Global virulence regulators of *Pseudomonas aeruginosa*

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Abstract

The main objective of the presented work was to integrate four putative regulatory virulence genes (*vqsR, gltR, 47D7, icsF*) of *Pseudomonas aeruginosa* into regulatory circuits and pathways using combined approach of functional genomics (microarrays), genetics *in silico* and various bioassays.

vqsR (virulence and quorum sensing regulator) encodes one of the major regulators of the cell-to-cell communication in *P. aeruginosa*. Inactivation of *vqsR* abrogated the production of the autoinducer molecules, which are known to be involved in the initiation of *P. aeruginosa* quorum sensing cascade. GeneChip experiments revealed downregulation of the whole battery of quorum sensing genes in the *vqsR* mutant and correspondingly the mutant was compromised in phenotypic traits that are under quorum sensing control. According to genome-wide transcriptional analyses, VqsR is also implicated in the modulation of the broad spectrum of virulence, iron-uptake and metabolic genes.

icsF (intracellular survival factor) encodes the key modulator of the expression of oxidative stress genes in *P. aeruginosa*. As oxidative stress response genes represent an effective tool to combat the harsh conditions in the intracellular compartments, the disruption of *icsF* inevitably has a negative effect on the intracellular survival of *P. aeruginosa* strain TB in polymorphonuclear neutrophils (PMNs). Besides the intracellular survival, the disruption of *icsF* also affects cell-to-cell communication and overall virulence of *P. aeruginosa* against *Caenorhabditis elegans*.

The preliminary experiments suggested an important role of *gltR* and *47D7* in *P. aeruginosa* virulence; however, complementation of these genes *in trans* did not restore the phenotype of the wild type strain, thus revealing that the observed phenotypes of the respective mutants are caused by a secondary genetic effect elsewhere in the genome.

Key-words: *Pseudomonas aeruginosa*, Quorum sensing, Intracellular survival

Kurzfassung

Primäres Ziel der vorliegenden Arbeit war, vier putativ regulatorische Gene (*vqsR, gltR, 47D7* und *icsF*) aus dem Genom von *Pseudomonas aeruginosa* genauer zu charakterisieren und in regulatorische Systeme einzuordnen. Angewandt wurde dazu eine Kombination aus funktioneller Genomanalyse (*microarrays*), *in silico* Genetik und verschiedene Bioassays.

vqsR (*virulence and quorum sensing regulator*) kodiert für einen der wichtigsten Regulatoren der Zell-Zell-Kommunikation *in P. aeruginosa*. Durch Inaktivierung dieses Gens wurde die Produktion der sog. *autoinducer* ausgeschaltet, die an der Initiierung der *quorum sensing* Kaskade in *P. aeruginosa* beteiligt sind. In *microarray* Experimenten wurde gezeigt, dass die Expression von *quorum sensing* Genen in einer *vqsR*-Mutante deutlich vermindert war. Dementsprechend fehlten der vqsR-Mutante auch charakteristische phänotypische Eigenschaften, die im Wildtyp-Stamm durch *quorum sensing* Systeme gesteuert werden. Nach den Ergebnissen einer genomweiten Transkriptionsanalyse ist das *vqsR*-Genprodukt zudem in die Modulation eines breiten Spektrums an Virulenzgenen, Eisen-Aufnahme und Stoffwechselgenen involviert.

icsF (*intracellular survival factor*) kodiert für einen Protein, das eine Schlüsselstellung in der Modulation der Expression von Genen bei oxidativem Stress einnimmt. Da Proteine, mit denen die Zelle oxidativem Stress begegnen kann, wirksame Werkzeuge zur Anpassung an die Umweltbedingungen in intrazellulären Kompartimenten von Eukaryonten darstellen, vermindert das Ausschalten von *icsF* erheblich die Fähigkeit des *P. aeruginosa* Stammes TB, in polymorphonuklearen neutrophilen Granulozyten (PMNs) zu überleben. Zusätzlich beeinträchtigt das Ausschalten von *icsF* auch die Zell-Zell-Kommunikation von *P. aeruginosa* und die generelle Virulenz gegenüber *Caenorhabditis elegans*.

