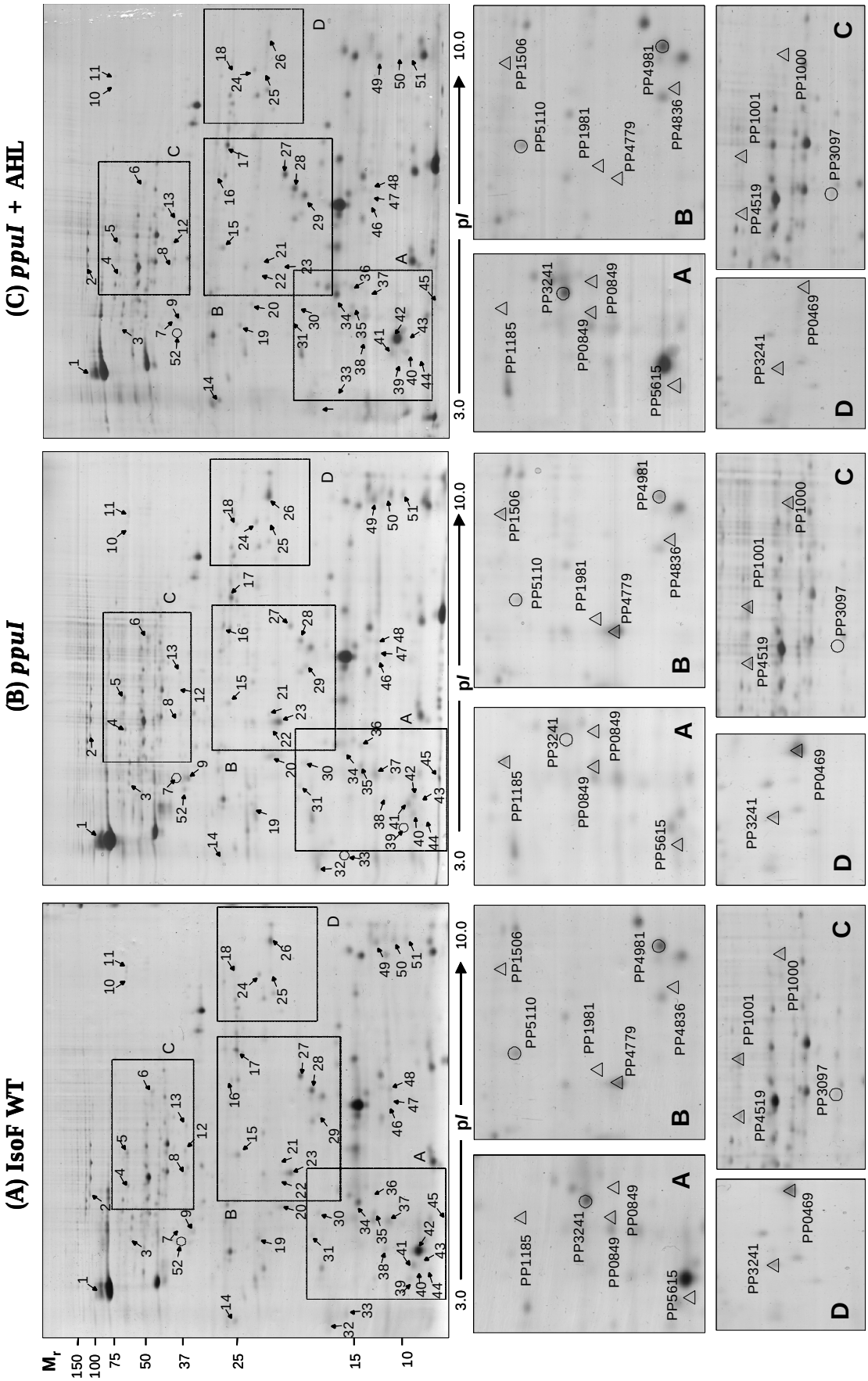

(A) IsoF WT
(B) ppuI
(C) ppuI + AHL
Mr
150
100
75
50
37
25
15
10
pI
3.0
10.0
A
B
C
D
PP1185
PP3241
PP0849
PP0469
PP5615
PP5110
PP1506
PP1981
PP4779
PP4981
PP4836
PP4519
PP1001
PP1000
PP3097

Table 9 Identification and functional category of the proteins, which are regulated by the *ppuI* sytem of *P.putida* IsoF and/or differentially expressed between cells grown as biofilm in silicone tubes and planktonic cultures. The regulation in the quorum-sensing comparison is referring to the wild type and the surface-induced genes are referred to the biofilm growth.

Protein Identification[a]	PPNo[a].	SpotNo[b]	regulation	Spot No[c]	regulation
Cell envelope and cellular processes					
outer membrane protein OmpH	PP1600			BF82	6,4
outer membrane protein H1	PP1185	QS30	-2	BF72	2,5
agglutination protein	PP4519	QS4	-2,3	BF14	1,8
cell division ABC transporter, ATP-binding protein FtsE	PP5110	QS15*	1,6		
Energy metabolism					
ornithine carbamoyltransferase, catabolic	PP1000	QS6*	-2	BF26	-5,5
arginine deaminase	PP1001	QS5	-2,3	BF15	13,9
bacterioferritin-associated ferredoxin, putative	PP1083			BF118	1,6
L-asparaginase II	PP2453			BF27	-8,6
thioredoxin	PP5215	QS40*	-5,1	BF92	-9,7
Hypothetical proteins					
conserved hypothetical protein	PP1224			BF32	3,6
conserved hypothetical protein	PP1518	QS24*	-1,6	BF123	-1,7
conserved hypothetical protein	PP3097	QS8*	1,8	BF33	21,5
conserved hypothetical protein	PP3241	QS34*	8,5	BF85	2,6
conserved hypothetical protein	PP4836	QS29*	-3,4	BF74	-5
conserved hypothetical protein	PP4981	QS28*	1,6	BF76	2,7
Protein fate					
chaperonin, 10 kDa	PP1360	QS36	-1,9	BF88	2,7
outer membrane lipoprotein carrier protein	PP4003	QS19	-2,2	BF63	5,1
Protein synthesis					
ribosomal protein L24	PP0465	QS46	-6,5	BF103	1,9
ribosomal protein L6	PP0469	QS26*	2,9	BF57	12,5

Purines, pyrimidines, nucleosides, and nucleotides					
nucleoside diphosphate kinase	PP0849	QS35	-2,4	BF89	30,9
adenylate kinase	PP1506	QS16	-2,4	BF48	4,4
AMP nucleosidase	PP4779	QS23*	2	BF107	present
Transport and binding proteins					
ABC transporter, periplasmic binding protein, putative	PP0112			BF53	5
outer membrane protein OprE3	PP0268			BF11	3
tricarboxylate transport protein TctC, putative	PP1418	QS7*	present	BF29	4
outer membrane protein OprF	PP2089	QS14*	1,8	BF66	6,1
ribose ABC transporter, periplasmic ribose-binding protein	PP2454	QS9	-3,5	BF30	6,8
outer membrane copper receptor OprC	PP4838			BF9	14,1
				BF10	34,8
cell division ABC transporter, ATP-binding protein FtsE	PP5110	QS15*	1,6		present
phosphate ABC transporter, periplasmic phosphate-binding protein	PP5329			BF41	-1,6
Unknown function					
hydrolase, haloacid dehalogenase-like family	PP0259			BF77	2,4
NifR3/Smm1 family protein	PP1981	QS21*	-1,7	BF62	-2,8

[a] Data generated from peptic mass maps were compared to the complete translated ORFs for *P. putida* KT2440
[b] Comparison of the proteins patterns of *P.putida* IsoF wild type and the *ppI* mutant
[c] Comparison of the proteins patterns of *P.putida* IsoF grown in silicon tubes and in planktonic culture
* Proteins identified by MALDI TOF peptide mass mapping and reported as QS regulated in previously published studies.

3.3.4 Impact of quorum-sensing on biofilm development

Quorum-sensing enables bacteria to respond as groups by sensing the actual cell density, a process that is facilitated in the biofilm mode of growth where bacterial cells are occurring in close proximity to each other. A comparison of the differentially expressed proteins in biofilm growth with the QS regulated proteins indicates a complex interplay between quorum-sensing and biofilm development. As biofilm formation has been shown to be regulated by quorum-sensing in different bacterial species including *P. aeruginosa* and *B. cenocepacia* (Davies *et al.*, 1998, Huber *et al.*, 2001) and it was found that the biofilm formed by *P. putida* IsoF differs from that formed by the *ppuI* mutant (Steidle et al., 2002), it was expected that some of the biofilm-differentiated proteins in *P. putida* represent QS regulated gene products. From 29 identified proteins differentially expressed in biofilm growth, 21 were found to be AHL-dependent. Nine proteins induced in biofilm growth were positively QS regulated, five were negatively QS regulated and repressed in biofilm growth. These 14 proteins might represent a direct intervention of quorum-sensing in biofilm development. Nine proteins were repressed by the QS system but induced in biofilm growths. There are two different effects taking part in the expression of these proteins, biofilm mode of growth and quorum-sensing. These genes are induced by the surface association in a stronger way than the repression lead by the QS system and that surface-induction seems to be overwhelming quorum-sensing regulation. However, further work will be required to address this issue in better detail.

From the surface-induced identified proteins 44% have also been regulated by quorum-sensing. It might be speculated that quorum-sensing induces the expression of proteins required for the maturation of *P. putida* IsoF biofilms. One of these proteins is the outer membrane protein OprF PP2089. Interestingly, PA1777 is the orthologous protein found in *P. aeruginosa* for PP2089. PA1777, which exhibits adhesin-like properties (Azghani *et al.*, 2002) was found to be QS regulated in both species and to be induced in the biofilm growth of *P. putida*.

4 Summary

In many bacteria quorum-sensing (QS) regulates a wide variety of phenotypes such as bioluminescence, antibiotic production, swarming motility, biofilm formation and production of virulence factors. The presented work comprises a global study of QS controlled and surface-induced genes of *Pseudomonas aeruginosa, Pseudomonas putida* and *Burkholderia cenocepacia* by comparative proteome analysis. Different extraction protocols for intracellular, surface and extracellular proteins were established and the expression patterns were compared by means of two-dimensional gel electrophoresis (2-DE).

P. aeruginosa utilizes two interrelated QS systems (*las* and *rhl*), which interact with additional regulators including Vfr and GacA, to regulate the expression of many phenotypes in a cell-density dependent manner. A global analysis of the QS regulon showed that 23.7% of all detected protein spots were differentially expressed between the PAO1 parent strain and the isogenic *lasI rhlI* double mutant. The surprisingly high number of QS regulated proteins relative to the number of regulated genes suggests that QS control also operates via post-transcriptional mechanisms. Twenty-seven proteins were identified that were previously reported to be QS controlled, among them several well-characterized virulence factors. Importantly, nineteen novel QS regulated proteins were identified, many of which are involved in iron utilization, suggesting a link between QS and the iron regulatory system.

For some of the identified proteins, phenotypic and immuno-chemical experiments were performed to verify if their expression was indeed QS regulated. It was shown that the expression of the flagellar capping protein FliD is reduced in the *lasI rhlI* double mutant. Arginine utilization under anaerobic conditions and iron uptake from haemoglobin were also found to be QS dependent. Additionally, it was shown that the ability to degrade lactoferrin is dependent on a functional QS system. Since the ability to scavenge iron from host proteins contributes to the infection process, these results are supporting the impact of QS on the pathogenic potential of *P. aeruginosa*.

Furthermore, it was shown that, albeit Vfr and GacA can be designated global regulators of gene expression in *P. aeruginosa,* their effect on QS controlled gene expression is conspicuously lower than expected. A comparative proteome analysis of PAO1 wild type, a PAO1 *gacA*, and a PAO1 *mvfR* mutant revealed that in total 3% (*gacA*) and 5% (*mvfR*) of all detected protein spots are differentially expressed. Additionally, the proportion of identified QS regulated proteins is significantly lower than the proportion of proteins affected by the QS regulon.

It is known that QS plays a significant role in the pathogenicity of *P. aeruginosa*. Strains TB121838 and TB10839, isolated from the sputa of two CF-patients that differ

dramatically in their pathogenic potential, were compared by 2-DE. Even though the strains are closely related, the degree of intraclonal variability is reflected in the percentage (4%) of differentially expressed proteins spots, these proteins were characterized by mass spectrometry. Thirteen out of 36 identified proteins had been reported as being QS regulated before and were found to be expressed at reduced levels or missing in TB121838 relative to TB10839. It was considered that the increased expression of these proteins could contribute to the pathogenic competence of TB10839. Further work is necessary to address this hypothesis.

A second part of this work addressed the identification of QS regulated proteins of *B. cenocepacia* H111, another important pathogen for persons suffering from cystic fibrosis. *B. cenocepacia* employs the *cep* QS system to control the expression of virulence factors as well as the formation of biofilms. The protein patterns of the intracellular, extracellular, and surface protein fractions of a signal molecule-deficient *cepI* mutant were compared with those of the parent strain H111. In total about 6% of the *B. cenocepacia* proteome was differentially expressed. These results indicate that QS represents a global regulatory system in *B. cenocepacia*. Nineteen proteins were identified by N-terminal sequence analysis, among them important factors such as AidA and the major subunit of type I pili, FimA.

Finally, surface-induced and quorum-sensing regulated proteins of *P. putida* IsoF were identified. There is some evidence that the *ppu* QS system is involved in biofilm development of *P. putida* IsoF. Therefore, a comparative analysis of the QS regulated and surface-induced proteins was performed for the surface-associated subproteome of *P. putida*. Biofilms were grown in silicone tubes under permanent medium flow.

In the global analysis of the *ppu* QS system 11% of the protein spots were differentially expressed between *P. putida* IsoF and the *ppuI* mutant. Twenty three proteins could be identified by mass spectrometry. These results indicate that the *ppu* system acts as a global regulatory system in this rhizosphere isolate. Moreover, it was shown that the biofilm mode of growth has a significant effect on protein expression. 26% of the proteins spots detected in the surface fraction were differentially expressed between biofilm and planktonic cultures. 29 proteins of different functional categories were identified verifying that bacteria growing in biofilms are indeed physiologically different from free-living organisms. Remarkably, from the identified proteins affected by the biofilm mode of growth 44% were consistently regulated by QS. This shows the stimulating impact that QS has on the maturation of biofilms in *P. putida* IsoF.

Zusammenfassung:

Bei vielen Bakterien wird eine Vielzahl von Phänotypen wie die Biolumineszenz, die Schwärmermotilität, die Bildung von Biofilmen, sowie die Produktion von Antibiotika und Virulenzfaktoren durch „Quorum Sensing“ (QS) kontrolliert. Im Rahmen dieser Arbeit wurde die Expression QS-kontrollierter und Oberflächen-induzierter Gene von *Pseudomonas aeruginosa*, *Pseudomonas putida* und *Burkholderia cepacia* mittels differentieller Proteom-Analyse untersucht. Es wurden verschiedene Verfahren zur Extraktion intrazellulärer, extrazellulärer und Oberflächen-Proteine etabliert; die mittels zweidimensionaler Gelelektrophorese (2-DE) erhaltenen Expressionsprofile wurden vergleichend untersucht.

P. aeruginosa nutzt zwei miteinander zusammenhängende QS-Systeme (*las* und *rhl*), die mit zusätzlichen Regulatoren wie Vfr und GacA interagieren, zur Regulation der Expression vieler Phänotypen in Abhängigkeit von der Zelldichte. Eine globale Analyse des QS-Regulons zeigte, dass 23,7 % aller detektierten Proteinspots im PAO1 Wildtyp-Stamm im Vergleich zur isogenischen *lasI rhlI* Doppelmutante unterschiedlich exprimiert werden. Die im Vergleich zu den entsprechend regulierten Genen überraschend hohe Zahl QS-regulierter Proteine deutet darauf hin, dass die Kontrolle durch QS auch post-transkriptionelle Mechanismen einschließt. 27 identifizierte Proteine waren schon als QS-kontrolliert beschrieben, darunter einige gut charakterisierte Virulenzfaktoren. Interessanterweise wurden auch 19 neue QS-regulierte Proteine identifiziert, von denen viele an der Nutzung von Eisen beteiligt sind. Dies könnte ein Hinweis auf eine Verbindung zwischen QS und dem Eisen-Regulationssystem sein. Für einige der identifizierten Proteine wurden phänotypische und immunochemische Experimente durchgeführt, um die Regulation ihrer Expression durch QS zu bestätigen. Es konnte gezeigt werden, dass die Expression des "*flagellar capping protein*" FliD in der *lasI rhlI* Doppelmutante reduziert ist. Auch der Abbau von Arginin unter anaeroben Bedingungen und die Aufnahme von Eisen aus Hämoglobin sind QS-abhängig. Zusätzlich wurde gezeigt, dass ein funktionierendes QS-System für den Lactoferrin-Abbau nötig ist. Da die Fähigkeit, Eisen aus Wirtsproteinen aufzunehmen, zum Infektionsprozess beiträgt, unterstreichen diese Ergebnisse den Einfluss von QS auf die Pathogenität von *P. aeruginosa*. Außerdem konnte nachgewiesen werden, dass der Einfluss von Vfr und GacA auf die Expression QS-regulierter Gene beträchtlich niedriger ist als erwartet, obwohl beide als globale Regulatoren der Genexpression bei *P. aeruginosa* beschrieben wurden. Eine vergleichende Proteomanalyse des PAO1 Wildtyps, einer PAO1 *gacA* sowie einer PAO1 *mvfR* Mutante zeigte, dass insgesamt 3 % (*gacA*) bzw. 5 % (*mvfR*) aller detektierten Proteinspots unterschiedlich exprimiert werden. Darüberhinaus ist der Anteil der in den Mutanten im Vergleich zum Wildtyp differentiell exprimierten QS-regulierten Proteine sehr gering. Es ist bekannt, dass QS eine bedeutende Rolle bei der Pathogenität von *P. aeruginosa* spielt. Die Stämme TB121838 und TB10839, die aus dem Sputum zweier CF-Patienten isoliert wurden und deren Pathogenität sich dramatisch unterscheidet, wurden mittels 2-DE verglichen. Obwohl diese Stämme sehr nahe miteinander verwandt sind, spiegelt sich das Ausmaß ihrer intraklonalen Variabilität in dem Prozentsatz (4 %) von unterschiedlich exprimierten Proteinen wieder. Diese Proteine wurden mittels Massenspektrometrie untersucht.

Bei 13 von 36 identifizierten Proteinen handelt es sich um bekanntermaßen QS regulierte Proteine, diese werden in TB121838 im Vergleich zu TB10839 in geringeren Mengen exprimiert

oder fehlen völlig. Die vermehrte Expression dieser Proteine könnte daher zur Pathogenität von TB10839 beitragen. Um diese Hypothese zu stützen, sind jedoch weiter-gehende Untersuchungen nötig.

Ein zweiter Teil dieser Arbeit befasste sich mit der Identifizierung von QS-regulierten Proteinen bei *B. cenocepacia* H111, einem weiteren wichtigen Krankheitserreger für CF-Patienten. *B. cenocepacia* nutzt das *cep* QS System, um die Expression von Virulenzfaktoren und die Biofilmbildung zu kontrollieren.

Die Proteinmuster der intrazellulären, extrazellulären und Oberflächenprotein-Fraktionen einer *cepI*-Mutante, die keine Signalmoleküle produzieren kann, wurden mit denen des H111 Wildtyps verglichen. Insgesamt wurden ca. 6 % des *B. cenocepacia* Proteoms unterschiedlich exprimiert. Diese Ergebnisse deuten darauf hin, dass QS ein globales Regulationssystem von *B. cenocepacia* darstellt. 19 Proteine wurden durch Sequenzieren des N-Terminus identifiziert, darunter wichtige Faktoren wie AidA und die Hauptuntereinheit der TypI Pili, FimA.

Zuletzt wurden noch Oberflächen-induzierte und QS-regulierte Proteine von *P. putida* IsoF identifiziert. Es gibt Hinweise darauf, dass das *ppu* QS-System an der Biofilmbildung von *P. putida* IsoF beteiligt ist. Daher wurde eine vergleichende Analyse der QS-regulierten Proteine und Oberflächen-induzierten Gene des Oberflächen-assoziierten Subproteoms von *P. putida* durchgeführt. Die Biofilme wurden in Silikonschläuchen unter ständigem Mediendurchfluss angezogen. Die globale Analyse des *ppu* QS-Systems ergab, dass 11 % der Proteinspots bei *P. putida* IsoF und der *ppuI* Mutante unterschiedlich exprimiert werden. 23 Proteine konnten massenspektrometrisch identifiziert werden. Diese Ergebnisse weisen darauf hin, dass das *ppu* System in diesem Rhizosphärenisolat ein globaler Regulator ist. Darüber hinaus konnte gezeigt werden, dass das Wachstum in Biofilmen einen bedeutenden Einfluss auf die Proteinexpression hat. 26 % der Proteinspots, die in der Oberflächenfraktion detektiert wurden, wurden von planktonischen und Biofilmkulturen unterschiedlich exprimiert. 29 Proteine unterschiedlicher funktionaler Kategorien wurden identifiziert, was bestätigte, dass Bakterien, die in Biofilmen wachsen, sich tatsächlich physiologisch von freilebenden Organismen unterscheiden. Auffälligerweise waren 44 % der durch das Wachstum in Biofilmen beeinflussten Proteine auch QS-reguliert. Dies zeigt den stimulierenden Einfluss von QS auf die Reifung von Biofilmen bei *P. putida* IsoF.

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6 Appendix

6.1 Identification of quorum-sensing regulated proteins in the opportunistic pathogen Pseudomonas aeruginosa by proteomics

Catalina Arevalo-Ferro, Morten Hentzer, Gerold Reil, Angelika Görg, Staffan Kjelleberg, Michael Givskov, Kathrin Riedel and Leo Eberl

Published in: Environmental Microbiology 5: 1350–1369 (2003)

Environmental Microbiology (2003) **5**(12), 1350–1369 doi:10.1046/j.1462-2920.2003.00532.x

Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics

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Summary

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic human pathogen which is responsible for severe nosocomial infections in immunocompromised patients and is the major pathogen in cystic fibrosis. The bacterium utilizes two interrelated quorum-sensing (QS) systems, which rely on *N*-acyl-homoserine lactone (AHL) signal molecules, to control the expression of virulence factors and biofilm development. In this study, we compared the protein patterns of the intracellular, extracellular and surface protein fractions of the PAO1 parent strain with those of an isogenic *lasI rhlI* double mutant by means of two-dimensional gel electrophoresis (2-DE). This analysis showed that the intensities of 23.7% of all detected protein spots differed more than 2.5-fold between the two strains. We only considered those protein spots truly QS regulated that were changed in the mutant in the absence of signal molecules but were rescued to the wild-type situation when the medium was supplemented with AHLs. These protein spots were characterized by MALDI-TOF peptide mapping. Twenty-seven proteins were identified that were previously reported to be AHL controlled, among them several well-characterized virulence factors. For one of the identified proteins, the serine protease PrpL, a biochemical assay was established to verify that expression of this factor is indeed QS regulated. Furthermore, it is shown that the quorum-sensing blocker C-30 specifically interferes with the expression of 67% of the AHL-controlled protein spots of the surface fraction, confirming the high specificity of the compound. Importantly, 20 novel QS-regulated proteins were identified, many of which are involved in iron utilization, suggesting a link between quorum sensing and the iron regulatory system. Two of these proteins, PhuR and HasAp, are components of the two distinct haem-uptake systems present in *P. aeruginosa*. In agreement with the finding that both proteins are positively regulated by the QS cascade, we show that the *lasI rhlI* double mutant grows poorly with haemoglobin as the only iron source when compared with the wild type. These results add haemoglobin utilization to the list of phenotypes controlled through QS in *P. aeruginosa*. The surprisingly high number of AHL-regulated proteins relative to the number of regulated genes suggests that quorum-sensing control also operates via post-transcriptional mechanisms. To strengthen this hypothesis we investigated the role of quorum sensing in the post-translational modification of HasAp, an extracellular protein required for the uptake of free and haemoglobin-bound haem.

Introduction

Pseudomonas aeruginosa is a ubiquitous environmental bacterium that is capable of infecting a wide variety of animals and plants. As human pathogen *P. aeruginosa* is the leading source of Gram-negative nosocomial infections (Van Delden and Iglewski, 1998) and causes chronic lung infections in approximately 90% of the individuals suffering from cystic fibrosis (Høiby and Frederiksen, 2000; Lyczak *et al*., 2000). Recently the genome of *P. aeruginosa* PAO1 was fully sequenced and 5570 open reading frames (ORF) were annotated (Stover *et al*., 2000). Approximately 5% of the genes encode known or

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putative virulence factor products that contribute to the pathogenicity of this bacterium (Wolfgang *et al*., 2003).

Expression of the majority of virulence factors is not constitutive but is regulated in a cell density-dependent manner. This form of gene regulation ensures that *P. aeruginosa* does not express pathogenic traits until the population has reached a critical density that suffices to overwhelm host defences and to establish the infection. Like many other Gram-negative bacteria, *P. aeruginosa* utilizes cell–cell communication systems that rely on diffusible *N*-acyl homoserine lactone (AHL) signal molecules to monitor the size of the population in a process known as quorum sensing (for reviews see Pesci *et al*., 1997; de Kievit and Iglewski, 2000; Camara *et al*., 2002). Two quorum-sensing systems have been identified in *P. aeruginosa*: the *las* system consisting of the transcriptional activator LasR and the AHL synthase LasI which directs the synthesis of *N*-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) and the *rhl* system consisting of RhlR and RhlI which directs the synthesis of *N*-butanoyl-homoserine lactone (C4-HSL). The two systems do not operate independently as the *las* system positively regulates expression of both *rhlR* and *rhlI*. Thus, the two quorum-sensing systems of *P. aeruginosa* are hierarchically arranged with the *las* system being on top of the signalling cascade. In complex interplays with additional regulators, including Vfr, GacA, RsaL, MvaT and RpoS, the quorum-sensing cascade regulates expression of a battery of extracellular virulence factors such as exoenzymes (elastase, alkaline protease), secondary metabolites (pyocyanin, hydrogen cyanide, pyoverdin) and toxins (exotoxin A) (Passador *et al*., 1993; Winson *et al*., 1995), and the development of biofilms (Davies *et al*., 1998; Yoon *et al*., 2002; Hentzer *et al*., 2003a). The importance of the cell–cell communication systems in the pathogenicity of *P. aeruginosa* has been investigated in various animal models and in all cases mutants defective in quorum sensing were substantially less virulent than the parent strains (Tang *et al*., 1996; Tan *et al*., 1999; Wu *et al*., 2001).

In a previous study, Whiteley *et al*. (1999) identified 35 QS-regulated genes by screening a library of mutants bearing random Tn*5-lacZ* insertions in the chromosome of a *lasI rhlI* double mutant for induction of β-galactosidase when the medium was supplemented with synthetic AHLs. The authors extrapolated that over 200 genes may be QS regulated in *P. aeruginosa* PAO1, comprising approximately 4% of the genome. More recently, the Affymetrix GeneChip® technology was used in three independent studies to systematically map the quorum-sensing regulon by transcriptomics (Hentzer *et al*., 2003b; Schuster *et al*., 2003; Wagner *et al*., 2003). The three studies showed that a strikingly high number of genes are QS regulated: Schuster *et al*. (2003) identified 315 positively and 38 negatively regulated genes, Wagner *et al*. (2003) identified 394 positively and 222 negatively regulated genes and Hentzer *et al*. (2003b) identified 163 positively regulated genes. When the results of the three studies are compared only 77 genes were invariably QS regulated and this set of genes was tentatively designated the 'general quorum-sensing regulon' (Hentzer *et al*., 2003b). Although these studies have generated an impressive amount of novel information on quorum sensing in *P. aeruginosa*, it has to be borne in mind that transcriptomics has some limitations. This global analysis tool only determines mRNA abundances and no conclusion on the levels and/or the post-translational modification state of the cellular proteins can be made (Gygi *et al*., 1999). However, previous work has provided evidence that the *las rhl* QS circuitry is also modulated at the post-transcriptional level by the RNA-binding protein RsmA (Pessi *et al*., 2001) and the *dskA* gene product (Jude *et al*., 2003). For the analysis of post-transcriptional modifications proteomics has become the method of choice, despite the fact that this method also has its limitations, most notably its comparably low sensitivity and the fact that the majority of proteins with membrane-spanning domains are lost (not resolved). In a recent study, Nouwens *et al*. (2003) compared the extracellular proteomes (the secretome) of the *P. aeruginosa* wild-type PAO1 with those of various QS-deficient mutants. Here we present an extended proteome analysis of the *P. aeruginosa* PAO1 quorum-sensing system. In addition to the secretome, we also analysed the subproteomes of the intracellular and surface-bound protein fractions.

Results

Growth of P. aeruginosa *and fractionation of cellular compartments*

To analyse the quorum-sensing regulon of *P. aeruginosa* we compared the protein expression pattern of the wild-type PAO1 with those of an isogenic *lasI rhlI* double mutant grown in the absence or presence of 2 μM C4- and 3-oxo-C12-HSL by two-dimensional gel electrophoresis (2-DE). We have recently employed the Affymetrix GeneChip® to map the QS regulon by transcriptomics (Hentzer *et al*., 2003b). In this study, we investigated the same pair of isogenic strains and used similar experimental protocols as in the study of Hentzer *et al*. (2003b). In accordance with the transcriptome studies of Hentzer *et al*. (2003b), Schuster *et al*. (2003) and Wagner *et al*. (2003), we observed that most QS controlled proteins are induced/repressed in the late exponential growth phase (data not shown). For this reason we have chosen an OD_{600} of 1.0 for our proteome analysis. Noteworthy, at this culture density proteolytic activity in the culture superna-

tant, which is known to be strictly QS regulated, was also maximal. The proteomes of the various cultures were separated into intracellular, extracellular and surface subproteomes prior to 2-DE as previously described (Riedel *et al*., 2003).

Comparative 2-DE analysis of the lasI rhlI *quorum-sensing system of* P. aeruginosa *and identification of differentially expressed proteins*

Proteins of the three cellular fractions were used for comparative 2-DE. To gain an overview a non-linear pH gradient from 3 to 10 was applied with samples of three independent growth experiments. For a more detailed analysis the pH-gradients 4–7 and 6–11 were used to increase the resolution of proteins with more acidic or basic isoelectric points. Comparison of gels and quantification of spot intensities were performed with the ImageMaster® software package. Spots with an increased or decreased intensity of more than 2.5-fold, averaged over six gels (two gels from each of three independent experiments), were recorded. As a further criterion we only considered those spots truly QS regulated that were changed in the mutant in the absence of signal molecules but were rescued to the wild-type situation in the presence of exogenous administered AHLs (2 μM of both C4- and 3-oxo-C12-HSL). Only proteins spots fulfilling all criteria were subjected to in-gel tryptic digestion and were characterized by MALDI-TOF peptide mapping.

Surface-bound proteins

Proteins present at the surface of bacteria often play a pivotal role in the interaction of the organism with its eukaryotic host. The analysis of this cellular compartment with a non-linear pH gradient 3–10 resulted in a total number of 415 protein spots (Fig. 1). Twenty-seven protein spots were only present in PAO1 (Table 1; *Supplementary material*, Table S1), of which six could by identified by mass spectrometry (MS, Table 2). Spot SF 51 corresponds to a cleavage fragment of DbpA, an RNA helicase, spot SF 72 was identified as a binding protein component of an ABC transporter (PA2204), and spot SF 87 represents Nuh, a non-specific ribonucleoside hydrolase. The three remaining protein spots SF 53, SF 54 and SF 70 are the products of the hypothetical genes PA5481, PA0122 and PA0315. While the PA0122 gene product encodes a putative haemolysin (49% identity to the Asp-haemolysin of *Aspergillus fumigatus*; Ebina *et al*., 1994), the functions of the other proteins are unknown. It is worth noting, however, that a PA0315 homologous protein was recently identified as QS regulated in the opportunistic human pathogen *Burkholderia cepacia* (Riedel *et al*., 2003).

The intensities of 52 protein spots were more than 2.5-fold reduced in the *lasI rhlI* double mutant relative to the wild type (Table 1; *Supplementary material*, Table S1). Seventeen of these proteins were identified by MS (Table 2). Spot SF 11 and SF 40 correspond to AotJ, an arginine/ornithine binding protein, spot SF 65 was identified as OprH, the outer membrane protein H1, spot SF 55 represents AprI, the alkaline proteinase inhibitor, spot SF 71 corresponds to PpiA, a peptidyl-prolyl *cis-trans* isomerase A, spot SF 5 was identified as periplasmic taurine-binding protein TauA, four spots SF 73, SF 77, SF 78 and SF 79 correspond to binding protein components of ABC transporters (PA2204, PA1342, PA4195) and spot SF 8 was identified as a putative periplasmatic phosphate ABC-transporter (PA5369). Six further proteins (SF 14, SF 43, SF 13, SF 44, SF 60, SF 56) with unknown function were identified (PA2659, PA3003, PA3611, PA4345, PA4739, PA5494). Importantly, nine of the characterized proteins have not yet been described as QS regulated. Furthermore, we also observed that eight proteins were exclusively expressed in the *lasI rhlI* double mutant and that 15 were upregulated in the mutant background (Table 1; *Supplementary material*, Table S1).

Extracellular proteins

Many of the previously identified QS-regulated genes encode for secreted proteins that often represent important virulence factors. In fact, a recent analysis of the PAO1 secretome showed that the production of several extracellular proteins is regulated by quorum sensing (Nouwens *et al*., 2003). Using a non-linear pH gradient of 3–10, 570 protein spots were detected in culture supernatants of PAO1 (Fig. 2). Of these 89 were absent in the *lasI rhlI* mutant, 58 proteins were at least 2.5-fold upregulated in the wild type, 43 proteins were downregulated by the quorum-sensing system and seven spots were exclusively expressed in the mutant strain (Table 1; *Supplementary material*, Table S1). The percentage of QS-regulated proteins of the secretome was significantly higher (36%) than those of the intracellular (17%) or surface (25%) protein fractions. Six of the identified exoproteins (Table 2) have previously been shown to be QS regulated by both transcriptomics (Hentzer *et al*., 2003b; Schuster *et al*., 2003; Wagner *et al*., 2003) and proteomics of culture supernatants (Nouwens *et al*., 2003). These proteins are: the elastase LasB (spots SN 94, SN 95, SN 98), the chitin-binding protein CbpD (spot SN 97), the endoprotease PrpL (spots SN 115, SN 117, SN 120, SN 121, SN 122, SN 158, SN 164), an aminopeptidase (PA2939, spot SN 32), and two proteins (PA0315, PA0423; spots SN161 and SN167, spot SN145) with unknown function. Moreover, we found that the copper transport

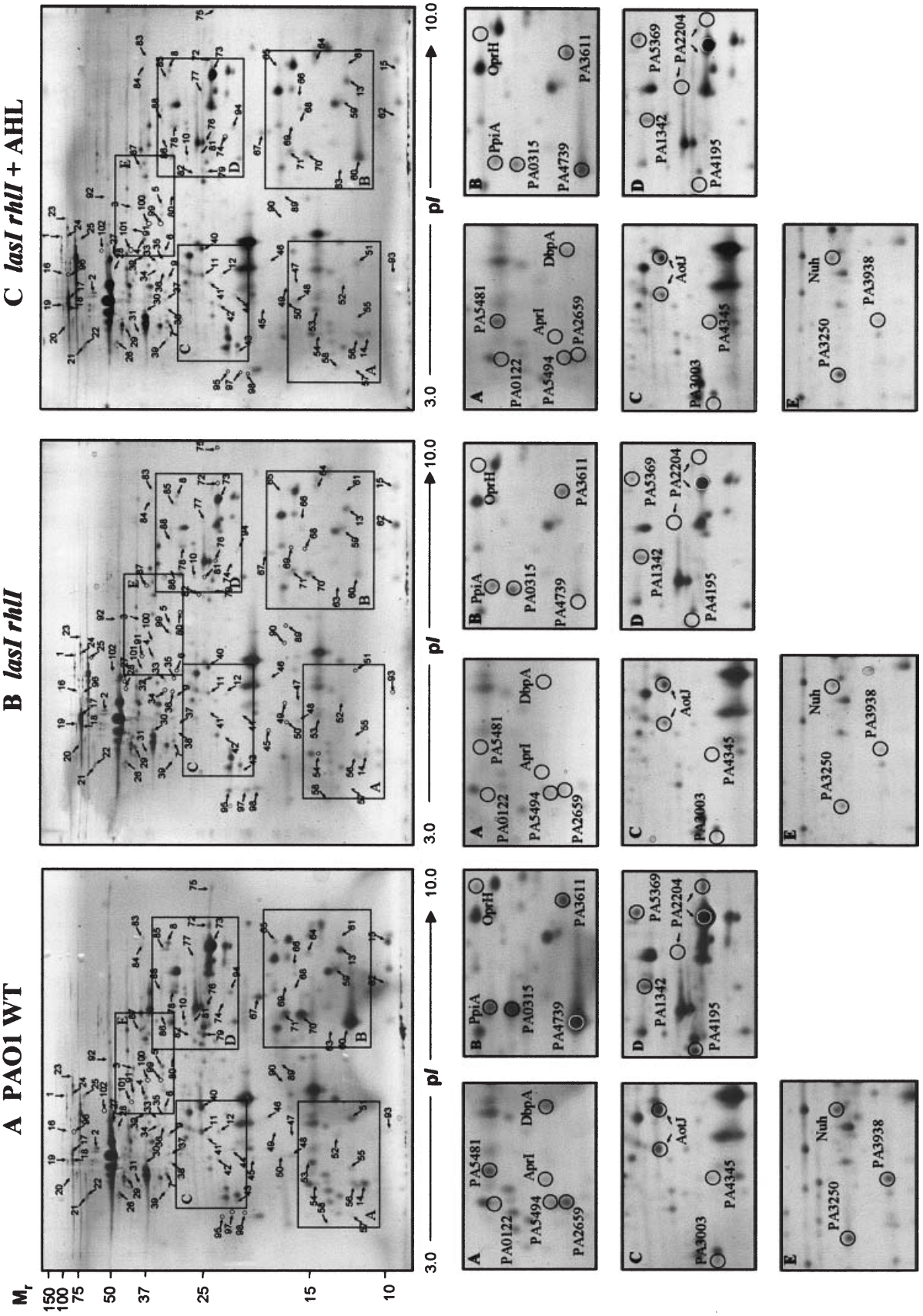

Fig. 1. Comparative two-dimensional gel electrophoresis (2-DE) of surface-bound proteins of the *Pseudomonas aeruginosa* wild-type PAO1 (A) and the *lasI rhlI* double mutant grown in the absence (B) or presence (C) of 2 μM 3-oxo-C12-HSL and C4-HSL. The proteins were separated on Immobiline Dry strips with non-linear pH gradients from 3 to 10 (Amersham Bioscience), followed by an SDS–PAGE on 13% polyacrylamide gels. Gels were stained with Coomassie brilliant blue G-250. Regions of interest are boxed (A–E) and shown below in better detail. Circles indicate positively QS-regulated spots.

Table 1. Global analysis of the *Pseudomonas aeruginosa* QS regulon by proteomics.

Cell compartment	Spot pattern	No. of spots	% of total spots
Surface	total number of spots[a]	415	100
	spots unique in PAO1 WT	27	6.5
	spots upregulated in PAO1 WT	52	12.5
	spots unique in *lasI rhlI*	8	1.9
	spots downregulated in PAO1 WT	15	3.6
Extracellular	total number of spots[a]	570	100
	spots unique in PAO1 WT	89	15.6
	spots upregulated in PAO1 WT	58	10.2
	spots unique in *lasI rhlI*	7	1.2
	spots downregulated in PAO1 WT	43	7.5
Intracellular	total number of spots[a]	986	100
	spots unique in PAO1 WT	57	5.8
	spots upregulated in PAO1 WT	23	2.3
	spots unique in *lasI rhlI*	4	0.4
	spots downregulated in PAO1 WT	83	8.4
In total	total number of spots[a]	1971	100
	spots unique in PAO1 WT	173	8.8
	spots upregulated in PAO1 WT	133	6.8
	spots unique in *lasI rhlI*	20	1.0
	spots downregulated in PAO1 WT	141	7.1

a. Number of spots determined per experiment (spots of wild type plus spots that are exclusively present in the *lasI rhlI* mutant).
The protein spot pattern of the wild-type PAO1 was compared with that of the *lasI rhlI* double mutant. The wild-type spot pattern was used as reference for protein matching with the ImageMaster® 2-DE analysis software. Only spots that differed in intensities by at least a factor of 2.5 and which could be rescued to the wild-type situation when the mutant was grown in the presence of 2 μM of each signal molecule were considered.

outer membrane porin OprC (spot SN 15) is downregulated in the wild type, consistent with the data of Schuster *et al*. (2003) who showed that transcription of *oprC* is under negative control by the QS system. Eight additional protein spots, corresponding to the arginine/ornithine binding protein AotJ (spots SN 112, SN 182), type B flagellin FliC (spots SN 37, SN 41, SN 42, SN 45, SN 181), and a periplasmic binding protein (PA5153, spot SN 129), were also identified by Nouwens *et al*. (2003). However, while these proteins were reported to be upregulated in QS-deficient mutant strains our data suggest that they are positively regulated by quorum sensing. A recent transcriptome study of genes expressed in biofilms of the wild-type PAO1 and the isogenic *lasI rhlI* double mutant (Hentzer *et al*., 2003a) demonstrated that expression of the haem acquisition gene *hasAp* is controlled by quorum sensing. In accordance, we observed that the intensities of the various HasAp spots (spots SN 110, SN 168, SN 190, SN 191, SN 192) are dependent on the quorum-sensing system (see below for a more detailed analysis). Most importantly, eight novel QS-regulated proteins falling into four classes were identified: (i) structural components of flagella, the flagellar capping protein FliD (spots SN 56, SN 58, SN 59, SN 61, SN 132, SN 133, SN 171) and the type 3 flagellar hook-associated protein FlgL (SN 80), (2) proteins involved in iron-uptake, the Fe(III)-pyochelin receptor FptA (SN 18), the ferripyoverdine receptor FpvA (SN 11), a TonB-dependent receptor (PA5505, SN 127) and the outer membrane haemin receptor PhuR (PA4710; SN 3, SN 4), (iii) the outer membrane protein OprF (spots SN 71, SN 73) which exhibits adhesin-like properties (Azghani *et al*., 2002), and (iv) one protein of unknown function (PA3785, spot SN 154).

Intracellular proteins

In total 986 spots could be detected in colloidal Coomassie stained gels when the intracellular fraction was analysed. Figure 3 shows a representative example of the protein pattern of the wild-type PAO1, the *lasI rhlI* double mutant and the double mutant grown in the presence of AHL signal molecules. Matching and comparing the respective 2-DE maps with the Imagemaster analysis software revealed that 168 proteins were differentially expressed (Table 1; *Supplementary material*, Table S1): 87 protein spots found with the wild type but missing in the *lasI rhlI* mutant, 23 protein spots with increased intensities in the wild type, four protein spots found with the *lasI rhlI* mutant but missing in the wild type and 83 protein spots with increased intensities in the *lasI rhlI* mutant. Many of the proteins characterized from the intracellular compartment have previously been reported as QS regulated (Table 2). In agreement with DNA-microarray experiments we identified an enoyl-CoA hydratase/isomerase (PA0745, spots IC 44, IC 71), the acetyl-coenzyme A synthetase AcsA (spot IC 136), the chitinase ChiC (spots IC 104, IC 110, IC 150), an acyl-CoA thiolase (PA2553, spot IC 63), a Clp-family ATP-dependent protease (PA3326, IC 22) and one protein with unknown function (PA1657, spot IC 14). Surprisingly, the phenazine biosynthesis protein (PA1900, spot IC 168), which was described as positively QS regulated by Schuster *et al*. (2003) was

Table 2. Identification of QS-regulated proteins by MALDI-TOF MS peptide mass mapping.

									DNA-Microarray[d]			2-DE[d]
PA No.[a]	Spot No.[b]	Protein identification[a]	Regulation	Theor. p*I*[c]	Theor. M_r[c] (kDa)	Pract. p*I*[c]	Pract. M_r[c] (kDa)	Sequence coverage (%)	Wagner *et al.* (2003)	Schuster *et al.* (2003)	Hentzer *et al.* (2003a, b)	Nouwens *et al.* (2003)
Surface-bound proteins												
PA0122	SF54	conserved hypothetical protein	↑	4.7	14.6	4.4	15.5	79.4	+	+	+	ND
PA0143	SF 87	non-specific ribonucleoside hydrolase Nuh	↑	7.0	37.5	7.4	36.6	26.3	–	+	–	ND
PA0315	SF 70	hypothetical protein	↑	6.6	15.5	7.6	16.3	55.2	+	–	–	ND
PA0455	SF 51	RNA helicase DbpA	↑	8.6	49.8	5.6	11.7	18.6	–	–	–	ND
PA0888	SF 11	arginine/ornithine binding protein AotJ	↑	6.4	28.0	5.4	25.0	53.3	–	–	–	+
	SF 40		↑	6.4	28.0	5.9	24.9	56.0	–	–	–	+
PA1178	SF 65	outer membrane protein H1 precursor OprH	↑	9.0	21.6	9.0	18.5	59.5	–	–	–	ND
PA1250	SF 55	alkaline proteinase inhibitor AprI	↑	5.3	14.0	4.7	12.0	51.9	+	+	+	ND
PA1342	SF 78	binding protein component of ABC transporter	↑	8.3	33.1	8.0	29.8	14.9	+	–	–	ND
PA2204	SF 72	binding protein component of ABC transporter	↑	9.1	29.3	9.8	24.0	57.1	–	–	±	ND
	SF 73		↑	9.1	29.3	8.9	24.0	60.4	–	–	±	ND
	SF 77		↑	9.1	29.3	8.6	24.0	50.4	–	–	±	ND
PA2659	SF 14	hypothetical protein	↑	4.9	11.2	4.4	10.6	59.8	–	–	–	ND
PA3003	SF 43	hypothetical protein	↑	4.7	22.5	4.1	22.2	38.8	–	–	–	ND
PA3227	SF 71	peptidyl-prolyl *cis-trans* isomerase A PpiA	↑	7.9	20.1	7.6	17.7	46.5	–	–	–	ND
PA3250	SF 4	hypothetical protein	↑	5.9	38.3	6.0	35.8	43.4	–	–	–	ND
PA3611	SF 13	hypothetical protein	↑	8.9	15.0	8.8	12.5	55.1	–	–	–	+
PA3938	SF 5	periplasmic taurine-binding protein TauA	↑	7.0	35.9	6.6	32.4	65.9	–	–	±	ND
PA4195	SF 79	binding protein component of ABC transporter	↑	7.0	29.5	7.2	24.5	61.6	–	–	–	ND
PA4345	SF 44	hypothetical protein	↑	5.1	22.2	5.0	22.4	28.6	–	–	–	ND
PA4739	SF 60	conserved hypothetical protein	↑	8.9	11.8	7.5	11.5	30.7	+	+	+	ND
PA5369	SF 8	hypothetical protein	↑	9.1	34.5	8.9	30.9	28.2	–	–	–	+
PA5481	SF 53	hypothetical protein	↑	5.4	17.4	4.8	15.7	61.3	–	+	+	ND
PA5494	SF 56	hypothetical protein	↑	5.2	10.8	4.3	11.7	16.7	–	–	–	ND

Table 2. *cont.*

PA No.[a]	Spot No.[b]	Protein identification[a]	Regulation	Theor. pI[c]	Theor. M_r[c] (kDa)	Pract. pI[c]	Pract. M_r[c] (kDa)	Sequence coverage (%)	DNA-Microarray[d] Wagner *et al.* (2003)	DNA-Microarray[d] Schuster *et al.* (2003)	DNA-Microarray[d] Hentzer *et al.* (2003a, b)	2-DE[d] Nouwens *et al.* (2003)
Extracellular proteins												
PA0315	SN 161	hypothetical protein	↑	6.6	15.5	7.1	14.2	40.0	+	–	–	–
	SN 167		↑	6.6	15.5	8.5	12.2	49.0	+	–	–	–
PA0423	SN 145	conserved hypothetical protein	↑	6.1	20.8	5.9	19.5	38.7	+	–	–	–
PA0852	SN 97	chitin-binding protein CbpD	↑	6.4	41.9	6.1	30.1	32.9	+	+	+	+
PA0888	SN 112	arginine/ornithine-binding protein AotJ	↑	6.4	28.0	5.3	24.8	53.3	–	–	–	+
	SN 182		↑	6.4	28.0	5.6	26.1	53.3	–	–	–	+
PA1087	SN 80	flagellar hook-associated protein type 3 FlgL	↑	4.6	46.8	3.9	31.8	38.0	–	–	–	–
PA1092	SN 37	flagellin type B FliC	↑	5.4	49.2	4.7	46.9	30.5	–	–	–	+
	SN 41		↑	5.4	49.2	4.8	44.8	33.2	–	–	–	+
	SN 42		↑	5.4	49.2	4.7	44.5	27.3	–	–	–	+
	SN 45		↑	5.4	49.2	4.7	43.6	22.5	–	–	–	+
	SN 181		↑	5.4	49.2	5.2	40.6	28.9	–	–	–	+
PA1094	SN 56	flagellar capping protein FliD	↑	6.5	49.4	5.7	39.4	33.3	–	–	–	–
	SN 58		↑	6.5	49.4	6.1	39.3	47.5	–	–	–	–
	SN 59		↑	6.5	49.4	6.0	39.3	40.3	–	–	–	–
	SN 61		↑	6.5	49.4	6.6	38.9	49.2	–	–	–	–
	SN 132		↑	6.5	49.4	4.6	22.3	30.0	–	–	–	–
	SN 133		↑	6.5	49.4	4.4	22.1	32.7	–	–	–	–
	SN 171		↑	6.5	49.4	4.1	12.0	16.5	–	–	–	–
PA1777	SN 71	outer membrane protein OprF	↑	5.0	37.6	4.5	34.5	48.0	–	–	–	–
	SN 73		↑	5.0	37.6	4.4	34.4	23.1	–	–	–	–
PA2398	SN 11	ferripyoverdin receptor FpvA	↑	5.4	91.2	6.5	68.2	31.4	–	–	–	–
PA2939	SN 32	aminopeptidase	↑	5.0	57.5	4.5	51.2	32.1	+	+	+	+
PA3407	SN 110	haeme acquisition protein HasAp	↑	4.3	20.9	3.7	24.1	23.5	–	–	±	–
	SN 168		↑	4.3	20.9	3.8	12.4	44.9	–	–	±	–
	SN 190		↑	4.3	20.9	3.8	18.0	44.9	–	–	±	–
	SN 191		↑	4.3	20.9	3.8	19.0	44.9	–	–	±	–
	SN 192		↑	4.3	20.9	3.8	21.0	44.9	–	–	±	–
PA3724	SN 94	elastase LasB	↑	6.3	53.7	6.7	30.2	32.9	+	+	+	+
	SN 95		↑	6.3	53.7	6.3	30.2	17.7	+	+	+	+
	SN 98		↑	6.3	53.7	6.5	30.0	34.7	+	+	+	+
PA3785	SN 154	conserved hypothetical protein	↑	6.3	17.0	7.4	16.3	58.2	–	–	–	–
PA3790	SN 15	copper transport outer membrane porin OprC	↑	6.0	79.3	6.1	68.1	12.3	–	+	–	–

PA4175	SN 115	Pvds-regulated endoprotease, lysyl class PrpL	↑	6.5	48.2	5.9	25.1	22.7	+	+	+	+
	SN 117		↑	6.5	48.2	6.5	24.9	29.9	+	+	+	+
	SN 120		↑	6.5	48.2	7.5	24.6	35.1	+	+	+	+
	SN 121		↑	6.5	48.2	7.0	24.6	26.4	+	+	+	+
	SN 122		↑	6.5	48.2	6.5	24.8	23.2	+	+	+	+
	SN 158		↑	6.5	48.2	8.7	14.7	10.6	+	+	+	+
	SN 164		↑	6.5	48.2	7.8	13.6	19.3	+	+	+	+
PA4221	SN 18	Fe(III)-pyochelin receptor FptA	↑	5.9	80.0	5.7	66.6	17.1	–	–	–	–
PA4710	SN 3	outer membrane haemin receptor PhuR	↑	5.7	84.7	6.7	86.1	15.8	–	–	–	–
	SN 4		↑	5.7	84.7	6.5	86.7	10.2	–	–	–	–
PA5153	SN 129	periplasmic-binding protein	↑	5.1	27.6	4.7	23.1	40.8	–	–	–	+
PA5505	SN 127	TonB-dependent receptor	↑	7.8	28.1	8.3	24.0	35.0	–	–	–	–
Intracellular proteins												
PA0745	IC 44	enoyl-CoA hydratase /isomerase	↑	6.0	29.9	6.4	26.2	34.9	+	–	–	ND
	IC 71		↑	6.0	29.9	5.7	27.1	38.6	+	–	–	ND
PA0887	IC 136	acetyl-coenzyme A synthetase AcsA	↑	5.9	71.8	6.4	67.8	29.6	–	+	–	ND
PA1657	IC 14	conserved hypothetical protein	↑	4.8	18.2	5.4	16.1	52.4	+	+	+	ND
PA1900	IC 168	phenazine biosynthesis protein	↑	5.9	19.0	6.2	18.9	56.2	–	+	–	ND
PA2300	IC 104	chitinase ChiC	↑	5.2	53.0	5.3	50.2	46. 6	+	+	+	ND
	IC 110		↑	5.2	53.0	5.2	49.8	48.9	+	+	+	ND
	IC 150		↑	5.2	53.0	5.4	50.6	50.3	+	+	+	ND
PA2553	IC 63	acyl-CoA thiolase	↑	5.7	41.4	6.3	37.9	63.9	+	–	–	ND
PA3326	IC 22	Clp-family ATP-dependent protease	↑	5.5	22.1	5.8	21.8	55.7	+	+	+	ND
PA4356	IC 89	xenobiotic reductase XenB	↑	5.1	37.8	5.2	36.4	51.4	–	–	–	ND
PA5078	IC 127	conserved hypothetical protein	↑	6.5	58.5	6.4	56.3	34.5	–	–	–	ND

a. Data generated from peptide mass maps were compared to the complete translated open reading frames (ORF) for *P. aeruginosa* PAO1 (http://www.pseudomonas.com).
b. Spot no. refers to numbers shown in Figs 1–3.
c. Isoelectric point and molecular mass obtained in the experiments (practical) and by calculation (theoretical).
d. Comparison of our data with previously published studies; +: accordant data; –: discordant data; ND: not determined.