Mutanten mit ausgeschaltetem *gltR*- bzw. *47D7*-Gen wiesen ebenfalls deutlich veränderte phänotypische Eigenschaften auf. Durch Komplementierung dieser Gene *in trans* wurde aber nicht der Phänotyp des Wildtyp-Stammes wiederhergestellt. Die Phänotypen der Mutanten waren also auf Sekundärmutationen an anderen Positionen im Genom zurückzuführen.

Schlüsselwörter: *Pseudomonas aeruginosa*, Quorum sensing, intrazelluläres Überleben

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1. Introduction

1.1. *Pseudomonas aeruginosa*

1.1.1. General information

Pseudomonas aeruginosa is one of the best-known members of the genus *Pseudomonas*, which comprises a group of Gram-negative polarly flagellated rods (Stanier *et al.,* 1966). In contrast to first attempts for taxonomy of pseudomonads, which were based on morphological properties of individual members, the current taxonomy of this genus is based on 16S rRNA sequences (Woese Fox, 1977). The most intensively studied members of genus *Pseudomonas* as for instance *P. putida*, *P. fluorescens*, *P. syringae* as well as *P. aeruginosa* belong to the group of γ-proteobacteria (Olsen *et al.*, 1994).

P. aeruginosa has a length in the range of 1.5-3 μm and a diameter of about 0.5-0.8 μm (Palleroni, 1986). One of the most noteworthy properties of this common aquatic organism is its ability to adapt and thrive in many ecological niches, from water and soil to plant and animal tissues (Hardalo and Edberg, 1997). In humans, this organism can colonize virtually any mucosal surface and can invade tissues and blood (Kulasekara and Lory, 2004). The exceptional competence of *P. aeruginosa* to colonize a wide variety of ecological niches is based on the ability of this bacterium to utilize a broad spectrum of organic compounds as food sources and its capability to survive for a long time under extremely harsh conditions where nutrients are limited.
The emergence of *P. aeruginosa* as a major opportunistic human pathogen during the past century may be a consequence of its resistance to the antibiotics and disinfectants that eliminate other environmental bacteria. *P. aeruginosa* is now a significant source of bacteraemia in burn victims, urinary-tract infections in catheterized patients, and hospital-acquired pneumonia in patients on respirators (Bodey *et al.*, 1983). It is also the predominant cause of morbidity and mortality in cystic fibrosis (CF) patients, whose abnormal airway epithelia allow long-term colonization of the lungs by *P. aeruginosa*. These infections are impossible to eradicate, in part because of the natural resistance of the bacterium to antibiotics, and ultimately lead to pulmonary failure and death (Stover *et al.*, 2000).

1.1.2. Genome organisation of *P. aeruginosa*

The ability of *P. aeruginosa* to thrive in the broad range of ecological niches was expected to be matched by a complex, highly regulated genomic repertoire. Not surprisingly, early estimates of the genomic size suggested a relatively large genome exceeding 6 million base pairs (Mb) (Schmidt *et al.,* 1996).

Few years ago, the complete genome of *P. aeruginosa* strain PAO1 was sequenced and published by Stover *et al.,* 2000 (http://www.pseudomonas.com), which confirmed the previous hypotheses about the large genome of this bacterium. At 6.3 Mb, the *P. aeruginosa* genome is markedly larger than most of the sequenced bacterial genomes. In fact, with 5,570 predicted open reading frames (ORFs), the genetic complexity of *P. aeruginosa* resembles that of the simple eukaryote *Saccharomyces cerevisiae*, whose genome encodes about 6,200 proteins (Ball *et al.*, 2000). In contrast, *P. aeruginosa* has only 30−40 % of the number of predicted genes present in the simple metazoans *Caenorhabditis elegans* and *Drosophila melanogaster* (Ewing and Green, 2000). The completion of the sequencing project of the *P. aeruginosa* PAO1 genome in 2000 and the results from the second *P. aeruginosa* genome sequencing of the strain PA14 clearly demonstrate that the ecological diversity is indeed reflected in the gene content (Kulasekara and Lory, 2004).