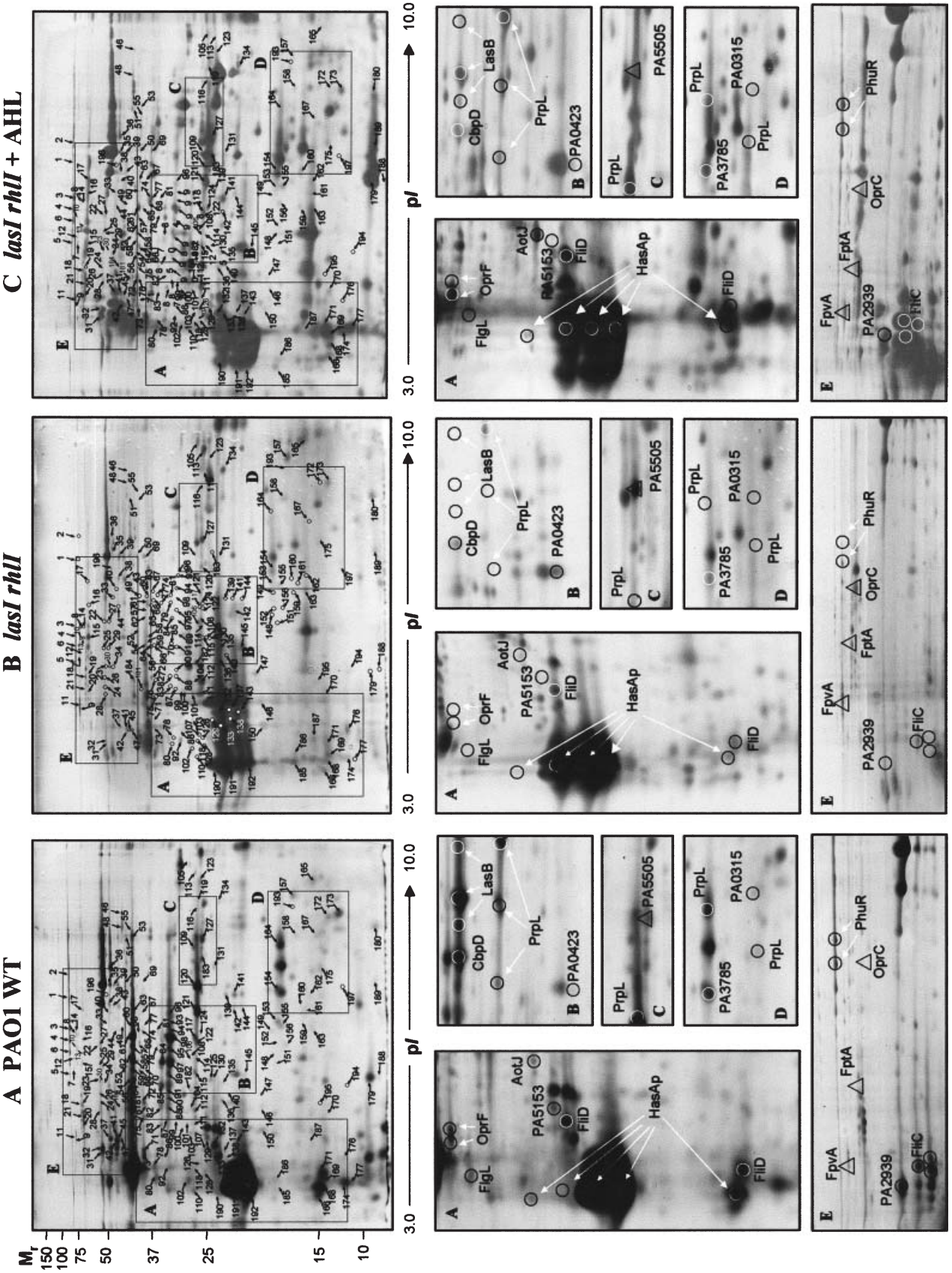

Fig. 2. Comparative 2-DE of extracellular proteins of the *P. aeruginosa* wild-type PAO1 (A), the *lasI rhlI* double mutant grown in the absence (B) or presence (C) of 2 μM 3-oxo-C12-HSL and C4-HSL. The proteins were separated on Immobiline Dry strips with non-linear pH gradients from 3 to 10 (Amersham Bioscience), followed by an SDS–PAGE on 13% polyacrylamide gels. Gels were stained with Coomassie brilliant blue G-250. Regions of interest are boxed (A–E) and shown below in better detail. Circles indicate positively QS-regulated spots; triangles indicate negatively QS-regulated spots.

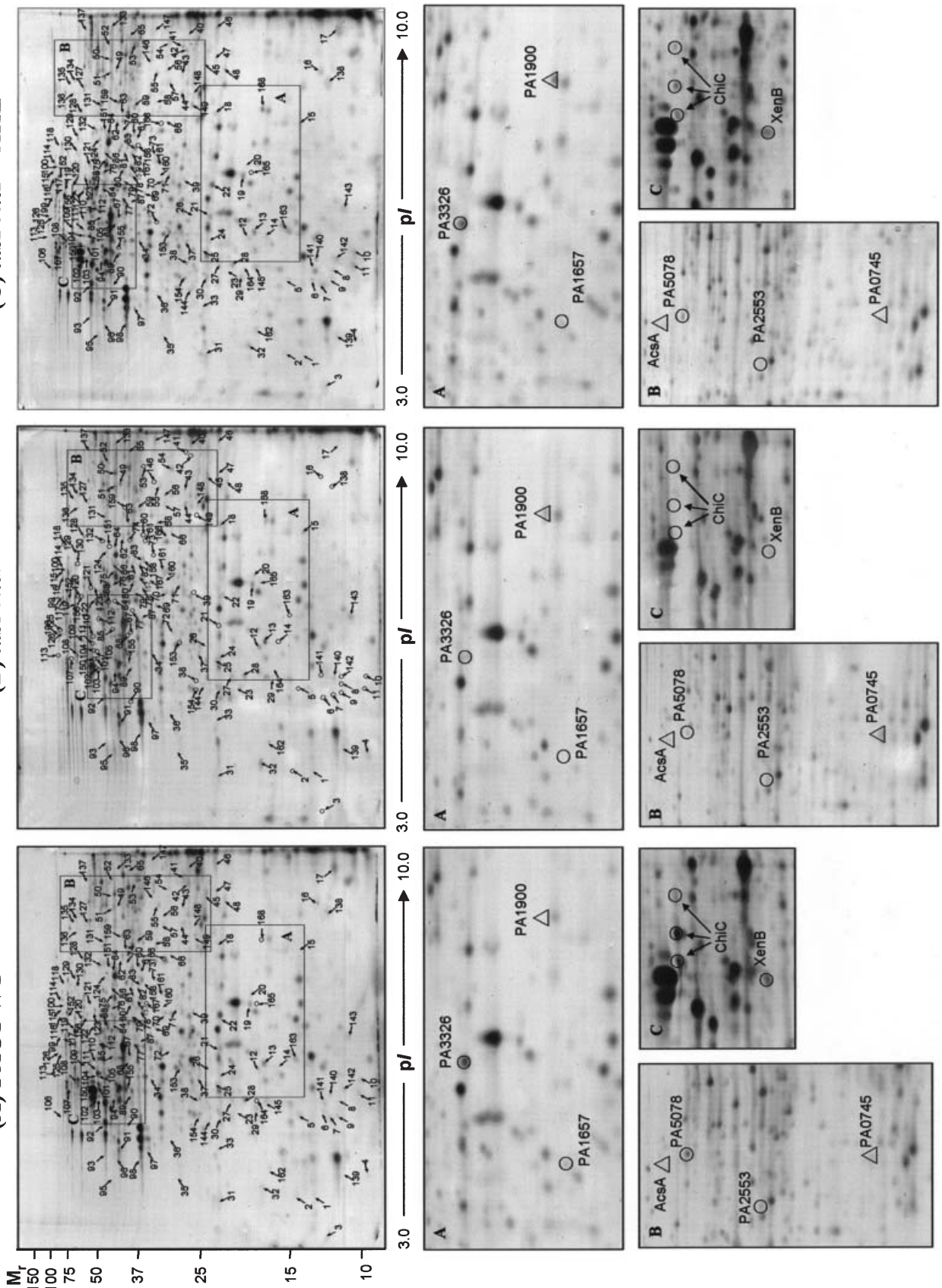

Fig. 3. Comparative 2-DE of intracellular proteins of the *P. aeruginosa* wild-type PAO1 (A), the *lasI rhlI* double mutant grown in the absence (B) or presence (C) of 2 μM 3-oxo-C12-HSL and C4-HSL. The proteins were separated on Immobiline Dry strips with non-linear pH gradients from 3 to 10, followed by an SDS–PAGE on 13% polyacrylamide gels. Gels were stained with Coomassie brilliant blue G-250. Regions of interest are boxed (A–C) and shown below in better detail. Circles indicate positively QS-regulated spots; triangles indicate negatively QS-regulated spots.

absent in the wild-type proteome, but strongly induced in the mutant when the growth medium was supplemented with AHL signal molecules (*Supplementary material*, Table S1; Fig. 3). Furthermore, two novel AHL-controlled proteins were characterized: PA5078 (spot IC 127), a hypothetical protein with 68% homology to the glucan biosynthesis protein OpgG of *Erwinia carotovora* (Page *et al*., 2001), and XenB (spot IC 89), a xenobiotic reductase.

Proteome analysis of furanone C-30 target genes

Using the GeneChip® microarray technology we have recently demonstrated that furanone C-30, a synthetic compound, which was developed on the basis of natural metabolites produced by the macroalgae *Delisea pulchra* (de Nys *et al*., 1993; Givskov *et al*., 1996), specifically inhibits the quorum-sensing cascade of *P. aeruginosa* (Hentzer *et al*., 2002, 2003b). To investigate whether C-30 would interfere with the expression of proteins that were identified as QS regulated in our study we compared the protein patterns of the wild-type PAO1 grown in the presence or absence of 10 μM C-30. As exemplified for the surface-protein fraction, we observed that the protein pattern of C-30-treated cells strongly resembled one of the *lasI rhlI* double mutants (*Supplementary material*, Fig. S1). A quantitative analysis revealed that expression of 67% of all QS-controlled surface protein spots were affected by the furanone (*Supplementary material*, Table S2). The presence of C-30 in the medium induced only very few changes in the expression profile of PAO1 that were not related to quorum sensing (less than 2.5% of all protein spots of the surface fraction), supporting the view that C-30 rather specifically interferes with AHL-mediated gene expression.

Lactoferrin degradation by the AHL-regulated endoprotease PrpL

Quorum-sensing mutants of *P. aeruginosa* were extensively analysed with respect to regulated phenotypes over the past few years. However, the recent functional genomics analyses revealed that an unexpected large number of genes are controlled by quorum sensing, suggesting that many yet unknown functions are AHL regulated. The detailed analysis of the regulation of these functions by phenotypic and/or biochemical assays will be of outmost importance to validate the results from the global analyses.

In agreement with previous functional genome analyses (Hentzer *et al*. 2003b; Nouwens *et al*. 2003; Schuster *et al*. 2003; Wagner *et al*. 2003), we showed that expression of the PrpL endoprotease, which is able to cleave lactoferrin, transferrin, elastin and casein (Wilderman *et al*. 2001), is AHL regulated. To validate this result we decided to test the wild-type and the *lasI rhlI* double mutant for PrpL activity. To this end concentrated culture supernatants of the two strains were incubated with lactoferrin as substrate. Degradation products were then analysed by SDS–PAGE (Fig. 4A). While the *lasI rhlI* double mutant did not exhibit any lactoferrin degrading activity (Fig. 4A, lane 2), almost complete hydrolysis of the substrate was observed with the wild type (Fig. 4A, lane 3). To verify that lactoferrin was exclusively hydrolysed by the

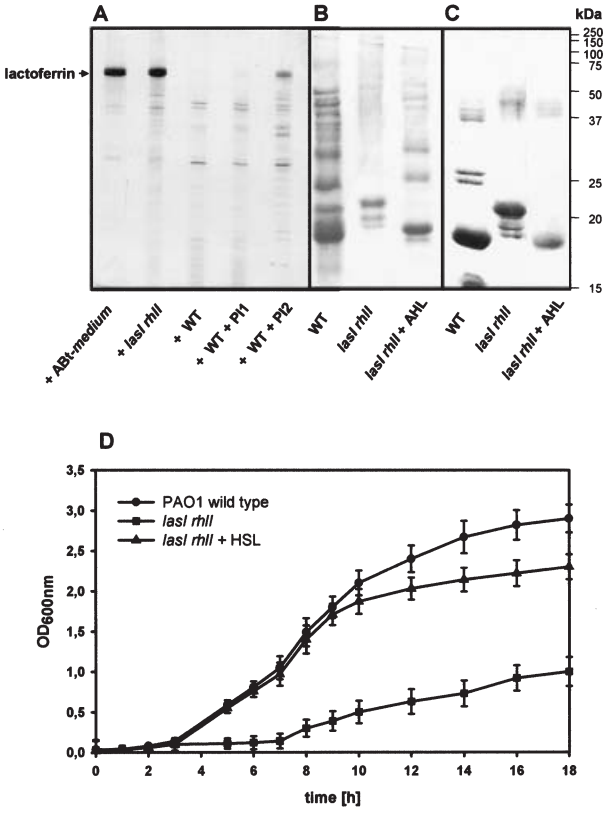

Fig. 4. A. Lactoferrin degradation by PrpL of *P. aeruginosa* is QS regulated. Lactoferrin was incubated with concentrated proteins from supernatants of the wild-type PAO1 or the *lasI rhlI* double mutant at 37°C for 1 h. To specifically inhibit metallo- or serine proteases 1 mM EDTA (PI1) or 4 mM Pefablock (PI2) were added respectively. Digestion products were analysed by SDS–PAGE. The protein band corresponding to the substrate lactoferrin is indicated by an arrow. (B and C) Western blot analysis of HasAp expression. The wild-type strain PAO1 and the *lasI rhlI* double mutant in the absence and presence of 2 μM 3-oxo-C12-HSL and C4-HSL were grown in 100 ml of ABt minimal medium supplemented with 10 mM citrate to an OD_{600} of 1.0. Proteins of the culture supernatant were then precipitated with 10% TCA, resolved by SDS–PAGE and probed with anti-HasA-antibodies derived from *Serratia marcescens* (Létoffé *et al*., 1994).
(B) shows the Coomassie stained SDS–PAGE; (C) shows the corresponding Western blot.
D. Growth of the PAO1 wild type and the *lasI rhlI* double mutant (in the absence or presence of 2 μM 3-oxo-C12-HSL and C4-HSL) in medium containing haemoglobin as the sole iron source. The curves represent the average from three independent experiments. Error bars represent the standard errors of the means.

serine protease PrpL and not by the QS-regulated metalloproteases LasA, LasB or AprA, the assays were carried out in the presence of EDTA, which specifically inhibits metalloprotease activity. As expected, no inhibition of lactoferrin degradation was found in the presence of EDTA (Fig. 4A, lanes 4 and 5). By contrast, the addition of the serine protease inhibitor Pefablock did lower lactoferrin hydrolysis. In conclusion, these data show that that expression of PrpL in *P. aeruginosa* is QS regulated.

Western blot analysis of HasAp *expression*

The quorum-sensing regulon of PAO1 has now been mapped by transcriptomics in three independent studies (Hentzer *et al*., 2003b; Schuster *et al*., 2003; Wagner *et al*., 2003). Furthermore, a proteome analysis was performed to identify QS-regulated proteins in the supernatant of PAO1 (Nouwens *et al*., 2003). Our proteome analysis shows a considerable concurrence with these studies. However, there are also several apparent discrepancies. A striking example is represented by HasAp, an extracellular protein that is required for the uptake of free and haemoglobin-bound haem (Létoffé *et al*., 1998). While our study suggests that the abundance of HasAp differs significantly between the supernatant and surface protein fractions of the PAO1 wild type and the *lasI rhlI* double mutant, neither the DNA-microarray analysis nor the secretome study of Nouwens *et al*. (2003) has identified this virulence factor as QS regulated (Table 2). To address this issue in better detail we analysed expression of HasAp in the two strains using the aid of anti-HasA-antibodies from *Serratia marcescens* (Létoffé *et al*., 1994), which are known to cross-react with the *P. aeruginosa* protein (Létoffé *et al*., 1998; Fig. 4B and C). With PAO1 one major band with an apparent molecular weight of 18 kDa and three additional weaker bands at approximately 26, 24 and 16 kDa were detected. In the case of the *lasI rhlI* double mutant the most abundant band has a molecular mass of 20.5 kDa and two additional bands at 19 and 18 kDa were observed. Importantly, when the *lasI rhlI* double mutant was cultured in medium supplemented with AHLs the protein pattern of the wild type was restored. These results are in good agreement with the 2-DE expression profile of the secreted HasAp where two spots (SN 190, SN 191; Fig. 2, subarea A; Table 2) appeared to be upregulated in the mutant while the abundance of three other spots (SN 110; SN 168; SN 192; Fig. 2, subarea A; Table 2) was reduced relative to the wild type. A comparison of the peptide masses derived from MS-analysis with the peptide masses calculated from a theoretical trypsin digestion (*Supplementary material*, Fig. S2) revealed that the most abundant spot of the *lasI rhlI* double mutant (SN 190, 20.5 kDa) corresponds to the unprocessed HasAp protein, while the major protein spot of the wild type (SN 192, 18 kDa) represents a processed form of HasAp that lacks several amino acid residues at the C-terminus (*Supplementary material*, Fig. S2).

The lasI rhlI *double mutant is defective in haem utilization.*

Our proteome analysis showed that the levels of active HasAp and PhuR are positively QS-regulated. Given that these two proteins are components of the two distinct haem-uptake systems present in *P. aeruginosa*, we speculated that the QS cascade might be involved in the regulation of haeme utilization. To test this hypothesis we grew the PAO1 wild type and the *lasI rhlI* double mutant in medium containing haemoglobin as the sole source of iron (Fig. 4D). When compared with the wild type the double mutant showed greatly reduced growth unless the medium was supplemented with AHLs. These results demonstrate that the ability to utilize haemoglobin as an iron source is QS controlled in *P. aeruginosa*.

Discussion

In this study we compared the protein patterns of the intracellular, extracellular, and surface protein fractions of the *P. aeruginosa* wild-type PAO1 with those of an isogenic *lasI rhlI* double mutant by means of 2-D gel electrophoresis. Our global analysis showed that 723 out of 1971 detected protein spots were differentially expressed (more than 2.5 fold up- or downregulated). Noteworthy, the spot pattern of the double mutant was only partly rescued when the cells were grown in medium containing AHL signal molecules. When only spots that could be restored were considered, 23.7% of all detected protein spots were found to be QS regulated (Table 1). Interestingly, some proteins were only weakly expressed in the wild-type strain while their expression was greatly stimulated when the *lasI rhlI* mutant was grown in the presence of AHL signal molecules. An example of such a protein is represented by the blue copper electron carrier azurin (Azu; PA4922), which has previously been identified as positively QS regulated in the secretome analysis of Nouwens *et al*. (2003).

Relative to the wild type the overall number of expressed proteins is significantly reduced in the *lasI rhlI* double mutant. This is most obvious in the secretome where about 14% of the protein spots detectable in the wild-type 2-DE picture are missing in the AHL-deficient strain. These data are congruent with previous studies, which have shown that inactivation of the QS system results in the reduced expression of many extracellular virulence factors including proteases, chitinase and lipase (Passador *et al*., 1993; Pearson *et al*., 1997) and the downregulation of the type II Xcp secretion system (Chapon-Herve *et al*., 1997).

The quorum-sensing regulon of *P. aeruginosa* PAO1 was recently mapped by transcriptomics using the Affymetrix GeneChip® in three independent studies (Hentzer *et al*., 2003b; Schuster *et al*., 2003; Wagner *et al*., 2003). To be able to compare the present proteome analysis with the transcriptome data, we used the same strains and grew them under similar conditions as in the study of Hentzer *et al*. (2003b). Of the 47 QS-regulated proteins identified in our study 11 of the corresponding genes were also found to be regulated at the transcriptional level by Hentzer *et al*. (2003b). Most notable, nine of these proteins are products of genes that were identified in all three transcriptome experiments. These nine genes encode PA0122 and PA1657, two conserved hypothetical proteins, the chitinase ChiC, the chitin binding protein CbpD, the elastase LasB, the Clp-family ATP-dependent protease PA3326, the PvdS-regulated endoprotease PrpL, an aminopeptidase (PA2939) and the alkaline protease inhibitor AprI. Chitinase ChiC and elastase LasB represent well-known AHL-controlled extracellular virulence factors that have been identified in various independent studies (Passador *et al*., 1993; Brint and Ohman, 1995; Latifi *et al*., 1995; Pearson *et al*., 1997; Whiteley *et al*., 1999). CbpD exhibits adhesin-like properties: the major 43 kDa protein is able to bind to chitin thereby protecting the protein against proteolytic degradation by the elastase LasB (Folders *et al*., 2000). PA2939 was recently characterized as a zinc-dependent metalloprotease, which cleaves amino acids from small peptides under nutrient limitation (Cahan *et al*., 2001). Appropriate substrates for the aminopeptidase might be delivered by the action of other extracellular proteases such as LasB, LasA, AprR and PrpL. PrpL exhibits strong homology with an endoprotease from *Enzymobacter lysogenes* and was shown to hydrolyse casein, elastin and lactoferrin (Wilderman *et al*., 2001). Previous work provided evidence that expression of PrpL is regulated by the alternative sigma factor PvdS (Wilderman *et al*., 2001; Lamont *et al*., 2002). We have used an enzymatic assay to clearly show that expression of PrpL is dependent on a functional quorum-sensing system (Fig. 4A). Clp-family ATP-dependent proteases are known as important regulatory enzymes, which are normally induced during many types of stress (Porankiewicz *et al*., 1999). They are responsible for the proteolytic removal of non-functional proteins caused by spontaneous denaturation, biosynthetic errors or accumulative mutations. The alkaline proteinase inhibitor AprI is a specific, high-affinity inhibitor of AprA, which is a member of the serralysin family of zinc-dependent metalloproteases (Feltzer *et al*., 2000). Serralysin inhibitors are thought to protect the cell from adventitious proteolysis during protease secretion (Létoffé *et al*., 1989).

One of the major differences between the transcriptome studies and our proteome analysis concerns the number of regulated functions, as the number of QS-regulated proteins (about 23.7% of all detected spots) is much higher than the number of regulated *P. aeruginosa* genes (2.9% in the study of Hentzer *et al*., 2003b, 6.3% in the study of Schuster *et al*., 2003 and 11.1% in the study of Wagner *et al*., 2003). Although some of the discrepancy is simply due to differences in the interpretation of microarray and proteome data (e.g. differences in the threshold levels used for the definition of QS-regulated genes or proteins), this explanation does not account for extend of the disagreement. In fact, the differences between the studies are even more striking when the restrictions of proteomics are considered. Low abundant proteins like many regulators, proteins with either a very high or a very low isoelectric point or with a high molecular mass as well as proteins with membrane-spanning domains are not readily displayed on 2-D PAGE. In addition, the number of samples that is manageable by 2-DE is restricted by the incommensurable higher experimental efforts inherent to this method when compared with transcriptomics using a commercially available DNA-microarray. As a consequence, transcriptome data are available for several time points along the growth curve of *P. aeruginosa* while our study is based on a single time point (late exponential phase, OD_{600} of 1.0), where most QS-regulated proteins were found to be affected. However, previous work has shown that some AHL-regulated genes are only induced at specific stages during growth (Whiteley *et al*., 1999; Schuster *et al*., 2003) and we therefore missed all those proteins in our analysis that are expressed either temporarily in the early growth phase or very late in the stationary phase.

There are also some notable disagreements between our secretome analysis and the one performed by Nouwens *et al*. (2003). While the latter study reported that the most abundant protein in the supernatant of PAO1 is the LasB protease, we found that under our experimental settings LasB was not among the dominant protein spots. By contrast, the major protein spots in our analysis were identified as HasAp, which was not at all detected by Nouwens *et al*. (2003). In total, 27 proteins were identified as AHL regulated in the study of Nouwens *et al*. (2003), of which only 10 were also identified in our study. Most strikingly, three of these 10 proteins were found to be oppositely regulated. These were identified as structural components of the *P. aeruginosa* flagellar apparatus FliC and FlgK and the arginine/ornithine binding protein AotJ. Although further work will be required to address this issue in better detail it is conceivable that some of the observed discrepancies are probably caused by differences in the experimental protocols. While in the study of Nouwens *et al*. (2003) complex Luria–Bertani medium was used we grew our strains in minimal medium supplemented with citrate

and Casamino acids. In fact, expression of several extracellular enzymes in *P. aeruginosa*, e.g. the LasB elastase (Ohman *et al*., 1980) or the lipase (Gilbert *et al*., 1991), have previously been demonstrated to be strongly dependent on medium composition.

Our results clearly show that inactivation of the *P. aeruginosa* PAO1 quorum-sensing system affects the cell's protein composition more strongly than the cell's transcriptome, indicating that a major part of AHL-dependent regulation occurs at the post-transcriptional level. Bearing in mind that LasR and RhlR are transcriptional regulators that after binding of their cognate AHL signal molecules activate or repress transcription of target genes this finding is rather unexpected. However, a recent transcriptome analysis revealed that seven of all AHL-induced genes code for proteins that are related to proteolysis, phosphorylation or glycosylation of proteins and that a further two encode known or putative chaperones (Wagner *et al*., 2003; Schuster *et al*., 2003). Hence, the QS circuitry controls transcription of several genes encoding proteins that in turn will affect expression of various proteins at the post-transcriptional level. For example, expression of the ATP-dependent Clp-protease PA3326 was shown to be AHL-controlled in all three transcriptome analyses as well as in the present proteome analysis. Members of this protease family are involved in post-translational protein modifications and often modulate the levels of various unrelated proteins (Porankiewicz *et al*., 1999). At present, no prediction can be made as to how many proteins are turned over by PA3326 in *P. aeruginosa*, as the specificity of this Clp-family protease is unknown. Another AHL-regulated protease that was previously shown to be involved in the post-transcriptional modification of proteins is LasB. Kamath *et al*. (1998) demonstrated that LasB cleaves the 16 kDa form of nucleoside diphosphate kinase (Ndk) to a truncated 12 kDa form. This smaller form is membrane associated and enhances formation of GTP, which is required for alginate synthesis. More recently, Cowell *et al*. (2003) provided evidence that LasB, together with the QS-regulated alkaline protease LasA, modulate the levels of the exotoxins ExoS and ExoT. Interestingly, although LasB is an extracellular protein, some activity is present intracellularly as processing of Ndk and the two exotoxins are thought to take place in the periplasm. Using a bioinformatic approach we searched all annotated proteins in the *P. aeruginosa* PAO1 genome for putative cleavage sites. This analysis revealed that approximately 63% of all PAO1 proteins are theoretic targets for LasB or/and LasA, supporting the idea that the two proteases play important roles in the processing and degradation of proteins in *P. aeruginosa*.

Post-transcriptional regulation is known to be multifacetted and can occur at various stages between transcription and protein maturation. In this study we have analysed expression of the haemophore HasAp in better detail, as several protein spots identified as HasAp showed dramatic variations in both their positions and intensities when the protein pattern of the wild-type PAO1 was compared with one of the *lasI rhlI* double mutants. HasAp is an iron-regulated extracellular haem-binding protein that is required for the utilization of haemoglobin iron (Létoffé *et al*., 1998). It has been suggested that expression of this haemophore is particularly important in the early stage of infection, when the haemoglobin concentration is low and thus a HasAp producing strain will out compete other colonizers by sequestering the available haem. In *P. aeruginosa* HasAp is synthesized as a 20.5 kDa preprotein that is activated by multiple C-terminal cleavages removing 15–21 amino acids. Only the processed form of the protein is able to bind haem. We have shown that the total amount of HasAp isoforms produced by the wild-type and the *lasI rhlI* double mutant does not differ significantly, confirming the results of the transcriptome analyses, which were unable to detect differences between the strains. However, in the wild type the most abundant HasAp band has a molecular mass of 18 kDa corresponding to the active haemophore form (Fig. 2, subarea A; Fig. 4B and C) while in the mutant the two dominant bands display molecular masses of 19 and 20.5 kDa indicative of inactive preforms (*Supplementary material*, Fig. S2). Thus, our data suggest that the production of functional HasAp haemophore by *P. aeruginosa* is QS regulated albeit indirectly by controlling a protease(s) that in turn is required for the processing of the HasAp preform. Although the protease(s) required for processing of the preprotein has not yet been identified it has been speculated that the extracellular proteases LasB, LasA and AprA, all of which are QS regulated, are involved in the process (Létoffé *et al*., 1998). In fact, HasAp contains potential cleavage sites for both LasB and LasA (*Supplementary material*, Table S3). Work under progress therefore aims at elucidating the roles of LasB and LasA in the processing of HasAp.

HasAp is a component of the *has* haem-acquisition locus, which in terms of genetic organization and protein identities is closely related to the homologous system in *S. marcescens* (Létoffé *et al*., 1998; Ochsner *et al*., 2000). In addition to the *has* system *P. aeruginosa* has a second haem-acquisition locus, *phu*, that consists of the *phuR* haemin receptor gene and the *phuSTUVW* operon encoding a typical ABC transporter. The proteins encoded by the *phu* locus show high similarity to the respective components of *Yersinia pestis* and *Yersinia enterocolitica* (Ochsner *et al*., 2000). The two systems operate in parallel and thus only inactivation of both systems causes severe defects in haem or haemoglobin utilization (Ochsner *et al*., 2000). Intriguingly, our proteome

analysis identified the outer membrane haemin-binding receptor PhuR as positively AHL-regulated, indicating that not only the *has* but also the *phu* haem acquisition system is regulated by the *lasI rhlI* QS circuitry. To address this issue we tested the wild-type and the *lasI rhlI* double mutant for growth on medium containing haemoglobin as the sole source of iron. These growth experiments clearly established that the ability to utilize haemoglobin as iron source is indeed QS-controlled in *P. aeruginosa*. This is the first time that an involvement of a QS system in the control of haemoglobin utilization has been demonstrated.

The intensity of two protein spots in the supernatant fraction that were identified as degradation products of the pyoverdin and pyochelin receptors FpvA and FptA (with molecular masses of 68 and 67 kDa), respectively, was increased in the *lasI rhlI* double mutant relative to the wild type (Table 2). As FpvA and FptA are outer membrane proteins with molecular masses of approximately 87 and 76 kDa (Poole *et al*., 1993; Ankenbauer and Quan, 1994), it is conceivable that the two proteins found in the culture supernatant are non-functional. Under iron limiting growth conditions, *P. aeruginosa* produces two major siderophores, pyoverdin and pyochelin which after complexing Fe(III) are taken up by the cell via the outer membrane receptors FpvA and FptA respectively. Given that both receptors appear to be degraded with increased rates in the *lasI rhlI* double mutant it appears possible that the mutant cells experience iron limitation despite the presence of excess iron in the medium. Interestingly, synthesis of the siderophore pyoverdin was demonstrated to be QS-controlled (Brint and Ohman, 1995; Latifi *et al*., 1995; Hentzer *et al*., 2003a). Previous work has shown that expression of proteins involved in iron-uptake is co-regulated via a hierarchical cascade of regulators including the ferric uptake regulator Fur and the alternative sigma factor PvdS (Ochsner *et al*., 1995; Ochsner *et al*., 2002). Most interestingly, expression of PvdS itself appears to be AHL-dependent, as this gene was recently found to be positively regulated by quorum sensing in biofilm-grown *P. aeruginosa* PAO1 cells (Hentzer *et al*., 2003a). Noteworthy in this context is the fact that expression of the AHL-regulated endoprotease PrpL (see above) is also controlled by the alternative sigma factor PvdS (Wilderman *et al*., 2001; Lamont *et al*., 2002). Thus expression of PrpL appears to be indirectly AHL-regulated via PvdS. In conclusion, these data support the hypothesis that the quorum-sensing regulatory system is integrated with the iron status of the cell as it has been proposed previously by Hassett *et al*. (1999). However, additional work will be required to unravel the interrelationship between quorum sensing and the iron regulatory system of *P. aeruginosa*.

Experimental procedure

Bacterial strains and growth conditions

The *P. aeruginosa* wild-type PAO1 (*Pseudomonas* genetic stock centre, http://www.pseudomonas.med.ecu.edu, strain PAO0001) and the *lasI rhlI* double mutant (Hentzer *et al*., 2003b) were routinely grown in ABt minimal medium (Clark and Maaløe, 1967) supplemented with 10 mM sodium citrate and 0.5% Casamino Acids under vigorous agitation at 37°C. For the 2-DE sample preparation the strains were grown to the late exponential growth phase (OD_{600} of 1.0). Gentamycin and tetracyclin were added as required at a final concentration of 60 μl ml^{-1}. Rescue experiments were performed by supplementing the growth medium with 2 μM 3-oxo-C12-HSL and 2 μM C4-HSL. The quorum-sensing blocker C-30 was used at a concentration of 10 μM. Growth was monitored spectrophotometrically by an Ultrospec Plus spectrophotometer (Amersham Bioscience) by measurement of optical density at 600 nm.

Analytical procedures

Total protein concentrations were determined according to the method of Bradford (1976). Next, 5 μl aliquots of the respective protein solution were mixed with 1 ml of Coomassie Plus protein assay reagent (Pierce) and the absorbance measured at 595 nm. The protein concentration was calculated using BSA as standard.

Analysis of lactoferrin degradation by PrpL

Lactoferrin degradation was measured as described by Wilderman *et al*. (2001). Briefly, the *P. aeruginosa* wild-type PAO1 and the *lasI rhlI* double mutant were grown in 200 ml ABt minimal medium supplemented with 10 mM citrate and 0.5% Casamino acids at 30°C for 16 h. Extracellular proteins in the culture supernatants were precipitated with ammonium sulphate, resolved and dialysed against 50 mM Tris-HCl, pH 7.5 for 6 h. Approximately 10 mg of lactoferrin (Sigma) was incubated at 37°C for 1 h with the extracellular proteins of PAO1 and the *lasI rhlI* double mutant in Tris-buffer (10 mM Tris-HCl, pH 7.5; 2 mM $CaCl_2$, and 40 mM KCl). Degradation products were separated by SDS–PAGE and stained with Coomassie brilliant blue. Metalloproteases (LasA, LasB and AprA) were inhibited by the addition of 1 mM EDTA and the serine protease PrpL was blocked by Pefablock (4 mM, Merck).

Haemoglobin utilization

Utilization of haemoglobin was tested by culturing *P. aeruginosa* strains in LBD medium (Luria Broth supplemented with 4 mM of the free iron chelator 2,2′ dipyridyl and 0.5 μM of bovine haemoglobin obtained from Sigma). Growth of the wild-type and the *lasI rhlI* double mutant (in absence and presence of 2 μM 3-oxo-C12-HSL and 2 μM C4-HSL) was monitored spectrophotometrically by an Ultrospec Plus spectrophotometer (Amersham Bioscience) by measurement of optical density at 600 nm.

Electrophoresis and immunological techniques

Proteins were analysed by standard SDS–PAGE (Laemmli, 1970) followed either by Coomassie blue staining or Western blot analysis. *S. marcescens* HasA antibodies (Létoffé *et al*., 1994) used in Western blot analysis for cross-reaction with HasAp were diluted 2000-fold.

Pre-fractionation of protein samples for 2-DE

Intracellular proteins. For the preparation of intracellular proteins 1 l cultures were harvested by centrifugation (5.000 r.p.m.; 4°C; 30 min) and the cells were washed in 0.9% NaCl solution. Next, 1 g of the cell pellet was resuspended in 7 ml 50 mM Tris-HCl buffer pH 7.5 supplemented with protease inhibitor (Protease Inhibitor Cocktail®, Roche). Cell lysis was performed by sonification and the cell debris was removed by centrifugation (15.000 r.p.m.; 1 h; 4°C). In order to improve focusing of proteins the lysates were extracted twice with phenol as described previously (Riedel *et al*., 2003). Then, 1 ml aliquots of the extracts were mixed thoroughly with phenol and incubated for 10 min at 70°C. The sample was cooled on ice for 5 min and the phases were then separated by centrifugation (5.000 r.p.m.; 10 min; 4°C). The aqueous phase was discarded and proteins were precipitated by adding 1 ml of ice-cold acetone. The sample was pelleted by centrifugation (15.000 r.p.m.; 20 min; 4°C) and the pellet was washed with 1 ml of ice-cold acetone. After a final centrifugation step (20 min; 15.000 r.p.m.; 4°C) the supernatant was removed and the pellet was air-dried for 1 h. The precipitate was finally solubilized in 200 µl of rehydration buffer [8 M urea, 2% (w/v) CHAPS, 15 mM DTT and 0.5% (v/v) IPG-buffer pH 3–10 or pH 4–7 respectively (Amersham Bioscience)].

Extracellular proteins. For the preparation of extracellular protein samples, cells of a 1 l culture were separated from the supernatant by centrifugation (4°C; 5.000 r.p.m.; 30 min). The proteins in the supernatant were precipitated with 10% w/v TCA at 4°C overnight. The precipitate was harvested by centrifugation (4°C, 5.000 r.p.m., 1.5 h), washed with ethanol, dried, resolved and extracted twice with phenol as described for the intracellular proteins. The final protein pellet (approximately 100 mg) was solubilized in 100 µl rehydration buffer.

Cell surface proteins. Surface-bound proteins were extracted as described by Nilsson *et al.* (2000). Cells of a 1 l culture were harvested by centrifugation (5.000 r.p.m.; 4°C; 20 min) and washed twice in 50 mM Tris-HCl, pH 7.5. Next, 4 g of the cell pellet was resuspended in 100 ml 0.2 M glycine hydrochloride, pH 2.2. The suspension was stirred at 20°C for 15 min and cells were removed by centrifugation (5.000 r.p.m.; 20 min; 4°C). The supernatant was neutralized with NaOH to pH 7.0 and the extracted proteins were precipitated with a threefold volume of acetone at –20°C overnight. The precipitate (200 mg) was harvested by centrifugation (4°C; 7.000 r.p.m.; 30 min), washed four times with ethanol, dried thoroughly and resolved in 4 ml of 50 mM Tris-HCl, pH 7.5. One millilitre aliquots were extracted twice with phenol as describe for the intracellular proteins. The final pellet (approximately 100 mg) was resuspended in 200 µl rehydration buffer.

Integrity of subcellular fractions

The purity of the subcellular fractions was assessed by identifying approximately 150 spots of each fraction via mass spectrometry. Ninety-two per cent of the 'surface proteins' were found to be either proteins associated with the cell surface, outer membrane proteins or periplasmic proteins. Only 6% were of cytoplasmic origin or extracellular proteins, 2% could not be assigned to any cellular compartment. Sixty per cent of the identified 'intracellular proteins' were known cytoplasmic proteins, 40% were either of different or unknown origin. Fifty-four per cent of the 'extracellular proteins' (referred to as the 'secretome') were identified as proteins containing a signal sequence and are therefore truly secreted proteins, 6% were of cytoplasmic origin, and 40% were outer membrane or periplasmic space proteins. This relatively high amount of contaminating proteins in the secretome fraction was described before in several other proteome studies (e.g. Nouwens *et al*., 2002, 2003) and is probably caused by the release of surface-associated and intracellular proteins by autolysis of cells during sample preparation.

Two-dimensional gel electrophoresis

For the first dimension samples were mixed with rehydration solution [8 M urea, 2% (w/v) CHAPS, 15 mM DTT and 0.5% (v/v) IPG-buffer pH 3–10 or 4–7, respectively (Amersham Bioscience)] resulting in a final protein amount of 200 µg protein in a volume of 350 µl. The isoelectric focusing was performed by using immobilized pH gradient (IPG) strips (Amersham Bioscience) as described elsewhere (Görg *et al*., 2000). The IPG strips (18 cm, pH 3–10 NL, pH 4–7, or pH 6–11) were rehydrated overnight at 30 V and focused for 3 h at 8000 V at 20°C under mineral oil. The IPG strips were then incubated for 10 min, in equilibration buffer I [6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS and 1% (w/v) dithiothreitol in 50 mM Tris-HCl buffer, pH 8.8] followed by equilibration buffer II [6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS and 4% (w/v) iodacetamide in 50 mM Tris-HCl buffer, pH 8.8]. After the equilibration step the strips were transferred to 22 cm × 22 cm 13% SDS–PAGE gels for the second dimension. Electrophoresis was performed at 150 V and 150 mA at 15°C for approximately 18 h. Protein spots were visualized by staining with Coomassie brilliant blue G-250 as described elsewhere (Neuhoff *et al*., 1988).

Data analysis

Three growth experiments were performed to increase the external reproducibility of our results, for the internal reproducibility two samples of every growth experiment were subjected to 2-DE. The gels were scanned with a densitometric ImageScanner (Amersham Bioscience) and the raw images were analysed using the Imagemaster 2-D gel analysis software version 4.0 (Amersham Bioscience). For each compar-

ison (wild type versus *lasI rhlI* mutant versus *lasI rhlI* mutant supplemented with 2 μM 3-oxo-C12-HSL and C4-HSL or wild type versus wild type plus 10 μM furanone C-30 respectively) six gels were analysed and compared. Only significant changes in spot intensity (more than 2.5-fold) were considered.

Protein identification by mass spectrometry

Sample preparation. In order to identify differentially expressed proteins the respective spots were excised from the 2-DE gel and subjected to in-gel digestion as described elsewhere (Shevchenko *et al*., 1996; Wilm *et al*., 1996) with minor modifications. The excised gel plugs were destained in a 50:50 mixture of 5 mM $(NH_4)HCO_3$, pH 7.8 and acetonitrile for 30 min at 37°C. Then the gel slices were shrunk with acetonitrile and dried by vacuum centrifugation or in a vacuum heater. For the tryptic digestion, 20 μg modified sequencing grade trypsin (Promega) dissolved in 100 μl 1 mM HCl and immediately before use diluted with 1500 μl 5 mM $(NH_4)HCO_3$, pH 7.8 was added to the dry gel pieces and incubated on ice for 1 h for re-swelling. After removing the supernatant, additional digestion buffer [10–30 μl 5 mM $(NH_4)HCO_3$, pH 7.8] was added and the digestion was continued at 37°C for 4–18 h. The supernatant of the digestion (peptide mixture) was spotted on MALDI plates coated with 0.25 μl α-cyano-4-hydroxycinnamic acid (1 mg ml^{-1} in acetonitrile) and air-dried (seed-layer).

Thereafter 0.5 μl of the digested sample was added to the slide and mixed with 0.5 μl matrix solution (15 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in 50% acetonitrile + 0.5% TFA) and air-dried for 10 min.

Mass spectrometry. An Ettan z2 MALDI-TOF (Amersham Biosciences) with an UV nitrogen laser (337 nm), delayed extraction, low mass rejection and harmonic reflectron was employed for peptide mass mapping in positive ion reflector mode. Ion acceleration voltage was 20 kV. For System calibration with a mass accuracy better than 30 p.p.m. a multi level external calibration was performed with peptide samples [angiotensin II (human), LHRH, fibrinopeptide A (human), ACTH 1–24 (human), ACTH 1–39 (human)]. Spectra were calibrated with internal calibration using trypsin autodigestion fragments (842.509 and 2211.101 Da) resulting in a mass accuracy better than 10 p.p.m. For each spectrum 200 single shots were accumulated.

Protein identification. The proteins were identified based on their peptide mass maps using the database search program Ettan MALDI-TOF Evaluation (Amersham Biosciences). A maximum of one missed enzymatic cleavage and modification of cysteins by carbamidomethylation and methionins by partial oxidation were considered during the searches. For database searches the non-redundant NCBI library and the updated *P. aeruginosa* genome sequences database of *P. aeruginosa* PAO1 from the *Pseudomonas* Genome Project (Stover *et al*., 2000) and *P. aeruginosa* Community Annotation Project (http://www.pseudomonas.com/SequenceDataU.asp) were used. Only identification results with an expectation rate of less than 0.3 were accepted.

Search for potential protease recognition motifs

In order to determine hypothetical recognition sites (GGG, GGL, GGA, GGV, GGI, or GGP for LasA and FVA, FLA, FIA, FYA or FFA for LasB) of the strictly QS-regulated proteases LasA and LasB in the complete set of *P. aeruginosa* proteins a PERL script was written to perform an exact string matching with the EMBOSS program *fuzzpro*.

Acknowledgements

We thank Cécile Wandersman for providing anti-HasA-antibodies, Dorothea Begert, Stefan Kirchner and Beate Schumacher for excellent technical assistance and Diego Riano-Pachon for his help with the computer-aided analysis of potential protease recognition sites. This work was funded by the Deutsche Forschungsgemeinschaft (RI 969/3-1), the Cystic Fibrosis Foundation Therapeutics, Inc., the Mukoviszidose e.V., the Danish Technical Research Council, the Plasmid foundation and the Villum Kann-Rasmussen Foundation.

Supplementary material

The following material is available from http://www.blackwell-publishing.com/products/journals/suppmat/mmi/mmi532/mmi532sm.htm

Fig. S1. Comparative 2-DE of surface proteins of the *Pseudomonas aeruginosa* PAO1 wild type (A), the lasI rhlI double mutant (B), and PAO1 grown in the presence of 10 μM furanone C-30. Proteins were separated and stained as described in the legend of Fig. 1. The resulting protein spot patterns were analysed with the Imagemaster-Software. Spots that are at least twofold up- or downregulated are shown in colours. Protein spots outlined in blue show no variation in intensities; yellow protein spots are absent in PAO1 WT but present in both the double mutant and the C-30 treated PAO1; protein spots shown in blue are exclusively present in PAO1 WT; the intensities of protein spots shown in pink are reduced in the double mutant as well as in PAO1 treated with C-30; the intensities of protein spots shown in green are increased in the double mutant as well as in PAO1 treated with C-30 relative to the wild type.

Fig. S2. Schematic drawing of expected HasAp protein fragments after digestion with trypsin. The observed mass fingerprints of spots SN 190, SN 191 and SN 192 correspond well with the calculated masses of trypsin fragments from the unprocessed HasAp protein (spot SN 190) and two processed HasAp variants (spots SN 191 and SN 192) that lack C-terminal amino acid residues (Létoffé *et al*., 1998). Peptides shown as bold lines were detected by mass spectrometry, masses of peptides shown as dotted lines were out of the detection range.

Table S1. Protein spots induced or repressed by the quorum-sensing system of *P. aeruginosa.* Only spots with an at least 2.5-fold change in signal intensity which were rescued to the wild-type level when the *lasI rhlI* double mutant was

grown in presence of 2 μM 2-oxo-C12-HSL and C4-HSL were considered. For each comparison six gels resulting from three independent protein extractions were analysed and compared.

Table S2. Protein spots induced or repressed by the quorum-sensing system of *P. aeruginosa* (only spots with an at least 2.5-fold change in signal intensity were considered) are compared with protein spots induced or repressed by the addition of 10 μM furanone C-30 to the growth medium of *P. aeruginosa* PAO1. For each comparison six gels resulting from three independent protein extractions were analysed and compared.

Table S3. Number of hypothetical cleavage sites of QS-sensing controlled exoproteases LasA and LasB in proteins identified as AHL-regulated in this study.

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6.2 Proteome analysis of intraclonal diversity of two Pseudomonas aeruginosa TB clone isolates

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Published in: Proteomics 4: 1241-1246 (2004)

Short Communication

Proteome analysis of intraclonal diversity of two *Pseudomonas aeruginosa* TB clone isolates

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Two strains, *Pseudomonas aeruginosa* TB10839 and TB121838, which belong to the TB clonal lineage, have been isolated from sputa of cystic fibrosis patients. Despite the fact that the strains are closely related, their pathogenic potential differs dramatically: while strain TB10839 is capable of proliferating in polymorphonuclear granulocytes, strain TB121838 is not. Comparative two-dimensional polyacrylamide gel electrophoresis coupled to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)-mass spectrometry was employed to map the extracellular, intracellular, and surface sub-proteomes of TB10839 and TB121838 and to identify differentially expressed proteins. About 4% of all detected protein spots were differentially expressed between both strains including absent or present spots and spots with a more than 2-fold changed intensity. This percentage reflects a relatively high degree of intraclonal variability. Many of the protein spots in TB10839 that were missing or expressed at lower levels in TB121838 were identified as quorum-sensing regulated virulence factors. It might be speculated that the increased expression of these proteins contributes to pathogenic competence of TB10839.

Received	1/10/03
Revised	5/12/03
Accepted	8/12/03

Keywords: Bacterial proteomics / Intraclonal diversity / Pathogenicity / *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, a ubiquitous environmental organism, is capable to thrive in diverse ecological niches and to infect a wide variety of animals, plants, and humans. As human pathogen, *P. aeruginosa* is the leading source of Gram-negative nosocomial and life-threatening infections and causes chronic lung infections in patients suffering from cystic fibrosis (CF) [1]. As many other Gram-negative bacteria *P. aeruginosa* utilizes cell-cell communication systems (also referred to as quorum-sensing) that rely on diffusible *N*-acyl homoserine lactone (AHL) signal molecules to control the expression of many virulence factors, *e.g.* proteases, chitinase, or siderophore production in a cell density-dependent manner (for review see [2]). Most CF patients become infected with a single clonal lineage, defining a set of genetically related strains. The genome of these clonal lineages often contains large islands, which comprise large hypervariable clusters of virulence genes encoding functions relevant for bacteria-host interactions, metabolic functions, or transporters [3]. The enormous ecological success of *P. aeruginosa* is probably due to its remarkable degree of genomic flexibility and phenotypic adaptation. It has been described that *P. aeruginosa* strains often undergo phenotypical changes during CF lung colonization. One

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Supporting information for this article is available on the WWW under www.proteomics-journal.de or from the author.

typical adaptation is the increased synthesis of alginate, which results in a characteristic mucoid phenotype [4, 5]. Furthermore, strains isolated from chronically infected CF patients generally produce lower amounts of virulence factor such as proteases, lipase, and hemolysin than their clonal relatives isolated at the onset of the infection [4]. Clonal divergence of *P. aeruginosa* caused by events like acquisition and loss of large blocks of DNA, large-scale inversions that affect the whole chromosomal structure, nucleotide substitutions, and other local variations of DNA has been described [6]. Recently, a proteome study of the intra- and interclonal diversity of *P. aeruginosa* suggested that the strain variability is mainly manifested in the secretome [7].

Two *P. aeruginosa* strains TB121838 and TB10839 have been isolated from the sputa of an 11-year old CF patient who rapidly deteriorated within three years until clinical stage IV (defined by Shwachman and Kulczycki, [8]) at the time of strain isolation, and of a 15-year old CF patient with stable stage II who in contrast experienced no pulmonary exacerbation in the three years before the time point of isolation [8]. Both isolates are genetically closely related [9]: (i) They share identical SNP genotypes in 15 out of 15 tested SNPs, (ii) both strains were 100% identical in the sequence of six completely sequenced genes (*oriC, citS, ampC, oprI, fliC, pilA)*, and (iii) the hybridization signals for 43 of 43 tested genes were comparable. On the other hand, the similarity value of the *Spe*I macrorestriction fragment analysis using UPGMA cluster was 0.14, reflecting a highly similar, but not identical fragment pattern that shows a difference of $\sim$ 300 kb in genome size. The two strains differ by up to 50% in their number of differentially regulated genes upon exposure to stress factors when hybridizing bacterial cDNAs with the 5500 genes-representing PAO1 microarrays. The most striking property of TB10839 is its ability to proliferate in polymorphonuclear granulocytes [9]. Moreover, TB10839 is highly cytotoxic for macrophages and adheres strongly to human airway epithelia and mucins. In the present study, we employed two-dimensional gel electrophoresis (2-DE) coupled to MS to identify differences in the protein expression profiles between the two strains.

For comparative proteome analysis the strains were grown to an OD_{600nm} of 2.5 in Luria Bertani (LB) medium, as many virulence factors are not expressed before the late exponential growth phase [10]. The proteomes of the various cultures were separated into intracellular, extracellular, and surface-associated sub-proteomes prior to 2-DE as previously described [11]. Protein samples derived from three independent growth experiments were used for 2-DE. Comparison of gels and quantification of spot intensities were performed with the Image-Master® software package. Spots with an increased or decreased intensity of more than twofold, averaged over six gels (two gels from each of three independent experiments), were recorded. Only protein spots fulfilling these criteria were subjected to in-gel tryptic digestion and were characterized by MALDI-TOF peptide mapping with the Ettan z2 MALDI-TOF (Amersham Biosciences, Uppsala, Sweden).

The analysis of the intracellular proteins with a pH gradient of 4 to 7 resulting in a total number of 920 protein spots for TB10839 and 930 protein spots for TB121838 is exemplarily depicted in Fig. 1. Ten protein spots were only present in TB121838, 5 spots exhibited higher, and 15 spots lower intensity when compared to TB10839 (supplementary Table SM1). In the surface-associated proteome 422 protein spots were found in TB10839 and 427 protein spots in TB121838 (supplementary Fig. SM1A). Of these spots, 2 were exclusively present in TB10839, 2 spots exhibited lower, and 3 spots higher intensity than the corresponding spots in TB121838 (supplementary Table SM1). Seven protein spots were only found in TB121838. 623 protein spots were detected in culture supernatants of TB10839 and 626 protein spots in TB121838 (supplementary Fig. SM1B). When compared to TB10839, 2 protein spots were absent, 2 and 3 protein spots showed at least twofold increased and decreased intensities, and 7 spots were exclusively expressed in TB121838 (supplementary Table SM1). In total, the intensity of $\sim$ 4% of all detected protein spots differs more than twofold between the two strains. Remarkably, the extracellular sub-proteome showed a higher diversity (5.5%) than the intra- and surface derived sub-proteomes (3.2% and 3.3%). These findings support the study of Wehmhöhner *et al.* [7] who demonstrated that particularly the secretome expression is a sensitive measure of *P. aeruginosa* strain variation.