Analysis of the complete genome sequence of *P. aeruginosa* revealed that this bacterium possesses numerous genes for transport, metabolism and growth on organic substrates, numerous iron-uptake systems as well as genes implicated in the enhanced ability to export various compounds (for instance enzymes and antibiotics) and four potential chemotaxis systems (Stover *et al.*, 2000). Consistent with its large genome and environmental adaptability, *P. aeruginosa* possesses one of the highest proportion of regulatory genes observed for a bacterial genome, which presumably modulate the diverse genetic and biochemical capabilities of this bacterium in changing environmental conditions. The number and variety of genes that allow *P. aeruginosa* to thrive in a wide range of environments places this organism as being truly ubiquitous, arguably one of the most developed bacterial species in terms of range of habitats it can occupy (Kulasekara and Lory, 2004).

1.1.3. *P. aeruginosa* and cystic fibrosis

P. aeruginosa is an opportunistic pathogen, which causes infections only in certain individuals with impaired host defenses as for instance in patients undergoing immunosuppressive therapies (cancer treatment), patients suffering from human immunodeficiency virus infections, patients with an extensive damage of primary barriers (burn wounds), and those with cystic fibrosis (CF) (Govan and Deretic, 1996). CF is an autosomal recessive disorder caused by mutations in a single gene on the long arm of chromosome 7 that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan *et al.,* 1989). After identification of the CF gene in 1989, the next years were associated with rapid expansion of knowledge regarding the structure and function of the CF gene product and the molecular mechanisms underlying the various phenotypic manifestations of the disease. CFTR protein was shown to interfere with chloride ion transport in CF patients thus resulting in the decreased secretion of mucins (Bear *et al.,* 1992; Tümmler and Kiewitz, 1999; Knowles and Boucher, 2002). This genetic defect leads to a number of medical problems and complications for the patients with CF, including infections of the lung with a broad spectrum of pathogens.

Chronic airway infection and the accompanying inflammatory response are clearly the major clinical problems for CF patients today. CF has a unique set of bacterial pathogens, including *Staphylococcus aureus, P. aeruginosa, Burkholderia cepacia, Haemophilus influenzae, Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*, that are frequently acquired in an age-dependent sequence (Gibson *et al.,* 2003).

P. aeruginosa is by far the most significant pathogen in CF. *P. aeruginosa* isolates from the lungs of CF patients are quite different from those causing infections in other settings. Their typical characteristics are not present in isolates causing initial infections but appear to be selected within CF airways. Whereas early isolates appear much like environmental isolates, later isolates are more resistant to antibiotics and are frequently mucoid (Burns *et al.,* 2001). Additional phenotypic changes in CF isolates of *P. aeruginosa* include the loss of flagella dependent motility (Luzar and Montie, 1985) and increased auxotrophy and tendency to form biofilms (Singh *et al.,* 2000; Thomas *et al.,* 2000). It has recently been reported that antibiotic-resistant phenotype variants of *P. aeruginosa* with an enhanced ability to

form biofilms arise at high frequency in the lungs of patients with CF (Drenkard and Ausubel, 2002).

A large genome of *P. aeruginosa* offers a potential for a tremendous ability of this bacterium to adapt to a wide variety of different environments, including the CF airway. *P. aeruginosa* isolated from CF sputa have even larger genomes than the laboratory strain, PAO1, suggesting that they have acquired new genes during their adaptation, in addition to alterations in those already present (Spencer *et al.,* 2003). A high frequency of hypermutability has been identified in *P. aeruginosa* isolates from patients with CF. This is likely caused by the milieu of the CF airway with large numbers of infecting organisms and compartmentalization of infection, combined with ineffective host defenses and ongoing antibiotic selective pressure (Oliver *et al.,* 2000, Gibson *et al.,* 2003).