Nineteen out of 78 differentially expressed protein spots were identified by MS on the basis of a predicted gene product in the genome database for *P. aeruginosa* PAO1 (http://www.pseudomonas.com; Table 1). For 13 additional spots peptide mass fingerprints were obtained which did not match with the available databases; the respective proteins are most likely specifically expressed in the TB clonal lineage. One protein that is exclusively present in TB121838 did not match with any ORF of PAO1 but was identified as ORF C62 [3]. The corresponding gene is located in a strain-specific gene island of *P. aeruginosa* clone C. Most interestingly, 12 spots correspond to gene products, which have recently been identified as AHL-controlled in *P. aeruginosa* PAO1 [10, 12, 13]. In complex interplays with additional regulators, including Vfr, GacA, RsaL, MvaT, and RpoS, the PAO1 quorum-sensing cascade regulates expression of a battery of extra-

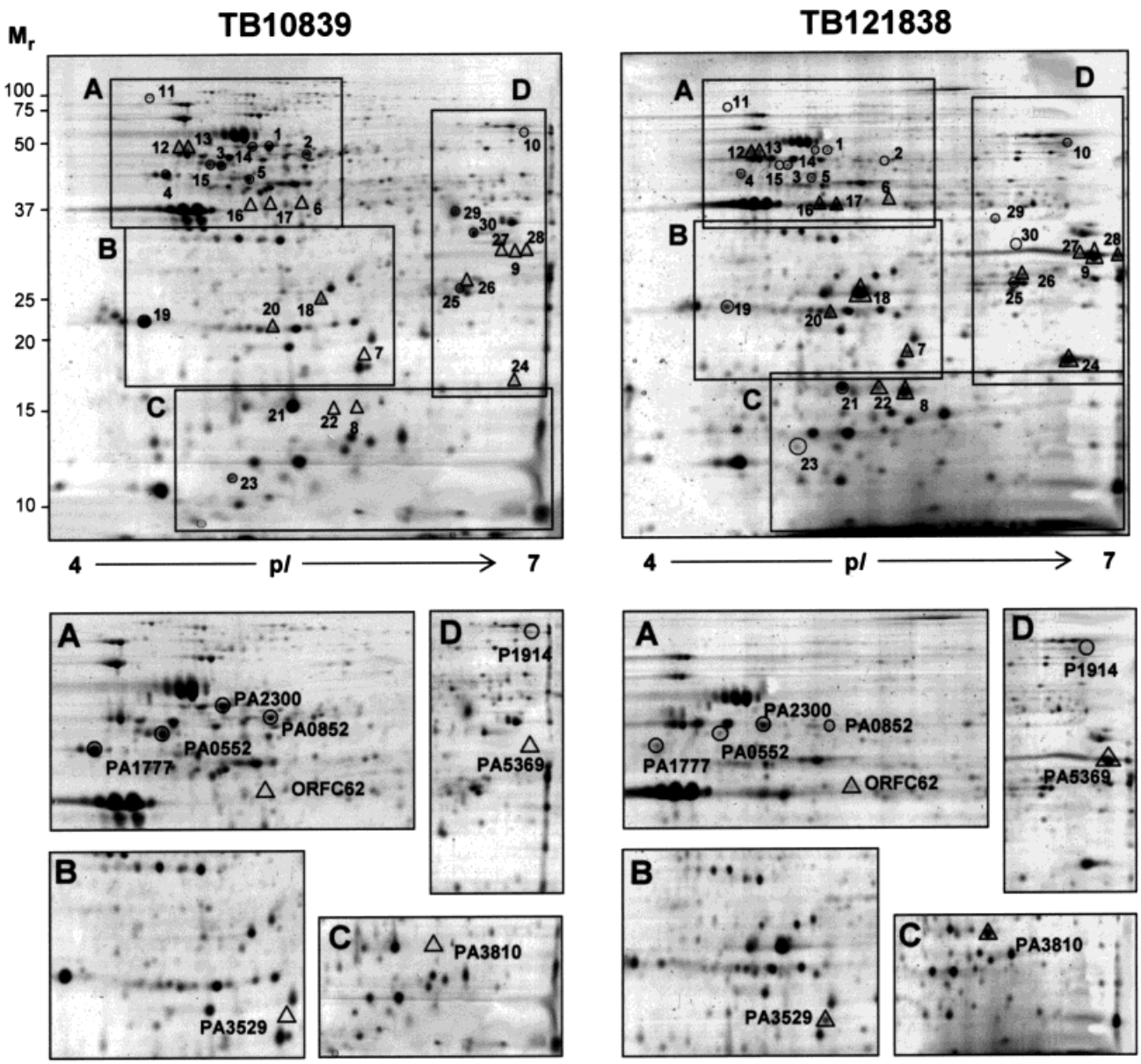

Figure 1. Comparative 2-DE of intracellular proteins of TB10839 and TB121838. The proteins were separated on Immobiline DryStrips with pH gradients from 4 to 7, followed by an SDS-PAGE on 13% polyacrylamide gels. Gels were stained with colloidal Coomassie. Differentially expressed proteins are marked by numbers. Regions with identified protein spots are boxed (A–D) and shown below in better detail. Circles indicate spots with increased intensity in TB10839; triangles indicate spots with decreased intensity in TB10839.

cellular virulence factors such as exoenzymes (elastase, alkaline protease), secondary metabolites (pyocyanin, hydrogen cyanide, pyoverdin), and toxins (exotoxin A) [14, 15] and the development of biofilms [16].

The fact that several of the identified proteins are known to be under control of the QS cascade of the organism prompted us to compare the two strains with respect to AHL-signal molecule production and expression of stringently AHL-regulated phenotypes, including extracellular proteolytic and chitinolytic activities and secreted siderophore pyoverdin. To this end, cultures of the two isolates were harvested at an OD_{600nm} of 1.5 (Fig. 2A) and the cell-free supernatants were further analyzed. To quantify the amounts of AHLs, we extracted the signal molecules as described recently [17] and incubated 2 μL of the extracts

Table 1. Identification of differentially expressed proteins by MALDI-TOF-MS peptide mass mapping

PA No.[a]	Spot ID[b]	Protein identification	Sequence coverage [%]	Expression[c]
PA0552	IC3	Phosphoglycerate kinase	20.4	↑
PA0623	SN6	Probable bacteriophage protein	37.5	↓
PA0852*	IC2	Chitin-binding protein CbpD	35.6	
	IC5		30.5	
	SN2		18.2	
	SN3		7.7	
	SN5		15.1	
PA1777*	IC4	Outer membrane protein OprF	21.1	↑
	SN1		18.3	
PA1914*	IC10	Conserved hypothetical protein	21.0	↑
PA2300*	IC1	Chitinase ChiC	29.4	↑
PA2328*	SN4	Hypothetical protein	10.3	+
PA3529	IC7	Single-stranded DNA-binding protein	33.1	−
PA3724*	SN7	Elastase LasB	13.2	+
PA3810	IC8	Heat shock protein HscA	9.7	−
PA4276	SF1	Secretion protein SecE	16.3	↑
PA4376	SF3	Nicotinate phosphoribosyl transferase	5.0	−
PA5369*	IC9	Hypothetical protein	31.3	−
ORF C62[d]	IC6	Hypothetical protein	13.8	−

a) Data generated from peptide mass maps were compared to the complete translated ORFs for *P. aeruginosa* PAO1 (http://www.pseudomonas.com)
b) Spot No. refers to numbers shown in Fig. 1, supplementary SM1A and B
c) Data refer to TB10839; ↑, increased expression; ↓, decreased expression; +, exclusively present; −, absent
d) Corresponds to gi24461585 [3]
* Recently reported as quorum-sensing regulated [10, 12, 13]

with the AHL-biosensor *Pseudomonas putida* (pKR-C12) [18], which is most sensitive for *N*-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL). This molecule is utilized by the *las* quorum-sensing system, which is on top of the *P. aeruginosa* quorum-sensing cascade. When compared to TB10839, TB121838 is producing significantly higher amounts of 3-oxo-C12-HSL (Fig. 2B). This is a rather unexpected finding, as our proteome analysis suggested that expression of several QS-regulated proteins is downregulated in TB121838. However, in support of our proteome analysis, which showed diminished synthesis of the LasB elastase and the ChiC chitinase, we observed that TB121838 indeed produced reduced levels of extracellular proteolytic and chitinolytic activities (Fig. 2C; data not shown). Most strikingly, the synthesis of the siderophore pyoverdin was completely abolished (Fig. 2D).

In this study, we compared the protein patterns of the intracellular, extracellular, and surface protein fractions of two closely related *P. aeruginosa* strains by means of 2-DE. Our global analysis showed that the expression rate of about 4% of all detected protein spots differed more than twofold between the two strains. However, we were unable to identify all the differentially expressed proteins by the aid of MS. Most likely, these proteins are specific for the TB clonal lineage and are thus neither present in the genome of the sequenced model strain PAO1, nor on the large chromosomal island present in *P. aeruginosa* clone C strains. We are currently attempting to identify these interesting proteins by *N*-terminal sequencing. However, it is conceivable that for an unambiguous identification additional data on TB-specific DNA sequences will be required.

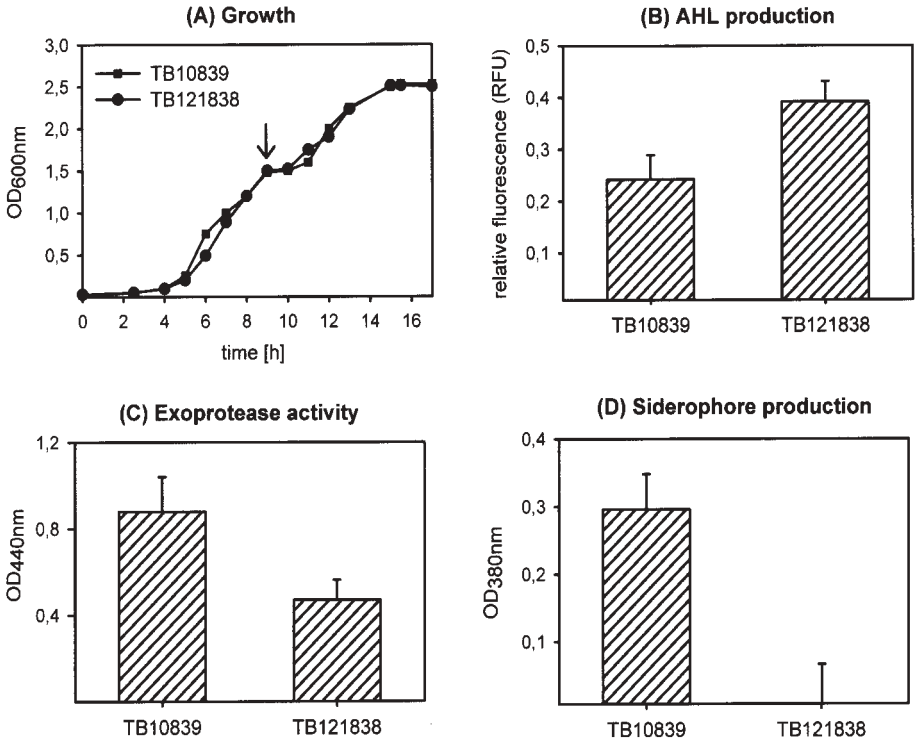

Figure 2. Comparison of growth, AHL production, and expression of quorum-sensing regulated phenotypes of TB10839 and TB121838 in LB medium. (A) Growth was measured spectrophotometrically at 600 nm. When the cultures reached an OD_{600} of 1.5 samples of spent culture supernatants were taken. (B) AHLs were extracted from the supernatant and quantified by the aid of the AHL monitor strain *P. putida* (pKR-C12) [18]. (C) Protease activities of spent culture supernatants were determined on azocasein as described recently [10]. (D) Presence of the siderophore pyoverdin in the supernatant was measured spectrophotometrically at 380 nm. The data represent mean values of three independent experiments. Error bars represent the standard errors of the means.

The most striking result of our proteome analysis was the finding that several QS-regulated proteins are expressed at reduced levels in TB121838 relative to TB10839. This result was further strengthened by a phenotypic characterization of the two strains that showed that expression of tightly QS-regulated phenotypes is indeed reduced in strain TB121838. Previous work has provided clear evidence that quorum sensing plays a pivotal role for the pathogenicity of *P. aeruginosa*. It was shown that AHL-deficient mutants were attenuated in a number of animal models including the neonatal mouse model of pneumonia [19] and a *Caenorhabditis elegans* model [20]. We therefore speculate that the reduced virulence of TB121838 may, at least in part, be a consequence of the downregulation of QS-regulated functions.

Even though expression of QS-regulated functions is clearly reduced in TB121838, the strain still synthesizes AHLs. In fact, measurements of the amounts of 3-oxo-C12-HSL revealed that TB121838 produces even elevated levels of this signal molecule relative to TB10839. We also characterized the different AHL species produced by the strains by thin-layer chromatography but apart from the fact that all detected AHL molecules were present in higher amounts in the supernatant of TB121838 no differences were observed (data not shown). These data may indicate that the amounts of AHLs produced do not directly correlate to the degree of quorum-sensing induction. In other words, the two strains may utilize different AHL threshold concentrations and thus population densities for triggering the quorum-sensing cascade. An alternative explanation could be that additional regulatory factors operating downstream of the *las rhl* quorum-sensing circuitry are different between the strains. Previous work has provided evidence that the QS cascade is modulated at the post-transcriptional level by the RNA-binding protein RsmA [21] and the *dksA* gene product [22]. A recent analysis of the *P. aeruginosa* PAO1 QS regulon by proteomics showed that several AHL-regulated proteins are subject to post-transcriptional regulation [13]. Work currently under way aims to test this possibility.

We thank Beate Schumacher and Angeliki Tsokou for excellent technical assistance. This work was funded by the Deutsche Forschungsgemeinschaft (RI 969/3-1).

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6.3 Analysis of the quorum-sensing regulon of the opportunistic pathogen Burkholderia cepacia *H111 by proteomics*

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Published in: Electrophoresis 24: 740-750 (2003)

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Analysis of the quorum-sensing regulon of the opportunistic pathogen *Burkholderia cepacia* H111 by proteomics

Burkholderia cepacia H111, an important pathogen for persons suffering from cystic fibrosis, employs a quorum-sensing (QS) system, *cep*, to control expression of virulence factors as well as the formation of biofilms. The QS system is thought to ensure that pathogenic traits are only expressed when the bacterial population density is high enough to overwhelm the host before it is able to mount an efficient response. In this study, we compared the protein pattern of the intracellular, extracellular, and surface protein fractions of an AHL-deficient *cepI* mutant with the one of the parent strain H111 by means of two-dimensional gel electrophoresis (2-DE). Our analysis showed that 55 proteins out of 985 detected spots were differentially expressed; these are expected to represent QS-controlled gene products. Addition of the respective signal molecules to the growth medium of the *cep* mutant fully restored the wild-type protein expression profile. In total about 5% of the *B. cepacia* proteome was downregulated and 1% upregulated in the *cepI* mutant, indicating that quorum sensing represents a global regulatory system. Nineteen proteins were identified with high confidence by *N*-terminal sequence analysis.

Keywords: *Burkholderia cepacia* / Microbial proteomics / Quorum sensing EL 5249

1 Introduction

The Gram-negative bacterium *Burkholderia cepacia*, which can be isolated from various habitats including soil, water and plant surfaces, was first described as a phytopathogen, associated with a soft rot of onion bulbs [1]. More recently, *B. cepacia* has emerged as an opportunistic pathogen of humans, particularly those with cystic fibrosis (CF) [2]. CF, the most common lethal inherited disease among the Caucasian population, is caused by mutations in the *cftr* gene, encoding a chloride channel [3, 4]. Impaired cystic fibrosis transmembrane conductance regulator (CFTR) function leads to high salt concentrations in epithelial secretions and the production of thick dehydrated mucus in the ducts of exocrine glands, *e.g.*, the airways of the lungs. The increased mucus viscosity impairs mucociliary and alveolar clearing, promoting colonization of the lung epithelium by opportunistic pathogenic bacteria [5, 6]. The major pathogen in adult CF patients is *Pseudomonas aeruginosa*, but over the past two decades *B. cepacia* emerged as another important pathogen, with prevalence rates in some CF centers of up to 40% [7]. *B. cepacia* is usually acquired late in the course of the disease, and the clinical outcome of a colonization is variable and unpredictable, ranging from asymptomatic carriage to a fulminated and fatal pneumonia, the so called 'cepacia syndrome' [2]. At present very little is known about the pathogenic mechanisms and virulence determinants of *B. cepacia*.

In *P. aeruginosa* as well as in many other Gram-negative bacteria expression of virulence factors is not constitutive but is regulated in a cell density-dependent manner. These regulatory systems ensure that pathogenic traits are only expressed when the bacterial population density is high enough to overwhelm the host before it is able to mount an efficient response. To monitor the size of the population *P. aeruginosa* utilizes a cell-cell communication system that relies on diffusible *N*-acyl homoserine lactone (AHL) signal molecules in a process known as quorum sensing (QS) [8–10]. Such communication systems depend on an AHL synthase, usually a member of the LuxI family of proteins, and an AHL receptor protein, which belongs to the LuxR family of transcriptional regulators. At low population densities cells produce a basal level of AHL *via* the activity of the AHL synthase. As the cell density increases, the diffusible AHL signal molecule accumulates in the growth medium. On reaching a critical

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Abbreviations: AHL, *N*-acyl-homoserine lactone; **QS**, quorum sensing

 0173-0835/03/0402–740 $17.50+.50/0

threshold concentration, the AHL binds to the cognate LuxR-type receptor protein, which in turn leads to the induction/repression of target genes. The pivotal role of quorum sensing for the pathogenicity of *P. aeruginosa* has been demonstrated in a number of animal models including the neonatal mouse model of pneumonia [11], the burned mouse model [12], and a *Caenorhabditis elegans* nematode model [13].

Recent work has identified a quorum-sensing system operating in *B. cepacia* consisting of the AHL synthase CepI, which directs the synthesis of *N*-octanoylhomoserine lactone (C_8-HSL) and, as a minor product, *N*-hexanoylhomoserine lactone (C_6-HSL) [14, 15], and CepR, which after binding of C_8-HSL is thought to activate or repress transcription of target genes. The *cep* system was shown to positively regulate biofilm formation, swarming motility, and production of extracellular proteolytic and chitinolytic activity, but to repress synthesis of the siderophore ornibactin [14, 16, 17]. As the *cep* system controls various apparently unrelated phenotypes it appears likely that it constitutes a global regulatory system. In this study, we employed proteomics to identify *cep*-controlled proteins in the clinical *B. cepacia* strain H111.

2 Materials and methods

2.1 Bacterial strains and growth conditions

The clinical isolate *Burkholderia cepacia* H111 [15, 18] and the *cepI* derivative H111-I [16] were routinely grown in AB minimal medium [19] supplemented with 10 mM sodium citrate under vigorous agitation at 30°C. For the 2-DE sample preparation the strains were grown to the stationary growth phase (OD_{600} of 2.0). Kanamycin was added as required at a final concentration of 100 $\mu L \cdot mL^{-1}$. Complementation of the *cepI* mutant was accomplished by the addition of 1 μM octanoylhomoserine lactone (C_8-HSL) to the growth medium. Growth was monitored spectrophotometrically by an Ultrospec Plus spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden) by measurement of optical density at 600 nm.

2.2 Analytical procedures

Total protein concentrations were determined according to the method of Bradford [20]. Five μL aliquots of the respective protein solution were mixed with 1 mL of Coomassie Plus protein assay reagent (Pierce, Rockford, IL, USA) and the absorbance measured at 595 nm. The protein concentration was calculated using BSA as standard.

2.3 Prefractionation of protein samples for 2-DE

2.3.1 Intracellular proteins

For the preparation of intracellular proteins 200 mL cultures were harvested by centrifugation (5000 rpm; 4°C; 20 min) and the cells were washed in 0.9% NaCl solution. One g of the cell pellet was resuspended in 2 mL 50 mM Tris/HCl buffer pH 7.5. Cell lysis was performed by sonification and the cell debris was removed by centrifugation (15 000 rpm; 1 h; 4°C). In order to improve focusing of proteins, the lysates were extracted twice with phenol extraction as described previously [21]. To this end, 1 mL aliquots of the extracts were mixed thoroughly with phenol and incubated for 10 min at 70°C. The sample was cooled on ice for 5 min and the phases were then separated by centrifugation (5000 rpm; 10 min; 4°C). The aqueous phase was discarded and proteins were precipitated by adding 1 mL of ice-cold acetone. The sample was pelleted by centrifugation (15 000 rpm; 20 min; 4°C) and the pellet was washed with 1 mL of ice-cold acetone. After a final centrifugation step (20 min; 15 000 rpm; 4°C) the supernatant was removed and the pellet was air-dried for 30 min. The precipitate was finally solubilized in 300 μL of lysis buffer (9.5 M urea, 2% w/v CHAPS, 0.8% w/v Pharmalyte pH 3–10 (Amersham Pharmacia Biotech, Uppsala, Sweden), 1% w/v dithiothreitol and 5 mM Pefabloc (Merck, Darmstadt, Germany)).

2.3.2 Extracellular proteins

For the preparation of extracellular protein samples, cells of a 1 L culture were separated from the supernatant by centrifugation (4°C; 5000 rpm; 20 min). The proteins in the supernatant were precipitated with 10% w/v TCA at 4°C overnight. The precipitate was harvested by centrifugation (4°C; 5000 rpm; 1.5 h), washed 4 times with ethanol and dried thoroughly. The protein pellet (100 mg) was solubilized in 100 μL lysis buffer as described for the intracellular protein fraction.

2.3.3 Cell surface proteins

Surface-bound proteins were extracted as described by Nilsson *et al.* [22]. Cells of a 1 L culture were harvested by centrifugation (5000 rpm; 4°C; 20 min) and washed twice in 50 mM Tris/HCl, pH 7.5. Four g of the cell pellet were resuspended in 100 mL 0.2 M glycine hydrochloride, pH 2.2. The suspension was stirred at 20°C for 15 min and cells were removed by centrifugation (5000 rpm; 20 min; 4°C). The supernatant was neutralized with NaOH to pH 7.0 and the extracted proteins were precipitated with a 3-fold volume of acetone at −20°C overnight. The pre-

cipitate (200 mg) was harvested by centrifugation (4°C; 12 000 rpm; 1 h), washed 4 times with ethanol, dried thoroughly and resolved in 200 μL lysis buffer as described above.

2.4 Two-dimensional gel electrophoresis

For the first dimension samples were mixed with rehydration solution (8 M urea, 2% w/v CHAPS, 15 mM DTT and 0.5% v/v IPG-buffer pH 3–10 (Amersham Pharmacia Biotech) resulting in a final protein amount of 200 μg protein in a volume of 350 μL. The isoelectric focusing was performed by using immobilized pH gradient (IPG) strips (Amersham Pharmacia Biotech) as described elsewhere [23]. The IPG strips (18 cm, pH 3–10 NL; pH 4–7; pH 6–11) were rehydrated overnight at 30 V and focused for 3 h at 8000 V at 20°C under mineral oil. The IPG strips were then incubated for 10 min, in equilibration buffer I (6 M urea, 30% w/v glycerol, 2% w/v SDS and 1% w/v dithiothreitol in 0.05 M Tris/HCl buffer, pH 8.8) followed by equilibration buffer II (6 M urea, 30% w/v glycerol, 2% w/v SDS and 4% w/v iodacetamide in 0.05 M Tris-HCl buffer, pH 8.8). After the equilibration step the strips were transferred to 22 cm × 22 cm 12.5% or 15% SDS-PAGE gels for the second dimension. Electrophoresis was performed at 150 V and 150 mA at 15°C for approximately 18 h. Protein spots were visualized by taining with Coomassie Brilliant Blue G-250 as described elsewhere [24].

2.5 Data analysis

The gels were scanned with a densitometric ImageScanner (Amersham Pharmacia Biotech) and the raw images were analyzed using the Imagemaster 2-D gel analysis software Version 4.0 (Amersham Pharmacia Biotech). For each comparison (wild-type *versus cepI* mutant *versus cepI* mutant supplemented with 1 μM C_8-HSL) six gels resulting from three independent protein extractions were analyzed and compared. Only significant changes in protein concentration (more than 4-fold) were taken into consideration.

2.6 Protein identification and database searches

For identification of proteins by *N*-terminal sequencing protein samples of up to 500 μg were separated by preparative 2-D gels, followed by semidry electroblotting to PVDF membranes and staining with Coomassie blue. The excised pieces of the PVDF membranes were subjected to an automated Edman degradation on a gas-phase sequencer (Applied Biosystems 477A) according to instructions from the manufacturer. The obtained *N*-terminal sequence was used for a TBLASTN search at the Sanger Centre, Cambridge, United Kingdom (http://www.sanger.ac.uk/Projects/Bcepacia/blastserver.shtml) and the corresponding open reading frame of the *B. cepacia* genome was identified. Protein sequences were used for homology searches in GenBank using the online BLAST search engine at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nihh.gov/).

3 Results

3.1 Growth of *B. cepacia* and prefractionation of cellular compartments

In order to characterize the quorum-sensing regulon of *B. cepacia* H111 we compared the protein expression pattern of the wild type with the one of the AHL-deficient *cepI* mutant H111-I by two-dimensional gel electrophoresis (2-DE). A maximum of information can be retrieved from 2-DE when protein samples are prefractionated on the basis of cellular location. The reduction of the sample complexity facilitates the resolution of spots, the increased loading capacity enables the detection of low-abundance proteins, and the additional knowledge about cellular topologies relieves the identification of differentially expressed proteins. The proteomes of H111 and H111-I were therefore separated into intracellular, extracellular, and surface sub-proteomes prior to 2-DE. Intracellular proteins were extracted by lysis of the harvested cells employing sonification and separation of the crude extract from the cell debris by centrifugation. Samples were phenol-extracted to reduce the amount of contaminating DNA and lipid compounds [21]. Extracellular proteins were concentrated by removal of whole cells using a combination of centrifugation, filtration, followed by protein precipitation and solubilization in sample buffer. Cell surface proteins were extracted by acid glycine as recently described by Nilsson *et al.* [22].

3.2 Comparison of intracellular protein profiles

To gain an overview, a nonlinear pH gradient from 3 to 10 was used with samples of three independent growth experiments. For a more detailed analysis the pH gradients 4–7 and 6–11 were applied to increase the resolution of proteins with more acidic or basic isoelectric points. In total 564 spots could be detected in colloidal Coomassie stained gels of H111. Figure 1 shows a representative example of the protein pattern of H111, H111-I, and H111-I supplemented with 1 μM C_8-HSL. Matching and comparing the respective 2-DE maps with the Image-

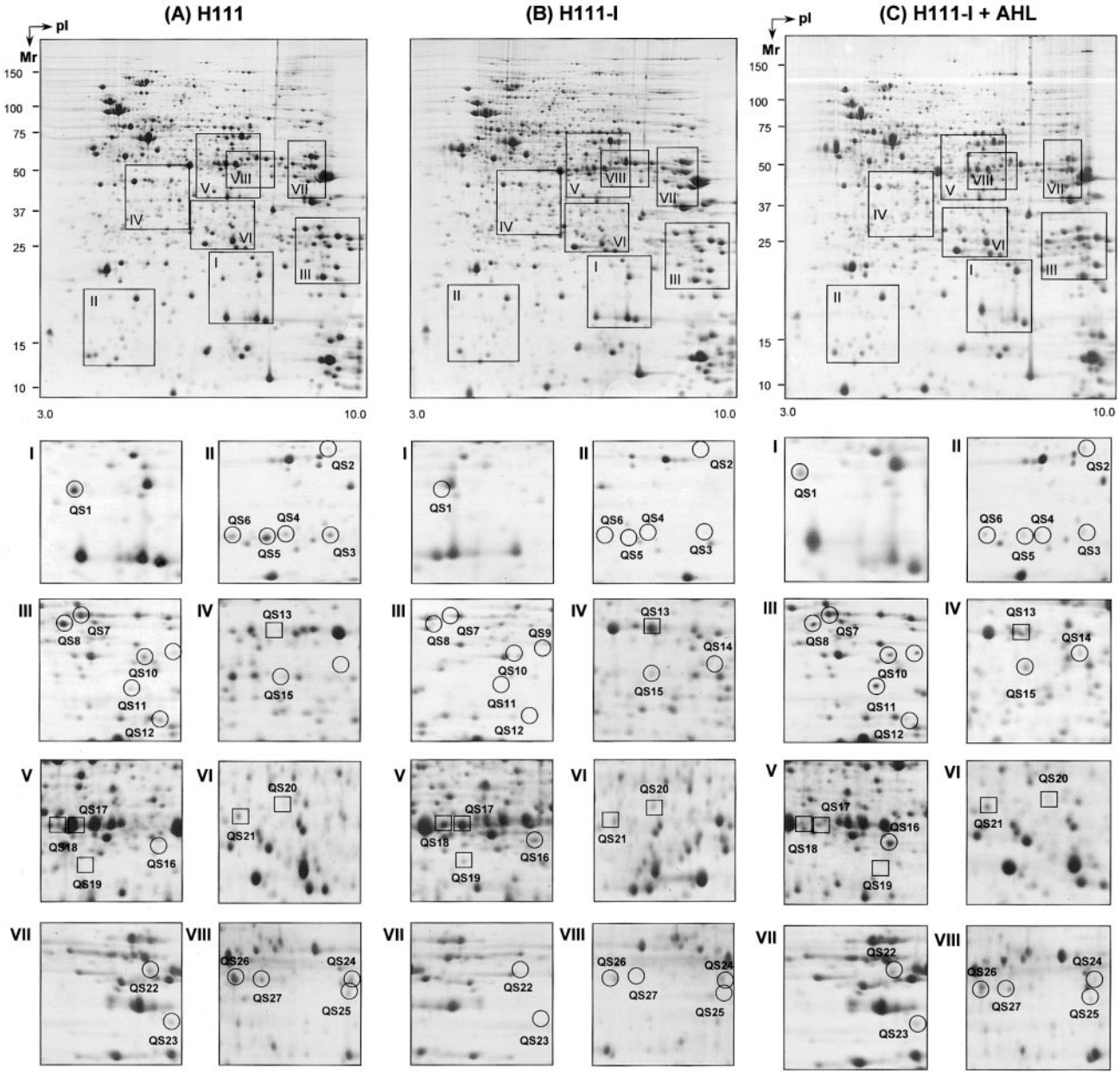

Figure 1. Comparative 2-DE of intracellular proteins of (A) the *B. cepacia* wild-type H111, (B) *cepI* mutant H111-I, and (C) H111-I complemented with 1 μM C_8-HSL. The crude protein extracts were separated on Immobiline Dry strips with different pH gradients (Amersham Pharmacia Biotech), followed by an SDS-PAGE on 12.5% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250. The outlined areas I–VIII indicate zones of the 2-DE gel that are shown below. Pictures of enlarged areas stem of 2-DE gels with either a broad pH-gradient (pH 3–10 nonlinear, areas depicting regions I, V, and VI) or a narrow pH-gradient (pH 4–7, II and IV; pH 6–11, III, VII and VIII). Encircled spots represent upregulated proteins in the wild-type strain; boxed spots represent upregulated proteins in the *cepI* mutant.

master analysis software revealed that 27 proteins are differentially expressed (Table 1). Only spots that are more than 4-fold up- or downregulated were considered. These protein spots were grouped into four categories (Table 2): (I) 12 protein spots found with the wild type but missing in the *cepI* mutant, (II) 1 proteins spot found with the *cepI* mutant but missing in the wild type, (III) 9 protein spots with decreased intensities in the *cepI* mutant, and (IV) 5 protein spots with increased intensities in the wild type. Importantly, addition of C_8-HSL to the growth me-

Table 1. Protein spots induced or repressed by the QS system of *B. cepacia*

Spot ID	Fraction[a)]	p*I*	M_r (kDa)	Intensity[b)] H111	Intensity[b)] H111-I	Intensity[b)] H111-I + C_8-HSL	Fold change[c)]
QS1[d)]	IC	6.6	17.9	100	4.8	97.2	+20.7
QS2[d)]	IC	5.3	20.7	100	–	83.3	/
QS3[d)]	IC	5.3	14.0	100	–	82.7	/
QS4[d)]	IC	5.0	14.0	100	–	84.5	/
QS5[d)]	IC	4.9	14.0	100	–	93.5	/
QS6[d)]	IC	4.6	14.0	100	–	89.7	/
QS7[d)]	IC	9.1	26.4	100	8.8	95.2	+11.3
QS8[d)]	IC	8.9	25.1	100	4.8	87.7	+20.6
QS9[d)]	IC	10.3	22.2	100	–	67.8	/
QS10[d)]	IC	9.9	20.9	100	–	84.0	/
QS11[d)]	IC	9.7	18.4	100	–	71.6	/
QS12	IC	10.1	16.8	100	–	91.2	/
QS13	IC	5.1	55.9	24.5	100	30.7	− 4.1
QS14	IC	5.6	49.7	100	–	76.2	/
QS15	IC	5.1	47.8	100	23.3	80.1	+ 4.3
QS16	IC	6.9	59.7	100	17.9	93.1	+ 5.7
QS17	IC	6.2	61.9	11.5	100	19.6	− 8.7
QS18	IC	6.0	62.1	8.0	100	10.5	−12.5
QS19	IC	6.2	56.1	–	100	–	/
QS20	IC	6.5	41.3	12.6	100	14.6	− 7.9
QS21	IC	6.0	39.5	15.2	100	21.1	− 6.5
QS22	IC	8.9	35.7	100	22.1	93.4	+ 4.5
QS23	IC	9.1	30.6	100	23.3	79.5	+ 4.3
QS24	IC	7.5	31.9	100	–	87.0	/
QS25	IC	7.5	30.3	100	–	78.5	/
QS26	IC	6.5	32.0	100	24.3	87.5	+ 4.1
QS27	IC	6.7	31.8	100	24.9	85.9	+ 4.0
QS28	EC	8.0	22.7	100	5.3	96.3	+18.6
QS29	EC	7.9	21.2	100	6.8	84.9	+14.7
QS30	EC	7.4	23.5	100	–	86.4	/
QS31	EC	5.8	19.5	100	–	82.4	/
QS32	EC	5.1	20.3	100	–	84.7	/
QS33[d)]	EC	6.5	18.0	100	–	90.8	/
QS34	EC	5.1	16.2	8.1	100	14.1	−12.4
QS35	EC	8.5	15.2	100	17.8	83.1	+ 6.8
QS36	EC	8.3	15.2	100	16.7	84.7	+ 4.9
QS37[d)]	EC	4.8	14.9	100	15.8	87.8	+ 6.3
QS38[d)]	EC	4.5	15.0	100	4.7	81.6	+22.7
QS39[d)]	EC	4.3	16.0	100	12.2	87.4	+ 8.2
QS40	EC	7.0	12.7	100	–	76.7	/
QS41	EC	7.0	11.9	100	–	71.3	/
QS42	EC	7.3	42.4	100	–	92.4	/
QS43	EC	7.4	36.5	100	24.3	72.4	+ 4.1
QS44	EC	7.0	31.9	8.5	100	13.3	−11.8
QS45	SF	7.8	19.3	17.5	100	24.4	− 5.7
QS46	SF	7.6	18.6	8.3	100	10.8	−12.0
QS47	SF	6.7	17.8	100	–	63.7	/
QS48	SF	7.1	15.9	10.6	100	17.4	− 9.4
QS49	SF	6.7	16.3	100	15.1	89.0	+ 6.6
QS50	SF	6.7	14.9	100	–	78.2	/
QS51	SF	7.0	13.2	100	–	86.7	/
QS52[d)]	SF	6.5	12.8	100	12.0	85.9	+ 8.3
QS53	SF	6.9	12.9	100	–	75.8	/
QS54[d)]	SF	6.3	18.1	100	–	91.2	/
QS55	SF	6.4	15.1	100	–	76.6	/

Table 1. Continued

Spot ID	Fraction[a)]	p*I*	M_r (kDa)	Intensity[b)] H111	Intensity[b)] H111-I	Intensity[b)] H111-I + C_8-HSL	Fold change[c)]
QS56	SF	5.1	16.4	100	–	86.9	/
QS57	SF	5.9	12.5	–	100	3.4	/
QS58	SF	5.8	23.6	100	–	67.9	/
QS59	SF	5.5	28.3	100	–	86.5	/
QS60[d)]	SF	4.3	15.1	100	–	84.9	/
QS61[d)]	SF	4.0	15.1	100	–	76.3	/
QS62	SF	3.8	15.2	100	–	77.5	/

Only spots with an at least 4-fold change in signal intensity were considered. For each comparison six gels resulting from three independent protein extractions were analyzed and compared.

a) Cell compartment: IC = intracellular; EC = extracellular; SF = surface

b) Relative protein expression levels in %, the intensity of the wild-type or mutant spots was used as a reference.

c) Fold change in protein expression calculated by the Imagemaster analysis software, positive numbers refer to proteins which are upregulated, negative numbers refer to proteins which are downregulated *via* the *B. cepacia* QS system.

d) Spots have been identified by *N*-terminal sequencing.

Table 2. Global analysis of the *B. cepacia* H111 QS regulon

Cell compartment	Spot pattern	No. of spots	% of total spots
Intracellular	Total number of spots (H111 wt)	564	100
	Spots unique in H111 wt	12	2.1
	Spots unique in H111-I	1	0.2
	Spots downregulated in H111-I	9	1.6
	Spots upregulated in H111-I	5	0.9
Extracellular	Total number of spots (H111 wt)	343	100
	Spots unique in H111 wt	7	2
	Spots unique in H111-I	0	0
	Spots downregulated in H111-I	8	2.3
	Spots upregulated in H111-I	2	0.6
Surface	Total number of spots (H111 wt)	84	100
	Spots unique in H111 wt	12	14.3
	Spots unique in H111-I	1	1.2
	Spots downregulated in H111-I	2	2.4
	Spots upregulated in H111-I	3	3.6
In total	Total number of spots (H111 wt)	985	100
	Spots unique in H111 wt	28	2.8
	Spots unique in H111-I	2	0.2
	Spots downregulated in H111-I	16	1.6
	Spots upregulated in H111-I	10	1.0

The protein spot pattern of the *B. cepacia* H111 wild type was compared with the one of the *cepI* mutant H111-I. The spot pattern of *B. cepacia* H111 wild type was used as reference for protein matching with the Imagemaster 2-D gel analysis software. Only spots different in intensity by at least the factor 4 were considered.

dium fully restored the wild-type protein profile indicating that the identified protein spots are indeed under the control of the *cep* quorum-sensing system (Table 1; Fig. 1C).

3.3 Comparison of extracellular protein profiles

As extracellular proteins are often important virulence factors, they represent a particularly interesting part of the bacterial proteome. We therefore analyzed the extracellular proteins from samples obtained from three independent growth experiments. Using a nonlinear pH gradient of 3–10, 343 protein spots were detected for the parent strain H111 (Fig. 2). Of these 7 were missing in the *cepI* mutant (category I), 8 proteins were at least 4-fold upregulated in the wild type (category III), and 2 proteins were downregulated by the quorum-sensing system (category IV; Tables 1 and 2). As for the intracellular proteins addition of respective signal molecules restored the expression profile of the wild type (Table 1, Fig. 2C).

3.4 Comparison of cell surface protein profiles

Proteins present at the surface of bacteria often play a prominent role for the pathogenesis. The analysis of surface proteins with a nonlinear pH gradient 3–10 resulted in a total number of 84 wild-type protein spots (Fig. 3), of which 12 proteins were exclusively produced in H111

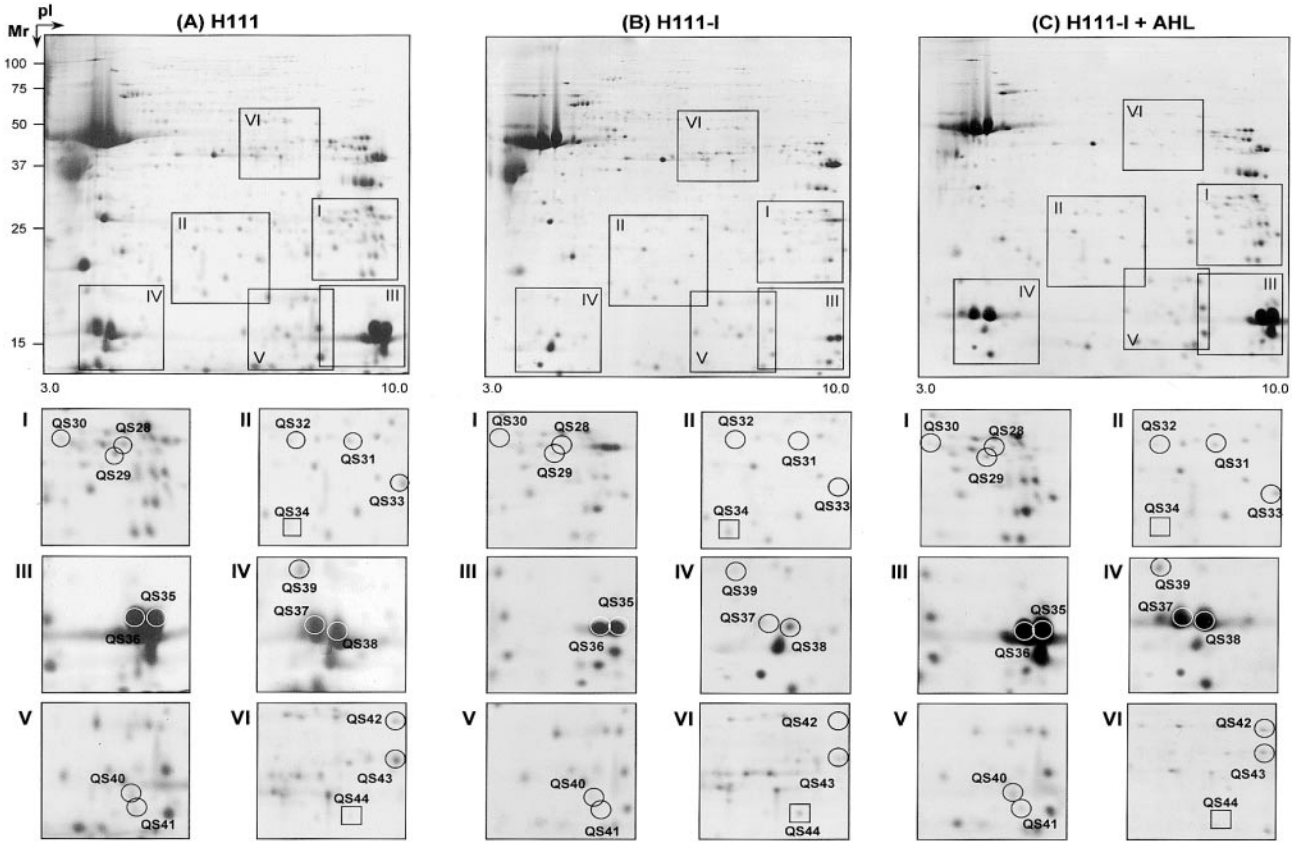

Figure 2. Comparative 2-DE of extracellular proteins of (A) the *B. cepacia* wild-type H111, (B) *cepI* mutant H111-I, and (C) H111-I complemented with 1 μM of C_8-HSL. The secreted proteins were separated on Immobiline Dry strips with a pH gradient from 3 to 10 (Amersham Pharmacia Biotech), followed by an SDS-PAGE on 15% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250. The outlined areas I–VI indicate zones of the 2-DE gel that are shown below. Encircled spots represent upregulated proteins in the wild-type strain; boxed spots represent upregulated proteins in the *cepI* mutant.

(category I; Tables 1 and 2) and 2 additional proteins were more than 4-fold upregulated in the wild type (category III). One protein was exclusively expressed in the *cepI* mutant (category II) and 3 further proteins were upregulated in H111-I (category IV). Interestingly the number of quorum sensing-regulated proteins was much higher in the surface protein fraction (25%) when compared to the intracellular and extracellular protein fraction (5%; Table 2). Comparable to the other two cell compartments the defect of the QS-mutant was fully restored by addition of C_8-HSL to the growth medium (Table 1, Fig. 3C).

3.5 Identification of quorum-sensing regulated proteins

As the sequence of the *B. cepacia* genome is not yet completed (http://www.sanger.ac.uk/Projects/Bcepacia/), an identification of the *cep*-regulated proteins by MALDI-TOF is not feasible. We therefore decided to determine the *N*-terminal amino acid sequences of major *cep*-regulated proteins (Table 3). For 19 out of 25 proteins sequences with a length of 9–22 amino acids were obtained. Searching the unfinished *B. cepacia* J2315 genome database (http://www.sanger.ac.uk/Projects/B_cepacia/) allowed the identification of the respective open reading frames (ORFs). The predicted amino acid sequences of these ORFs were then used for a BLASTP search in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). The results of these analyses are summarized in Table 3.

Three spots derived from the intra- (QS1), extracellular (QS33) and surface-bound (QS54) protein fraction identified an ORF with high similarity to AidA of *Ralstonia solanacearum* AW1 (53% identity). *aidA* (autoinducer dependent) is located upstream of the *sol* quorum-sensing locus of the organism and was shown to be regulated by the LuxR homologue SolR [25]. However, the function of this protein in *R. solanacearum* is unknown.

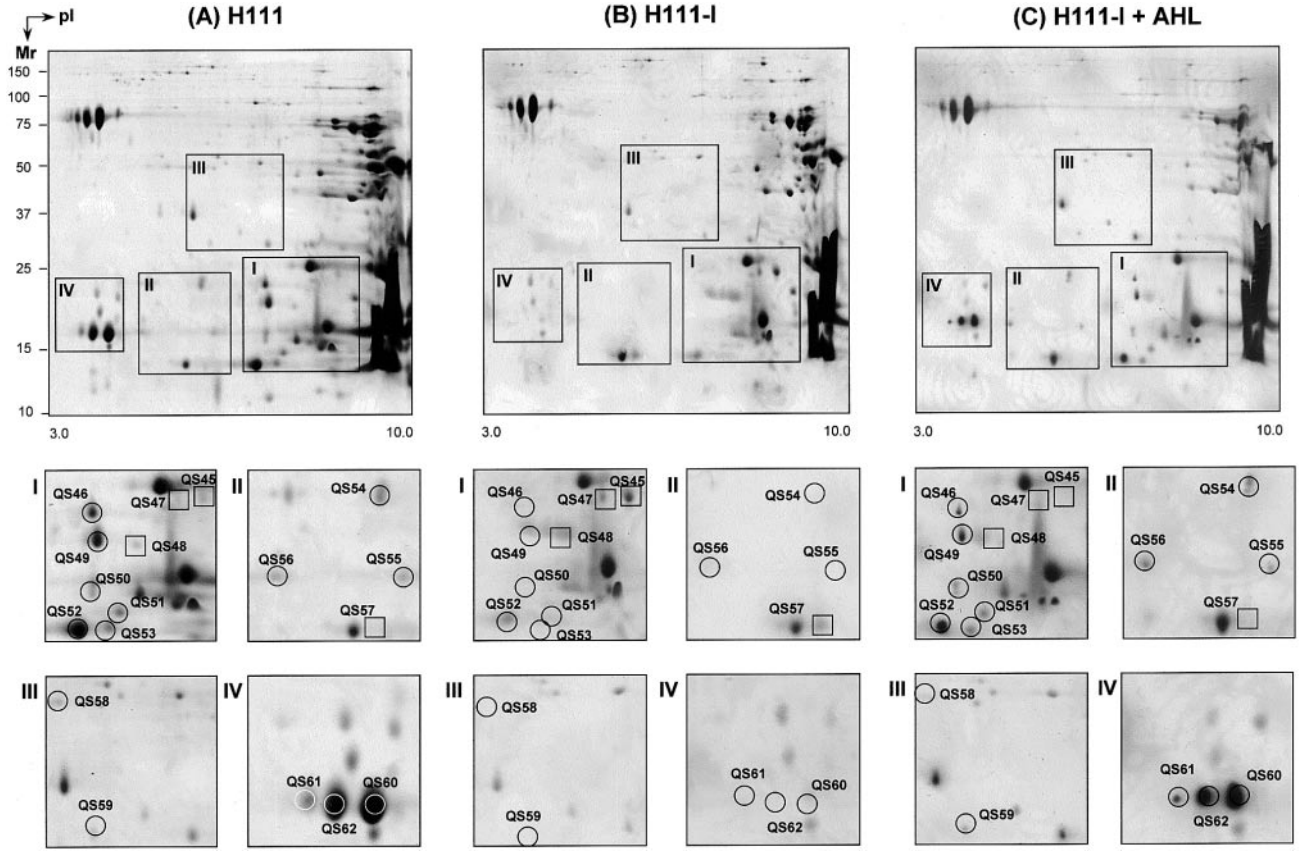

Figure 3. Comparative 2-DE of surface-bound proteins of (A) the *B. cepacia* wild-type H111, (B) *cepI* mutant H111-I, and (C) H111-I complemented with 1 μM of C_8-HSL. Proteins were separated on Immobiline Dry strips with a pH gradient from 3 to 10 (Amersham Pharmacia Biotech), followed by an SDS-PAGE on 15% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250. The outlined areas I–IV indicate zones of the 2-DE gel that are shown below. Encircled spots represent upregulated proteins in the wild-type strain; boxed spots represent upregulated proteins in the *cepI* mutant.

Six other spots present in cytosolic (QS5, 6), secreted (QS37, 38) and surface (QS60, 61) protein fractions corresponded to an ORF with strong similarity (50% identical amino acids) to FimA, the major subunit of type I pili of *Escherichia coli* [26]. The fact that this protein exists in isoforms with different p*I* suggests that it is subject to post- or cotranslational modification. For *E. coli* it has been demonstrated that type I pili are important virulence factors as these surface structures mediate specific adherence to mammalian host tissues, such as the surface of the urinary tract [27, 28]. More recently it was shown that type I pili also play an important role in the formation of *E. coli* biofilms on abiotic surfaces [29]. As biofilm formation of *B. cepacia* is controlled by the *cep* system [16], it is tempting to speculate that FimA is involved in this process.

The analysis of spots QS2 and QS9 identified two ORFs with striking similarity to oxidoreductases of *R. solanacearum* GMI1000. QS2 has 85% identity with RSc0754 encoding a putative peroxidase and QS9 has 84% identity with *sodB* encoding a probable superoxide dismutase [30]. While the theoretical p*I* of the identified *B. cepacia* peroxidase fits reasonably well with the observed value for QS2, the theoretical p*I* of the putative *B. cepacia* superoxide dismutase (SOD) differs significantly from the one observed for QS9. For *P. aeruginosa* it has been shown that expression of genes, which are essential for relieving oxidative stress, are QS-controlled [31]. Mutants defective in AHL-mediated communication showed decreased expression of two superoxide dismutases, SodA and SodB, and the major catalase KatA. Furthermore, it has been shown that production of the superoxide dismutase SodB is significantly increased in *P. aeruginosa* biofilms [21, 32]. Therefore, our results may indicate that the *cep* QS system provides a regulatory link between surface colonization and the development of resistance against oxidative stress.

Table 3. Identification of *cep*-regulated proteins in *B. cepacia* H111 by *N*-terminal sequencing

Spot ID	Fraction[a)]	*N*-terminal sequence	p*I*/M_r		Similarity (organism)	Identity[c)] (%)	Ref.
			Practical[b)]	Theoretical[b)]			
QS1 QS33 QS54	IC EC SF	SRVTDVLVSFDTETIL	6.6/17.9 6.1/18.9 6.5/18.0	6.3/18.1	AidA (*Ralstonia solanacearum* AW1)	53	[25]
QS2	IC	SLRLGDIAPDFSPE	5.3/20.7	5.8/23.8	Probable peroxidase RSC0754 (*R. solanacearum* GMI1000)	85	[30]
QS3; QS4	IC	ADSQTSSNRAGEFSIPP	5.3; 5.0/14.0	5.2/13.9	Hypothetical protein RSC3288 (*R. solanacearum* GMI1000)	40	[30]
QS5; QS6 QS37; QS38 QS60; QS61	IC EC SF	AGTGTINFTGEIVAGACG	4.9; 4.6/14.0 4.8; 4.5/14.9; 15.0 4.3; 4.0/15.1	6.1/17.3	FimA (*Escherichia coli*)	49	[26]
QS7	IC	DPLTDSVKKRGVLRVG	9.1/26.4	9.3/28.9	Probable binding protein component of ABC transporter PA0314 (*Pseudomonas aeruginosa*)	54	[33]
QS8	IC	DTKVSNPQAASLRESLTR GVAP	8.9/25.1	5.8/60.5	Thermolysin metallopeptidase (*Bacillus anthracis*)	27	[35]
QS9	IC	AHTLPPLPYA	10.3/20.0	5.6/21.0	Superoxide dismutase SodB (*R. solanacearum* GMI1000)	84	[30]
QS10	IC	LADKCKLDSKPGQ	9.9/20.9	10.1/23.1	Ribosomal 30S subunit S4 RpsD (*R. solanacearum* GMI1000)	88	[30]
QS11	IC	LVAQAPQPPVAPVATAIT	9.7/18.4	9.4/19.2	Probable signal peptide protein RSC0900 (*R. solanacearum* GMI1000)	59	[30]
QS39	EC	GLLSFIKEAGEK	4.0/17.0	4.5/16.3	Hypothetical protein RSC2148 (*R. solanacearum* GMI1000)	45	[30]
QS52	SF	EARVFFVEP	6.5/12.8	6.7/14.2	Hypothetical protein PA0315 (*P. aeruginosa*)	50	[33]

a) Cell compartment, IC = intracellular; EC = extracellular; SF = surface
b) Isoelectric point and molecular mass obtained in the experiments (practical) and by calculation (theoretical)
c) Based on complete protein sequences obtained by TBLASTN search (http://www.sanger.ac.uk/Projects/Bcepacia/blastserver.shtml)

Spot QS7 identified an ATP-binding protein that is most similar to PA0314 of *P. aeruginosa* (54% identical amino acids) [33]. This protein is thought to be part of a hypothetical type I secretion system. We have demonstrated that expression of an ABC-exporter in *Serratia liquefaciens* is regulated by the *swr* QS system that operates in this organism [34]. This exporter mediates secretion of a metalloprotease, a lipase and S-layer protein.

Spot QS8 corresponded to an ORF with relatively weak similarity (27% identical amino acids) to a thermolysin metallopeptidase of *Bacillus anthracis* [35]. Both the p*I* and the M_r of spot QS8 differed significantly from the theoretical values predicted for the protein encoded by the corresponding *B. cepacia* ORF. The determined amino acid sequence of spot QS8 is homologous to position 27–48 of this ORF (100% identity of a stretch of 22 amino acids), indicating that the analyzed spot represents a degradation product of the protein. In fact, in silicio analysis revealed that removal of 32.9 kDa corresponding to 304 amino acids from the *C*-terminus of an *N*-terminal truncated protein would give rise to a product with a predicted M_r of 25 094 and p*I* of 8.8, which is very close to the observed M_r of 25 100 and p*I* of 8.9. In several bacteria expression of extracellular proteolytic enzymes are controlled by quorum sensing [14, 34, 36]. However, QS8 was found in the intracellular protein fraction and is therefore unlikely to represent the extracellular protease that was previously shown to be *cep*-regulated [14, 16].

Two other intracellular proteins showed significant homology (59 and 88% identical amino acids) to a signal peptide protein RSC0900 (QS11) and the ribosomal 30S subunit S4 RpsD (QS10) of *R. solanacearum* [30]. The amino acids derived from the remaining four protein spots (QS3, QS4, QS39, and QS52) corresponding to the hypothetical proteins RSC3288, RSC2148 and PAO315 of *R. solanacearum* [30] and *P. aeruginosa* [33] did not display any significant similarity to proteins with known function.

4 Discussion

Employing 2-DE we have shown that the *cep* quorum-sensing system of *B. cepacia* H111 controls expression of at least 55 different proteins out of 585 detected spots. Remarkably, the protein spot pattern of the wild-type strain can be fully restored by addition of the respective signal molecule to the growth medium of an AHL-deficient mutant. Thereby 5% and 1% of the proteome are up- and downregulated, respectively. This result indicates that QS represents a global gene regulation system in *B. cepacia* and is consistent with previous findings showing that a number of apparently unrelated functions including the production of extracellular hydrolytic enzymes, swarming motility, biofilm formation, and synthesis of the siderophore ornibactin are *cep*-regulated [14, 16, 17]. Transcriptional activation of target gene expression is thought to require binding of the CepR to palindromic sequences, so-called *cep* boxes, in the promoter regions target genes. A search for sequences resembling the *cep* box identified a putative CepR binding site upstream of *aidA* (data not shown). No obvious binding sites could be found upstream of the ORFs encoding *cep*-regulated proteins. There are several possible explanations for this discrepancy: (i) the regulation of the identified ORFs differ between strain H111 used in this study and the sequenced strain J2315, (ii) the identified genes are present within operons and thus the actual promoter region could be far away, and (iii) the identified proteins are not directly regulated by CepR but are further downstream in regulatory cascade. Work currently under way aims to test these possibilities.

We thank Christina Hoboth and Beate Schumacher for excellent technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft (RI 969/3-1, EB 252/1-3).

Received August 7, 2002

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6.4 Protein spots induced or repressed in the PAO1 gacA *mutant and PAO1* mvrf *mutant.*

Only spots with at least 2.0-fold change in signal intensity were considered. For each comparison three gels resulting from three independent protein extractions were analysed and compared.

	Wild type			gacA			mvfr		
	Intensity[a]	pI[d]	Mr kD[d]	Intensity[a]	fold change[b]	Regulation	Intensity[a]	fold change[c]	Regulation
Intracellular proteins									
IC1	0,77	6,3	34,3	0,31	-2,5	down	-		absent
IC2	2,85	6,6	55,7	0,62	-4,6	down	-		absent
IC3	6,29	5,7	17,5	2,88	-2,2	down	-		absent
IC4	1,92	5,9	11,1	0,39	-5,0	down	2,6	1,4	
IC5	2,15	5,7	20,5	2,48	1,2		1,025	-2	down
IC6	1,85	5,1	41,3	2,14	1,2		0,92	-2	down
IC7	0,71	5,7	41,4	0,35	-2,0	down	0,9	1,2	
IC8	1,90	5,7	50,8	1,34	-1,4		0,3	-6,9	down
IC9	1,62	4,9	32,3	2,93	1,8		0,4	-4,0	down
IC10	0,23	5,1	28,6	0,50	2,2	up	0,5	2,4	up
IC11	0,52	4,9	15,2	0,08	-6,4	down	-		absent
IC12	3,41	4,6	22,0	4,39	1,3		1,2	-2,8	down
IC13	0,49	6,1	16,7	1,03	2,1	up	-		absent
IC14	4,99	4,9	33,6	-		absent	12,3	2,5	up
IC15	1,82	5,6	73,3	2,38	1,3		0,9	-2,1	down
IC16	4,00	5,6	13,5	2,02	-2,0	down	2,0	-2,0	down
IC17	7,95	5,8	13,9	6,34	-1,3		3,5	-2,3	down
IC18	5,80	6,1	20,5	7,85	1,4		2,7	-2,1	down
IC19	2,06	6,6	55,2	0,63	-3,3	down	-		absent
IC20	7,78	5,4	21,1	8,73	1,1		3,8	-2,0	down
IC21	9,81	5,6	21,0	12,60	1,3		4,9	-2,0	down
IC22	1,70	5,0	15,1	0,14	-12,0	down	3,2	1,9	
IC23	6,15	4,8	33,5	-		absent	12,9	2,1	up
IC24	1,01	5,5	73,6	1,34	1,3		0,2	-4,2	down
IC25	3,35	5,7	14,4	4,67	1,4		1,7	-2,0	down
IC26	0,11	5,2	52,6	0,05	-2,2	down	-		absent
IC27	0,23	5,3	52,9	0,06	-4,2	down	-		absent
Surface-bound proteins									
SF1	3,81	9,2	15,9	1,3	-2,95	down	4,8	1,26	
SF2	0,99	9,1	25,2	5,2	5,25	up	10,6	10,72	up
SF3	5,19	7,8	26,6	7,4	1,43		2,6	-2,00	down
SF4	5,31	7,2	16,4	7,2	1,36		2,4	-2,24	down
SF5	0,37	6,9	20,1	1,1	2,87	up	5,6	15,06	up
SF6	5,37	8,6	21,9	4,8	-1,11		1,3	-4,28	down
SF7	7,54	7,8	25,9	8,9	1,18		3,7	-2,03	down
SF8	8,12	8,1	29,7	7,3	-1,11		1,0	-7,92	down
SF9	2,03	5,9	35,1	1,0	-2,09	down	1,0	-2,03	down
SF10	0,12	5,2	35,3	0,2	1,90		2,9	24,05	up
SF11	-	-	-	-		-	2,4		present
SF12	0,12	5,4	26,3	0,7	5,62	up	9,7	78,18	up

SF13	1,64	4,0	21,9	6,5	3,93	up	0,8	-2,12	down
SF14	-	-	-	1,0		present	9,7		present
SF15	1,91	6,7	16,0	2,6	1,38		-		absent
SF16	6,75	4,3	14,1	1,6	-4,18	down	2,0	-3,33	down
SF17	3,05	7,6	15,7	2,7	-1,13		1,2	-2,46	down
SF18	4,03	7,6	14,9	4,3	1,07		2,0	-2,03	down
SF19	8,35	7,5	11,3	7,0	-1,19		0,8	-10,80	down
SF20	8,22	7,6	30,2	7,0	-1,18		2,3	-3,56	down
SF21	8,36	7,7	36,6	6,6	-1,26		4,0	-2,10	down
SF22	1,85	8,1	34,4	2,2	1,19		0,9	-2,05	down
SF23	6,70	4,4	13,5	5,6	-1,19		-		absent
SF24	4,38	7,4	36,7	1,1	-3,91	down	1,3	-3,39	down
Extracellular proteins									
SN1	6,50	6,5	49,5	2,15	-3,0	down	5,50	-1,2	
SN2	2,00	6,0	50,2	0,95	-2,1	down	12,70	6,4	up
SN3	10,03	9,8	17,4	3,40	-3,0	down	5,13	-2,0	down
SN4	1,20	4,6	23,4	1,20	1,0		0,54	-2,2	down
SN5	1,12	4,5	20,0	0,55	-2,0	down	1,03	-1,1	
SN6	0,50	7,1	18,3	1,20	2,4	up	0,49	-1,0	
SN7	2,36	6,4	17,5	2,46	1,0		1,21	-2,0	down
SN8	7,80	6,6	24,7	3,40	-2,3	down	8,24	1,1	
SN9	0,70	8,4	34,8	0,84	1,2		1,43	2,0	up
SN10	0,97	8,2	30,2	1,06	1,1		0,49	-2,0	down
SN11	0,88	8,2	27,1	0,99	1,1		0,41	-2,1	down
SN12	11,12	4,6	43,0	10,46	-1,1		4,29	-2,6	down
SN13	10,70	4,5	43,1	8,60	-1,2		5,23	-2,0	down
SN14	7,20	5,9	25,0	3,64	-2,0	down	2,17	-3,3	down
SN15	5,23	6,1	31,0	2,54	-2,1	down	2,64	-2,0	down
SN16	7,05	5,7	34,0	2,06	-3,4	down	3,47	-2,0	down
SN17	5,06	5,0	50,2	4,50	-1,1		1,65	-3,1	down
SN18	4,54	4,6	54,6	3,76	-1,2		1,57	-2,9	down
SN19	2,21	5,9	33,0	0,84	-2,6	down	1,09	-2,0	down
SN20	0,96	5,5	22,3	0,98	1,0		0,35	-2,7	down
SN21	1,10	4,9	22,5	0,54	-2,0	down	0,37	-3,0	down
SN22	0,40	5,9	50,1	0,56	1,4		2,64	6,6	up
SN23	0,31	5,8	12,4	0,27	-1,1		0,15	-2,1	down
SN24	2,68	6,0	39,9	0,94	-2,9	down	1,29	-2,1	down
SN25	0,33	8,0	36,0	0,35	1,1		0,99	3,0	up
SN26	7,26	4,8	33,1	3,70	-2,0	down	2,30	-3,2	down
SN27	7,17	4,5	33,0	3,64	-2,0	down	2,48	-2,9	down
SN28	2,38	6,2	87,6	0,76	-3,1	down	1,15	-2,1	down
SN29	2,46	6,0	87,6	0,86	-2,9	down	1,19	-2,1	down
SN30	7,42	5,5	26,5	2,64	-2,8	down	3,20	-2,3	down

[a] Normalized protein expression levels determined by the ImageMaster® analysis software version 4.0

[b] Fold change in protein expression calculated by the ImageMaster® analysis software, positive numbers refer to proteins induced and negative numbers refer to proteins repressed in the PAO1-gacA mutant when compared with the PAO1-Wild type

[c] Fold change in protein expression calculated by the ImageMaster® analysis software, positive numbers refer to proteins induced and negative numbers refer to proteins repressed in the PAO1-mvfr mutant when compared with the PAO1-Wild type

[d] Isoelectric point and molecular mass obtained in the experiment.

6.5 Identification and spot signal intensities of induced or repressed proteins in the biofilm growth on glass wool of P. putida *IsoF*

Appendix 5A Protein spots induced or repressed in the biofilm growth on glass wool of *P. putida* IsoF. Only spots with at least 1.5-fold change in signal intensity were considered. For each comparison three gels resulting from three independent protein extractions were analysed and compared.

	BF[a]	PK[a]	fold change[b]	pI[c]	MW (kd)[c]	Regulation
1	44,9	114,1	-2.544	9,0	53,6	Down
2	142,0	258,1	-1.818	5,5	51,7	Down
3	4,9	172,6	-35.521	5,1	47,6	Down
4	202,0	98,9	2.043	5,2	43,6	Up
5	105,6	39,8	2.655	7,5	39,8	Up
6	86,4	34,8	2.484	7,9	40,4	Up
7	175,3	25,6	6.858	7,3	37,6	Up
8	13,0	30,5	-2.350	8,7	40,6	Down
9	12,2	24,2	-1.982	8,2	42,4	Down
10	255,5	628,2	-2.458	6,7	39,1	Down
11	119,8	357,6	-2.986	6,4	39,0	Down
12	9,7	142,7	-14.636	6,5	35,9	Down
13	14,2	40,9	-2.887	6,2	36,1	Down
14	-	21,4	-	4,9	36,9	Absent
15	38,9	14,8	2.630	5,0	35,8	Up
16	203,4	23,7	8.594	4,9	35,6	Up
17	90,7	5,8	15.706	4,7	35,5	Up
18	523,1	124,4	4.204	4,5	33,4	Up
19	452,6	214,9	2.106	4,3	33,3	Up
20	-	229,2	-	4,0	32,9	Absent
21	5,3	65,7	-12.472	5,9	27,6	Down
22	-	11,2	-	6,8	31,5	Absent
23	-	7,9	-	7,1	31,6	Absent
24	-	23,1	-	8,0	29,9	Absent
25	8,6	73,9	-8.575	8,1	29,1	Down
26	7,4	141,3	-19.159	8,1	32,3	Down
27	24,5	92,5	-3.770	8,4	32,0	Down
28	-	11,9	-	8,6	31,5	Absent
29	-	0,0	-	8,5	30,2	Absent
30	50,3	113,3	-2.253	9,0	34,1	Down
31	19,1	307,4	-16.066	9,5	34,2	Down
32	81,3	156,1	-1.920	9,4	23,8	Down
33	79,9	40,1	1.993	8,9	24,5	Up
34	11,9	37,4	-3.146	8,7	24,9	Down
35	13,1	101,0	-7.723	8,4	24,1	Down
36	9,4	36,9	-3.928	8,2	22,6	Down
37	15,6	121,7	-7.819	9,0	19,9	Down
38	28,7	183,6	-6.394	8,1	19,7	Down
39	-	64,6	-	7,8	22,1	Absent
40	-	22,1	-	7,7	21,0	Absent
41	-	46,1	-	7,3	22,0	Absent

42	-	42,7	-	7,3	20,5	Absent
43	5,8	12,2	-2.096	7,2	22,1	Down
44	81,6	13,5	6.047	6,8	22,6	Up
45	176,1	33,2	5.306	6,7	19,8	Up
46	13,8	79,5	-5.750	6,2	21,8	Down
47	113,2	37,5	3.021	5,6	22,6	Up
48	79,0	9,0	8.794	9,6	17,3	Up
49	194,5	8,6	22.661	9,2	16,1	Up
50	79,9	26,2	3.046	8,6	16,1	Up
51	16,0	80,7	-5.046	8,1	15,8	Down
52	-	33,8	-	8,3	17,3	Absent
53	-	37,1	-	7,6	16,5	Absent
54	-	30,8	-	7,4	15,2	Absent
55	221,0	102,3	2.160	6,7	14,9	Up
56	12,0	83,3	-6.953	6,4	14,8	Down
57	14,0	133,2	-9.481	5,2	15,6	Down
58	84,6	17,0	4.981	5,2	18,4	Up
59	191,4	97,2	1.969	5,5	18,6	Up
60	95,2	21,9	4.353	6,5	18,0	Up
61	189,3	91,9	2.060	6,9	18,1	Up
62	3,6	55,8	-15.709	8,2	18,5	Down
63	5,0	92,0	-18.489	8,4	18,5	Down
64	3,9	55,6	-14.273	9,0	12,0	Down
65	11,5	47,1	-4.096	8,5	13,7	Down
66	48,7	257,8	-5.291	8,3	11,5	Down
67	92,1	199,7	-2.167	6,5	10,6	Down
68	411,7	113,4	3.631	6,5	11,5	Up
69	148,9	3,0	48.844	7,1	12,8	Up
70	23,8	5,7	4.203	6,7	12,8	Up
71	5,6	109,9	-19.472	5,7	12,8	Down
72	65,8	9,2	7.186	4,7	10,7	Up
73	112,8	44,5	2.532	4,3	12,6	Up
74	-	157,8	-	4,0	12,1	Absent
75	-	91,9	-	3,6	12,4	Absent
76	2,8	-		8528,0	13070,0	Present
77	63,7	-		8182,0	13901,0	Present
78	5,5	-		8058,0	12895,0	Present
79	37,8	-		7894,0	31261,0	Present
80	45,6	68,7	-1.507	8,6	53,2	Down
81	153,7	92,5	1.662	7,5	45,4	Up
82	89,9	48,8	1.844	5,6	28,9	Up
83	159,2	243,0	-1.526	5,8	29,0	Down
84	11,9	20,9	-1.762	8,7	18,3	Down
85	154,4	235,1	-1.523	7,3	11,3	Down

[a] Normalized protein expression levels determined by the ImageMaster® analysis software version 4.0

[b] Fold change in protein expression calculated by the ImageMaster® analysis software, positive numbers refer to proteins induced and negative numbers refer to proteins repressed in the biofilm growth on glass wool of *P. putida* IsoF

[c] Isoelectric point and molecular mass obtained in the experiment.

Appendix 5B: Identification of induced and repressed proteins in the biofilm growth on glass wool of *P. putida* IsoF by MALDI-TOF MS peptide mass mapping and Orthologous proteins in P. aeruginosa.

PP No[a].	Spot No[b]	PP Description	Regulation	Theor. pI[c]	Theor. M (kDa)[c]	Pract. pI[c]	Pract M (kDa)[c]	Sequence coverage (%)	Ortholog in *P. aeruginosa*[d]		
									PA No.	PA description	Identity (%)
PP4187	1	2-oxoglutarate dehydrogenase, lipoamide dehydrogenase component	2,5	5,9	49,9	9,0	53,6	24,9			
PP3753	3	transcriptional regulator, AraC family	35,5	10,0	40,2	5,1	47,6	35,9			
PP5413*	4	ATP synthase F1, beta subunit	-2,0	4,9	49,3	5,2	43,6	72,9	PA5554	ATP synthase beta chain (atpD)	90,4%
PP1141*	7	branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein	-6,9	6,0	39,4	7,3	37,6	35,0	PA1074	branched-chain amino acid transport protein BraC (braC)	62,6%
PP2089*	18	outer membrane protein OprF	-4,2	4,7	37,0	4,5	33,4	68,3	PA1777	outer membrane protein OprF precursor (oprF)	64,6%
PP4716	34	phosphoglucosamine mutase	3,1	5,7	47,7	8,7	24,9	18,4	PA4749	phosphoglucosamine mutase (glmM)	80,9%
PP2909	38	conserved hypothetical protein	6,4	5,4	37,2	8,1	19,7	15,7			
PP0797*	49	Conserved hypothetical protein	-22,7	6,3	20,0	9,2	16,1	32,4	PA2169	hypothetical protein {Pseudomonas aeruginosa PAO1}	54,4%
PP1185*	55	outer membrane protein H1	-2,2	6,3	21,5	6,7	14,9	30,8	PA1178	outer membrane protein H1 precursor (oprH)	55,4%
PP4780	56	acyl-CoA dehydrogenase, putative	7,0	6,2	59,8	6,4	14,8	9,1	PA3972	probable acyl-CoA dehydrogenase	79,7%
PP2501	57	phage integrase, putative	9,5	9,6	53,2	5,2	15,6	14,1			
PP0237	66	sulfonate ABC transporter, periplasmic sulfonate-binding protein SsuA	5,3	8,6	34,5	8,3	11,5	10,9	PA3445	conserved hypothetical protein	81,1%
PP4187	80	2-oxoglutarate dehydrogenase, lipoamide dehydrogenase component	1,5	5,9	49,9	8,6	53,2	28,9	PA1587	lipoamide dehydrogenase-glc (lpdG)	84,1%
PP1258	85	epimerase, putative	1,5	5,8	33,7	7,3	11,3	16,6	PA1268	hypothetical protein	68,6%

[a] Data generated from peptide mass maps were compared to the complete translate ORF for *P. putida* (http://www.tigr.org)
[b] Spot No. refers to numbers shown in Fig. 13.
[c] Isoelectric point and molecular mass obtained in the experiments (practical) and from the databases(theorical)
[d] Comparison of our data against *P. aeruginosa* translated ORF database.

6.6 Orthologous proteins between P. aeruginosa *and* P. putida.

All identified proteins in *P. putida* IsoF were compared against the *P. aeruginosa* PAO1complete translated ORFs.

PP No[a]	PA No[b]	PA description	Identity(%)	Similarity(%)
PP5160	PA0309	hypothetical protein	62,4%	76,0%
PP5110	PA0374	cell division protein FtsE (ftsE)	87,3%	92,7%
PP4998*	PA0402	aspartate carbamoyltransferase (pyrB)	89,8%	94,6%
PP4981*	PA0423	conserved hypothetical protein	59,0%	76,3%
PP1418*	PA0754	hypothetical protein	85,1%	90,1%
PP1429*	PA0764	negative regulator for alginate biosynthesis MucB (mucB)	58,8%	72,3%
PP2338*	PA0792	propionate catabolic protein PrpD (prpD)	81,8%	88,9%
PP4491	PA0871	pterin-4-alpha-carbinolamine dehydratase (phhB)	86,4%	94,1%
PP4486*	PA0888	arginine/ornithine binding protein AotJ (aotJ)	60,7%	71,2%
PP1224	PA0974	conserved hypothetical protein	57,0%	63,0%
PP1141*	PA1074	branched-chain amino acid transport protein BraC (braC)	62,6%	75,1%
PP1185*	PA1178	outer membrane protein H1 precursor (oprH)	55,4%	70,1%
PP1258	PA1268	hypothetical protein	68,6%	81,1%
PP2453	PA1337	glutaminase-asparaginase (ansB)	78,3%	84,9%
PP1071*	PA1342	probable binding protein component of ABC transporter	69,7%	78,3%
PP4187	PA1587	lipoamide dehydrogenase-glc (lpdG)	84,1%	91,2%
PP3241*	PA2134	hypothetical protein	46,4%	64,1%
PP0797*	PA2169	hypothetical protein	54,4%	73,5%
PP4003	PA2614	periplasmic chaperone LolA (lolA)	64,4%	79,3%
PP0268*	PA2760	probable outer membrane protein	62,2%	74,7%
PP1981	PA3129	conserved hypothetical protein	75,1%	84,0%
PP4539	PA3225	probable transcriptional regulator	80,5%	88,9%
PP1099	PA3266	cold acclimation protein B (capB)	70,6%	88,2%
PP2463	PA3266	cold acclimation protein B (capB)	69,1%	82,4%
PP0237	PA3445	conserved hypothetical protein	81,1%	89,2%
PP1083	PA3530	conserved hypothetical protein	72,2%	79,2%
PP1600	PA3647	probable outer membrane protein precursor	77,4%	84,9%
PP1518	PA3675	hypothetical protein	65,7%	77,3%
PP1506	PA3686	adenylate kinase (adk)	73,1%	81,0%
PP4836*	PA3785	conserved hypothetical protein	56,2%	69,3%
PP4838*	PA3790	outer membrane protein OprC (oprC)	71,5%	80,5%
PP0849	PA3807	nucleoside diphosphate kinase (ndk)	72,3%	80,9%
PP5165	PA3931	conserved hypothetical protein	85,7%	94,4%
PP4779	PA3970	AMP nucleosidase (amn)	83,5%	90,9%
PP4780	PA3972	probable acyl-CoA dehydrogenase	79,7%	86,1%
PP0504	PA4067	outer membrane protein OprG precursor (oprG)	65,0%	75,9%
PP0469	PA4248	50S ribosomal protein L6 (rplF)	90,4%	94,4%
PP0465	PA4252	50S ribosomal protein L24 (rplX)	90,4%	94,2%
PP1360	PA4386	GroES protein (groES)	92,7%	97,9%
PP0961	PA4453	conserved hypothetical protein	59,9%	74,4%
PP0954	PA4460	conserved hypothetical protein	54,5%	68,2%
PP4716	PA4749	phosphoglucosamine mutase (glmM)	80,9%	89,9%
PP4726	PA4760	DnaJ protein (dnaJ)	72,7%	79,0%
PP4870*	PA4922	azurin precursor (azu)	60,2%	76,6%

PP4874	PA4932	50S ribosomal protein L9 (rplI)	57,4%	66,2%
PP5078	PA5038	3-dehydroquinate synthase (aroB)	77,0%	84,1%
PP1001	PA5171	arginine deiminase (arcA)	83,0%	91,9%
PP1000	PA5172	ornithine carbamoyltransferase, catabolic (arcB)	92,0%	96,7%
PP0259	PA5177	probable hydrolase	76,8%	85,9%
PP5215	PA5240	thioredoxin (trxA)	81,3%	91,6%
PP0196	PA5252	probable ATP-binding component of ABC transporter	78,6%	86,8%
PP5294	PA5334	ribonuclease PH (rph)	87,1%	92,9%
PP5329*	PA5369	hypothetical protein	78,3%	83,9%
PP0112*	PA5505	probable TonB-dependent receptor	80,8%	86,9%
PP5413	PA5554	ATP synthase beta chain (atpD)	90,4%	93,4%
PP2089*	PA1777	outer membrane protein OprF precursor (oprF)	64,6%	76,2%
PP2454	PA1946	binding protein component precursor of ABC ribose transporter (rbsB)	76,9%	89,9%

[a] Data generated from peptide mass maps were compared to the complete translate ORF for *P. putida* (http://www.tigr.org)
[b] Data generated from peptide mass maps were compared to the complete translate ORF for *P. aeruginosa* PAO1 (http://www.pseudomonas.com)
[c] Identity refer to the proportion of identical aminoacids between the two sequences.
[d] Similarity refer to the proportion of similar aminoacids between the two sequences, according to a substitution matrix (BLOSUM62).
* Quorum-sensing regulated proteins reported in previously published studies.

6.7 Identification of induced or repressed proteins in the biofilm growth on silicone tubes of P. putida

Identification of induced or repressed proteins in the biofilm growth on silicone tubes of *P. putida* IsoF by MALDI-TOF MS peptide mass mapping and Orthologous proteins in *P. aeruginosa*.

PP No[a]	Spot No[b]	PP description	Regulation	Theor. pI[c]	Theor. M (kDa)[c]	Pract pI[c]	Pract M (kDa)[c]	Sequence coverage (%)	Ortholog in *P. aeruginosa*[d]		
									PA No.	PA description	Identity (%)
PP0112[e]	BF53	ABC transporter, periplasmic binding protein, putative	5,0	6,9	27,7	7,5	24,1	20,3	PA5505	probable TonB-dependent receptor	80,8%
PP0259	BF77	hydrolase, haloacid dehalogenase-like family	2,4	5,1	25,4	7,0	15,1	14,5	PA5177	probable hydrolase	76,8%
PP0268[e]	BF11	outer membrane protein OprE3	3,0	5,8	47,8	4,9	47,7	21,2	PA2760	probable outer membrane protein	62,2%
PP0465	BF103	ribosomal protein L24	1,9	10,1	11,3	6,7	12,6	35,6	PA4252	50S ribosomal protein L24 (rplX)	90,4%
PP0469	BF57	ribosomal protein L6	12,5	9,7	19,2	9,6	19,1	20,3	PA4248	50S ribosomal protein L6 (rplF)	90,4%
PP0849	BF89	nucleoside diphosphate kinase	30,9	5,5	15,0	5,1	12,7	43,3	PA3807	nucleoside diphosphate kinase (ndk)	72,3%
PP1000[f]	BF26	ornithine carbamoyltransferase, catabolic	-5,5	5,9	37,9	7,3	38,5	12,5	PA5172	ornithine carbamoyltransferase, catabolic (arcB)	92,0%
PP1001[f]	BF15	arginine deaminase	13,9	5,6	46,4	6,1	46,5	22,1	PA5171	arginine deiminase (arcA)	83,0%
PP1083	BF118	bacterioferritin-associated ferredoxin, putative	1,6	5,1	7,6	6,1	35,3	44,4	PA3530	conserved hypothetical protein	72,2%
PP1185[e]	BF72	outer membrane protein H1	2,5	6,3	21,5	5,1	16,2	30,8	PA1178	outer membrane protein H1 precursor (oprH)	55,4%
PP1224	BF32	conserved hypothetical protein	3,6	7,7	28,7	5,8	29,5	15,7	PA0974	conserved hypothetical protein	57,0%
PP1360	BF88	chaperonin, 10 kDa	2,7	5,4	10,2	5,6	13,3	38,1	PA4386	GroES protein (groES)	92,7%
PP1418[e]	BF29	tricarboxylate transport protein TctC, putative	4,0	6,2	35,0	4,9	32,1	22,4	PA0754	hypothetical protein	85,1%
PP1506	BF48	adenylate kinase	4,4	5,6	23,2	6,7	25,8	18,5	PA3686	adenylate kinase (adk)	73,1%
PP1518	BF123	conserved hypothetical protein	-1,7	9,1	23,8	9,2	20,4	24,1	PA3675	hypothetical protein	65,7%

PP1600	BF82	outer membrane protein OmpH	6,4	9,4	18,8	9,4	14,5	16,8	PA3647	probable outer membrane protein precursor	77,4%
PP1981	BF62	NifR3/Smm1 family protein	-2,8	7,0	35,5	6,5	20,3	21,7	PA3129	conserved hypothetical protein	75,1%
PP2089[e]	BF66	outer membrane protein OprF	6,1	4,7	37,0	4,3	23,0	10,8	PA1777	outer membrane protein OprF precursor (oprF)	64,6%
PP2453[f]	BF27	L-asparaginase II	-8,6	7,0	38,6	6,9	36,4	26,0	PA1337	glutaminase-asparaginase (ansB)	78,3%
PP2454	BF30	ribose ABC transporter, periplasmic ribose-binding protein	6,8	5,8	33,7	4,9	30,9	15,4	PA1946	binding protein component precursor of ABC ribose transporter (rbsB)	76,9%
PP3097	BF33	conserved hypothetical protein	21,5	6,8	69,2	5,8	32,4	10,9			
PP3241[e]	BF85	conserved hypothetical protein	2,6	5,8	18,2	5,3	14,0	21,1	PA2134	hypothetical protein	46,4%
PP4003	BF63	outer membrane lipoprotein carrier protein	5,1	8,8	25,3	5,7	20,6	27,2	PA2614	periplasmic chaperone LolA (lolA)	64,4%
PP4519	BF14	agglutination protein	1,8	5,5	50,4	5,7	45,8	16,2			
PP4779	BF107	AMP nucleosidase	present	6,9	57,6	9,7	13,7	14,1	PA3970	AMP nucleosidase (amn)	83,5%
PP4836[e]	BF74	conserved hypothetical protein	-5,0	6,0	17,4	6,6	16,1	24,8	PA3785	conserved hypothetical protein	56,2%
PP4838[e]	BF9	outer membrane copper receptor OprC	14,1	5,9	74,5	5,9	72,6	7,1	PA3790	outer membrane protein OprC (oprC)	71,5
	BF10		34,8	5,9	74,5	6,2	72,4	10,6			
PP4981[e]	BF76	conserved hypothetical protein	2,7	7,9	22,1	7,0	16,4	36,8	PA0423	conserved hypothetical protein	59,0%
PP5215	BF92	thioredoxin	-9,7	5,0	11,7	4,4	10,5	43,1	PA5240	thioredoxin (trxA)	81,3%
PP5329[e]	BF41	phosphate ABC transporter, periplasmic phosphate-binding protein	-1,6	9,1	34,9	9,0	31,0	22,3	PA5369	hypothetical protein	78,3%

[a] Data generated from peptide mass maps were compared to the complete translate ORFs for *P. putida* IsoF (http://www.tigr.org)
[b] Spot No. refers to numbers shown in Fig. 16.
[c] Isoelectric point and molecular mass obtained in the experiments (practical) and from the databases(theorical)
[d] Comparison of our data against *P. aeruginosa* translated ORFs database.
[e] Quorum-sensing
[f] Biofilm Sauer *et al.* (2001)

6.8 Protein spots induced or repressed in the biofilm growth on silicone tubes of P. putida *IsoF.*

Only spots with at least 1.5-fold change in signal intensity were considered. For each comparison three gels resulting from three independent protein extractions were analysed and compared.

	PK[a]	BF[a]	Fold change[b]	pI[c]	Mr kD[c]	Regulation
1	2839,2	1502,8	-1,9	4,0	55,4	Down
2	54,7	-	-	3,9	44,0	Absent
3	16,1	-	-	4,8	67,8	Absent
4	2,0	10,0	5,1	5,1	80,6	Up
5	2,8	15,1	5,5	5,2	79,9	Up
6	1,7	22,5	13,4	5,3	79,7	Up
7	1,3	18,0	14,4	5,4	72,6	Up
8	2,1	55,8	26,3	5,6	72,4	Up
9	4,8	67,3	14,1	5,9	72,6	Up
10	1,3	44,4	34,9	6,2	72,4	Up
11	2,3	6,9	3,0	4,9	47,7	Up
12	2,5	26,7	10,6	5,1	47,7	Up
13	6,3	30,5	4,8	5,3	47,4	Up
14	10,8	19,9	1,8	5,7	45,8	Up
15	2,7	38,0	13,9	6,1	46,5	Up
16	410,2	158,9	-2,6	5,6	54,7	Down
17	346,2	9,9	-35,0	7,0	53,7	Down
18	2,6	1,2	-2,2	6,8	48,4	Down
19	4,4	-	-	6,9	42,7	Absent
20	6,4	158,6	24,9	4,0	35,6	Up
21	0,5	2,9	6,4	5,2	36,0	Up
22	11,1	28,2	2,5	5,5	35,5	Up
23	33,0	133,3	4,0	5,8	35,5	Up
24	6,0	12,2	2,0	6,4	34,1	Up
25	2,3	-	-	6,5	31,8	Absent
26	82,8	15,1	-5,5	7,3	38,5	Down
27	22,2	2,6	-8,6	6,9	36,4	Down
28	1,3	13,5	10,6	4,8	31,9	Up
29	2,4	9,7	4,0	4,9	32,1	Up
30	3,9	26,6	6,8	4,9	30,9	Up
31	3,1	8,1	2,6	5,1	29,7	Up
32	13,0	46,4	3,6	5,8	29,5	Up
33	0,5	10,9	21,5	5,8	32,4	Up
34	1,2	18,3	15,5	5,9	32,0	Up
35	0,5	16,9	36,8	6,2	32,2	Up
36	0,8	12,6	15,9	6,5	32,0	Up
37	20,2	-	-	6,3	29,3	Absent
38	29,1	-	-	6,5	29,2	Absent
39	43,3	-	-	6,7	29,2	Absent
40	664,6	56,0	-11,9	8,8	29,2	Down
41	8,4	5,3	-1,6	9,0	31,0	Down
42	0,8	-	-	4,6	26,0	Absent
43	3,5	-	-	4,9	25,7	Absent
44	33,8	7,5	-4,5	4,9	26,0	Down

45	19,5	-	-	5,6	23,4	Absent
46	1,7	43,3	25,9	5,5	25,5	Up
47	2,9	-	-	5,9	26,0	Absent
48	3,0	13,2	4,4	6,7	25,8	Up
49	2,5	17,7	7,1	7,0	25,5	Up
50	5,0	41,1	8,2	7,0	24,3	Up
51	1,1	-	-	7,2	26,6	Absent
52	5,5	24,4	4,5	7,5	25,8	Up
53	21,1	104,6	5,0	7,5	24,1	Up
54	201,0	57,4	-3,5	7,8	24,0	Down
55	30,5	18,9	-1,6	8,2	24,0	Down
56	1,0	10,0	10,2	8,1	22,7	Up
57	6,7	83,3	12,5	9,6	19,1	Up
58	90,5	33,0	-2,7	8,9	19,1	Down
59	0,0	-	-	8,2	17,8	Absent
60	4,8	-	-	8,2	18,5	Absent
61	11,4	43,8	3,8	7,8	20,1	Up
62	30,8	10,8	-2,8	6,5	20,3	Down
63	7,0	35,3	5,1	5,7	20,6	Up
64	131,5	64,1	-2,1	3,7	24,2	Down
65	11,6	34,6	3,0	4,0	23,3	Up
66	2,1	13,1	6,1	4,3	23,0	Up
67	1,6	34,7	21,6	4,5	19,5	Up
68	12,4	44,3	3,6	3,3	15,4	Up
69	20,8	47,2	2,3	3,6	15,4	Up
70	31,6	111,5	3,5	4,4	15,7	Up
71	6,6	14,0	2,1	4,9	16,6	Up
72	7,0	17,1	2,5	5,1	16,2	Up
73	27,9	54,0	1,9	6,4	16,7	Up
74	75,0	15,1	-5,0	6,6	16,1	Down
75	221,3	99,9	-2,2	6,9	15,9	Down
76	49,2	133,9	2,7	7,0	16,4	Up
77	10,5	25,3	2,4	7,0	15,1	Up
78	17,6	9,6	-1,8	7,0	17,3	Down
79	251,0	130,8	-1,9	7,3	17,0	Down
80	213,3	26,1	-8,2	7,7	17,1	Down
81	4,7	121,7	26,1	9,4	14,9	Up
82	28,7	183,7	6,4	9,4	14,5	Up
83	11,2	44,5	4,0	5,5	15,3	Up
84	31,1	79,9	2,6	5,4	14,7	Up
85	28,7	73,9	2,6	5,3	14,0	Up
86	17,0	65,4	3,9	5,0	13,4	Up
87	9,6	28,7	3,0	5,2	13,4	Up
88	7,1	19,5	2,8	5,6	13,3	Up
89	3,0	90,6	30,1	5,1	12,7	Up
90	5,6	25,0	4,5	5,1	12,1	Up
91	106,3	51,6	-2,1	4,8	10,5	Down
92	82,2	8,4	-9,7	4,4	10,5	Down
93	61,7	482,3	7,8	4,7	10,9	Up
94	23,8	62,6	2,6	4,4	11,1	Up
95	8,6	62,0	7,2	4,6	10,4	Up
96	2,8	18,5	6,5	4,2	11,4	Up
97	26,1	7,2	-3,6	4,2	10,1	Down

98	29,0	70,0	2,4	5,0	9,1	Up
99	274,7	557,0	2,0	6,1	10,1	Up
100	75,7	292,4	3,9	7,3	10,0	Up
101	22,3	110,0	4,9	7,5	9,2	Up
102	4,7	22,9	4,8	7,0	12,5	Up
103	4,1	8,0	2,0	6,7	12,6	Up
104	-	5,9	-	5,1	47,7	Present
105	-	46,6	-	5,9	25,6	Present
106	-	12,8	-	4,9	20,8	Present
107	-	39,4	-	9,7	13,7	Present
108	-	64,5	-	9,4	13,2	Present
109	-	12,2	-	9,7	12,7	Present
110	-	25,9	-	9,7	11,9	Present
111	-	252,8	-	9,4	10,2	Present
112	-	56,1	-	5,6	19,0	Present
113	-	104,4	-	9,7	10,4	Present
114						
115	5,3	3,1	-1,7	7,1	48,1	Down
116	60,5	38,2	-1,6	5,3	37,1	Down
117	3,1	4,8	1,5	5,7	36,0	Up
118	24,6	39,1	1,6	6,1	35,3	Up
119	94,7	155,8	1,6	6,3	35,3	Up
129	20,8	11,8	-1,8	6,2	36,9	Down
121	6,1	3,5	-1,7	5,7	25,6	Down
122	24,9	46,2	1,9	8,3	23,4	Up
123	43,2	25,9	-1,7	9,2	20,4	Down
124	42,2	27,3	-1,5	8,0	20,0	Down
125	98,0	59,0	-1,7	4,4	20,7	Down
126	27,5	42,1	1,5	4,8	12,7	Up
127	164,4	98,9	-1,7	7,8	10,1	Down
128	115,0	63,1	-1,8	6,6	13,0	Down
129	230,2	116,5	-2,0	7,3	13,0	Down
130	16,2	28,9	1,8	7,7	35,7	Up

[a] Normalized protein expression levels determined by the ImageMaster® analysis software version 4.0

[b] Fold change in protein expression calculated by the ImageMaster® analysis software, positive numbers refer to proteins induced and negative numbers refer to proteins repressed in the biofilm growth on silicone tubes of *P. putida* IsoF

[c] Isoelectric point and molecular mass obtained in the experiment.

6.9 Identification of quorum-sensing regulated proteins of P. putida *IsoF.*

Identification of quorum-sensing regulated proteins of *P. putida* IsoF by MALDI-TOF MS peptide mass mapping and Orthologous proteins in *P. aeruginosa*.

PP No[a]	Spot No[b]	*P. putida* Protein Identification	Regulation	Theor pI[c]	Theor M (kDa)[c]	Pract pI[c]	Pract M (kDa)[c]	Sequence coverage (%)	Ortholog in *P. aeruginosa*[d]		
									PA No.	PA description	Identity (%)
PP0465	QS46	ribosomal protein L24	-6,5	10,1	11,3	6,7	13,3	35,6	PA4252	50S ribosomal protein L24 (rplX)	90,4%
PP0469	QS26	ribosomal protein L6	2,9	9,7	19,2	9,5	19,8	20,3	PA4248	50S ribosomal protein L6 (rplF)	90,4%
PP0849	QS35	nucleoside diphosphate kinase	-2,4	5,5	15,03	4,9	14,1	43,3	PA3807	nucleoside diphosphate kinase (ndk)	72,3%
PP1000[f]	QS6	ornithine carbamoyltransferase, catabolic	-2,0	5,9	37,9	7,0	39,3	12,5	PA5172	ornithine carbamoyltransferase, catabolic (arcB)	92,0%
PP1001[f]	QS5	arginine deaminase	-2,3	5,6	46,4	6,0	47,6	22,1	PA5171	arginine deiminase (arcA)	83,0%
PP1185[e]	QS30	outer membrane protein H1	-2,0	6,3	21,5	4,9	16,5	30,8	PA1178	outer membrane protein H1 precursor (oprH)	55,4%
PP1360	QS36	chaperonin, 10 kDa	-1,9	5,4	10,2	5,2	14,1	38,1	PA4386	GroES protein (groES)	92,7%
PP1418[e]	QS7	tricarboxylate transport protein TctC, putative	present	6,2	35,0	4,6	32,9	22,4	PA0754	hypothetical protein	85,1%
PP1506	QS16	adenylate kinase	-2,4	5,6	23,2	6,8	26,7	18,5	PA3686	adenylate kinase (adk)	73,1%
PP1518	QS24	conserved hypothetical protein	-1,6	9,1	23,8	9,0	21,4	24,1	PA3675	hypothetical protein	65,7%
PP1981	QS21	NifR3/Smm1 family protein	-1,7	7,0	35,5	5,6	21,7	21,7	PA3129	conserved hypothetical protein	75,1%
PP2089[e]	QS14	outer membrane protein OprF	1,8	4,7	37,0	3,2	26,1	10,8	PA1777	outer membrane protein OprF precursor (oprF)	64,6%
PP2454	QS9	ribose ABC transporter, periplasmic ribose-binding protein	-3,5	5,8	33,7	4,6	31,7	15,4	PA1946	binding protein component precursor of ABC ribose transporter (rbsB)	76,9%
PP3097	QS8	conserved hypothetical protein	1,8	6,0	69,2	5,6	32,9	10,9			
PP3241[e]	QS34	conserved	8,5	5,8	18,2	5,1	14,9	21,1	PA2134	hypothetical protein	46,4%

		hypothetical protein									
PP4003	QS19	outer membrane lipoprotein carrier protein	-2,2	8,8	25,3	4,5	22,2	27,2	PA2614	periplasmic chaperone LolA (lolA)	64,4%
PP4519	QS4	agglutination protein	-2,3	5,5	50,4	5,4	47,2	16,2			
PP4779	QS23	AMP nucleosidase	2,0	6,9	57,6	5,7	19,1	14,1	PA3970	AMP nucleosidase (amn)	83,5%
PP4836[e]	QS29	conserved hypothetical protein	-3,4	6,0	17,4	6,6	16,5	24,8	PA3785	conserved hypothetical protein	56,2%
PP4981[e]	QS28	conserved hypothetical protein	1,6	7,9	22,1	7,0	16,8	36,8	PA0423	conserved hypothetical protein	59,0%
PP5110	QS15	cell division ABC transporter, ATP-binding protein FtsE	1,6	10,3	24,81	5,9	25,5	16,9	PA0374	cell division protein FtsE (ftsE	87,3%
PP5215	QS40	thioredoxin	-5,1	5,0	11,7	4,1	11,1	43,1	PA5240	thioredoxin (trxA)	

[a] Data generated from peptide mass maps were compared to the complete translate ORFs for *P. putida* IsoF (http://www.tigr.org)
[b] Spot No. refers to numbers shown in Fig. 18.
[c] Isoelectric point and molecular mass obtained in the experiments (practical) and from the databases(theorical)
[d] Comparison of our data against *P. aeruginosa* translated ORFs database.
[e] Quorum-sensing
[f] Biofilm Sauer *et al.* (2001)

6.10 Protein spots induced or repressed by the quorum-sensing system of P. putida IsoF.

Only spots with at least 1.5-fold change in signal intensity which were rescued to the wild-type level when the *ppuI* mutant was grown in presence of 2µM 2-oxo-C12-HSL were considered. For each comparison six gels resulting from three independent protein extractions were analysed and compared.

	Isof BF[a]	ppu BF[a]	ppu + BF[a]	fold change [b]	pI[c]	Mr kD[c]	Regulation
1	353,8	782,9	437,0	-2,2	3,6	60,5	Down
2	15,7	33,2	17,7	-2,1	5,3	71,9	Down
3	11,1	17,1	13,5	-1,5	4,5	46,0	Down
4	23,3	54,2	35,8	-2,3	5,4	47,2	Down
5	44,0	100,0	32,3	-2,3	6,0	47,6	Down
6	17,6	34,8	18,3	-2,0	7,0	39,3	Down
7	11,7	-	3,1	-	4,6	32,9	Present
8	12,8	7,0	6,9	1,8	5,6	32,9	Up
9	30,8	106,8	-	-3,5	4,6	31,7	Down
10	5,8	12,7	-	-2,2	9,1	47,7	Down
11	2,2	5,9	2,8	-2,7	8,8	47,9	Down
12	19,8	3,2	7,3	6,3	6,1	32,8	Up
13	15,0	5,4	5,8	2,7	6,4	32,7	Up
14	49,9	28,2	57,2	1,8	3,2	26,1	Up
15	54,8	33,7	45,6	1,6	5,9	25,5	Up
16	15,8	37,4	7,5	-2,4	6,8	26,7	Down
17	16,4	49,2	7,3	-3,0	7,2	26,4	Down
18	3,5	14,4	-	-4,2	9,7	23,7	Down
19	28,5	63,8	34,7	-2,2	4,5	22,2	Down
20	15,0	34,5	10,2	-2,3	5,0	20,3	Down
21	27,9	48,7	37,2	-1,7	5,6	21,7	Down
22	4,5	28,6	15,2	-6,3	5,4	21,6	Down
23	61,3	134,3	2,2	-2,2	5,7	19,1	Down
24	30,2	47,9	31,5	-1,6	9,0	21,4	Down
25	6,3	21,1	10,6	-3,3	9,1	19,9	Down
26	83,5	238,8	37,2	-2,9	9,5	19,8	Down
27	155,8	75,1	132,6	2,1	7,2	17,5	Up
28	158,8	96,0	123,8	1,7	7,0	16,8	Up
29	17,7	37,0	15,2	-2,1	6,6	16,5	Down
30	20,2	41,4	10,7	-2,1	4,9	16,5	Down
31	16,6	30,8	7,6	-1,9	4,5	16,9	Down
32	53,8	118,7	17,5	-2,2	3,1	15,9	Down
33	17,1	-	-	-	3,1	15,3	Present
34	187,4	22,1	50,9	8,5	5,1	14,9	Up
35	24,8	58,8	5,3	-2,4	4,9	14,1	Down
36	18,3	35,3	7,2	-1,9	5,2	14,1	Down
37	70,4	116,6	6,9	-1,7	4,7	13,4	Down
38	32,4	59,2	3,7	-1,8	4,3	13,5	Down
39	21,0	-	-	-	3,7	12,1	Present
40	10,1	51,2	3,1	-5,1	4,1	11,1	Down
41	73,1	13,1	25,4	5,6	4,0	11,8	Up
42	569,9	87,1	106,3	6,5	4,3	11,5	Up
43	78,5	2,4	5,3	32,3	4,2	10,9	Up

44	8,8	41,2	2,6	-4,7	3,9	10,7	Down
45	85,1	16,3	32,8	5,2	4,8	9,7	Up
46	5,7	36,8	35,8	-6,5	6,7	13,3	Down
47	7,6	49,5	47,2	-6,5	6,8	13,3	Down
48	16,0	65,4	53,7	-4,1	7,0	13,2	Down
49	45,4	176,8	73,2	-3,9	9,6	13,7	Down
50	13,2	63,8	16,2	-4,8	9,6	12,6	Down
51	30,8	74,4	27,6	-2,4	9,6	11,8	Down
52	-	40,700	-	-	4,5	31,9	Absent

[a] Normalized protein expression levels determined by the ImageMaster® analysis software version 4.0

[b] Fold change in protein expression calculated by the ImageMaster® analysis software, positive numbers refer to proteins which are up-regulated, negative numbers refer to proteins which are down-regulated via the *P. putida* IsoF QS regulatory circuit.

[c] Isoelectric point and molecular mass obtained in the experiment.

6.11 Publications list

Arevalo-Ferro, C., Hentzer, M., Reil, G., Angelika, Görg., Kjelleberg, S., Givskov, M., Riedel, K., Eberl, L. 2003. Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. *Env. Microbiol.* **5**:1350–1369

(major experimental part, analysis of data and writing of the document together with Dr. K. Riedel and Prof. Dr. L. Eberl).

Arevalo-Ferro, C., Buschmann, J., Reil, G., Görg, A., Wiehlmann, L., Tümmler, B., Eberl, L. Riedel, K. 2004. Proteome analysis of intraclonal diversity of two *Pseudomonas aeruginosa* TB clone isolates. *Proteomics*. **5**. Online

(experimental part, analysis of data together with J. Buschmann, writing of the document together with J. Buschmann, Dr. K. Riedel and Prof. Dr. L. Eberl).

Riedel, K., Arevalo-Ferro, C., Reil, G., Görg, A., Lottspeich, F., Eberl, L. 2003. Analysis of the quorum-sensing regulon of the opportunistic pathogen *Burkholderia cepacia* H111 by proteomics. *Electrophoresis*. **24**:740–750

(surface-bound extraction protocol, part of experiments related with surface proteins fraction, partially writing of the document together with Dr. K. Riedel and Prof. Dr. L. Eberl).

Acknowledgements

This work was performed at the Institute of Microbiology of Technische Universität München from July 2001 to June 2004.

I am very grateful to Prof. Dr. K.-H. Schleifer for giving me the opportunity to pursue my doctoral thesis in his institute, for the excellent working conditions provided, and especially for being my doctor father.
I would like to sincerely thank Dr. Kathrin Riedel for the valuable supervision, guidance and support she gave me during this time, for giving critical observations since the beginning of the work, for letting me be her first PhD student and taking care of my long first days in Germany. (I would have never died hungry)
I thank also Prof. Dr. Leo Eberl who accepted me in his research group, for his collaboration and interest in my work, his optimistic visits to our lab and his good ideas for the writing of the papers.
I greatly acknowledge Prof. Dr. A. Görg for the cooperation in which we were able to identify our proteins and to learn so much.
I also acknowledge Dr. G. Reil for the protein identification, his motivation and enormous patience.
My special thanks to: Beate Schumacher for her friendly and extraordinary help.
Dorle Bergert whose smile and assistance are both important in the lab. Frau Leeb for her support with all my EXTRA “bureaucratique” documents and trying hard to make me speak German.
Herr Josef Reischenbeck for his help building up all kind of desired glass tubes and “Stopfen” for the biofilm war, Helga Widhopf and Elisabeth Maier who made desperate efforts to get German words out of me.
I would also like to thank all my colleagues and friends that helped me to cope more easily with the distance:
Birgit Huber for her sincere friendship (una amistad transparente como el agua), her frankness and more than I can say in these two lines. Also for reading this document word by word and willing to make corrections of all kind of things.
Manuela Köthe for her incredible black humor that I really enjoyed and Anette Steidle for teaching me the “Bohnen spiel” and to join with the “Putida” interest.
Julia Buschmann for her friendship, the nice work together, the nice results we got, for helping me with my Deutsch Hausaufgaben and keep helping until the last page of this work.

Nydia "y" Arnoud for all the afternoons I spent in their place of safety, to Christian Zeit for providing buen cafecito any time. A Micaela Grimoldi por una amistad que sólo los niños puden dar… Y a sus padres también.
The so called Isos, Thomas Behr and his very precise answers, Marko Pavlekovic for taking so much care of me (the "big, good and old" Marko), Katie Fichtl for "the sunshine and the Irishfolk concerts", Katrin Zwirglmaier for trying so hard to learn Spanish and for conspiring in English, André Mehlen for the good atmosphere around him and his charming blue eyes, Giulio Petroni for staying as long as he could in the never ending nights while I was writing this document, Johannes Fried (Giovanni) for the good pasta, the wine and the long skating ways, Barbara Wunner-Füßl and Sibylle Schadhauser. To Johannes and Chrissie for nice dinners and talks.
The "Diplomanden and Ex-Diplomanden"of the Eberl´s group, Christopher Bruns for taking care of Kermit and Piggy, Melanie Kinzner, Christian Winderl, Julia Wopperer, Rike Feldman her party mood and sweet ways, especially to Robert Bauer for the pleasant talks and his gorgeous British ways.
To the faraway Wagner's especially Holger Daims for giving me his hand to make the small step over the ocean. The students (GPs) and HiWis that made the life so easy and such good parties, to Elena and her peacefully smile, to Gloria Lutzny and Andreas Bauer, to Martina, Isabel, Gigi and Andy for all the work they made and more, and to Angie for standing with me next to the fight against the biofilm growths and the biofilm harvesting,
and, the most important, to Diego for holding on all this way and all this distance and helping me so long and so much.
Finally, to my parents for all the love and support they have given.

Cell-cell communication
group