1.2. Pathogenic lifestyle of *P. aeruginosa*

Just as varied as the clinical diseases caused by *P. aeruginosa*, this typical nosocomial pathogen possesses a large variety of both cell-associated and extracellular virulence factors. It is important to realize that the pathogenesis of *P. aeruginosa* is not related to a single virulence factor, but to the precise and delicate interplay between different factors, leading from efficient colonization and biofilm formation, to tissue necrosis, invasion and dissemination through the vascular system, as well as activation of both local and systemic inflammatory responses (Van Delden, 2004).

1.2.1. Cell-associated virulence factors

The first step in *P. aeruginosa* infections is the adherence to and colonization of host epithelial surfaces. The primary *P. aeruginosa* adhesins for respiratory mucins are the flagella cap protein (Arora *et al.,* 1998) and flagellin (Feldman *et al.*, 1998; Lillehoj *et al.,* 2002). Flagellum driven motility has been reported to enhance the efficiency of surface colonization by *P. aeruginosa* (O´Toole and Kolter, 1998a) as well as *P. fluorescens* (O´Toole and Kolter, 1998b). However, many *P. aeruginosa* strains isolated from chronically infected CF airways do not produce flagella, thus indicating that flagella plays a role only in the early step of infection and longer term maintenance of the organism in this milieu involves other factors (Mahenthiralingam *et al.,* 1994). The other factors implicated in the adherence of *P. aeruginosa* to host epithelium include type IV pili, covered by galactose-binding or mannose-binding lectins, which account for 90 % of the adherence capacity (Ramphal *et al.,* 1984; de Bentzmann *et al.*, 1996; Hahn, 1997), fimbrias (Vallet *et al.,* 2001) the outer membrane porin OprF (Azghani *et al.,* 2002), lipopolysaccharide and alginate (Gilboa-Garber, 1996; D´Argenio, 2004). The ability of *P. aeruginosa* to attach to abiotic surfaces, to host tissues, or to each other, and the subsequent differentiation of the microorganisms into biofilm, can be considered a major virulence trait in a variety of infections (Watnik and Kolter, 2000).

1.2.2. Extracellular virulence factors

P. aeruginosa produces several extracellular products that after the initial colonization can cause extensive tissue damage, bloodstream invasion, and dissemination. In vivo studies have shown that *P. aeruginosa* mutants defective in the production of individual factors are less pathogenic*;* however, the relative contribution of a given factor may vary with the type of infection (Nicas and Iglewski, 1985).

Alkaline protease is an important extracellular virulence factor implicated in the corneal infections (Howe and Iglewski, 1984), degradation of the components of the complement (Hong and Ghebrehiwet, 1992) and hydrolysis of fibrin and fibrinogen (Shibuya *et al.,* 1991).
Exotoxin A catalyses the ADP-ribosylation and inactivation of elongation factor 2, leading to inhibition of protein biosynthesis and cell death (Wick *et al.,* 1990). Purified exotoxin A is lethal for laboratory animals, thus confirming its role as a major virulence factor of *P. aeruginosa* (Woods and Iglewski, 1983).
LasA and LasB elastases are responsible for destruction of protein elastin, which accounts for a significant part of human lung tissue and is an important component of blood vessels (Galloway, 1991). Both elastases, LasB and LasA, have been found and their transcription has been demonstrated *in vivo* in the sputum of CF patients (Storey *et al.,* 1992, Jaffar-Bandjee *et al.,* 1995). LasB elastase is implicated not only in the degradation of elastin and another tissue components (Heck *et al.,* 1986), but it also interferes with host defense mechanisms (Van Delden, 2004).
Phospholipase C, which represents one of the two *P. aeruginosa* hemolysins, has been shown to induce vascular permeability, organ damage and death in animal models as well as to contribute to the release of inflammatory mediators (Berk *et al.,* 1987; Konig *et al.,* 1996). Rhamnolipid, which is the second *P. aeruginosa* hemolysin is a rhamnose-containing glycolipid biosurfactant with detergent-like structure believed to be responsible for dissolving of the lung surfactant phospholipids, making them more accessible to cleavage by phospholipase C (Liu, 1974). It is hypothesized that both hemolysins, phospholipase C and rhamnolipid, may act synergistically to breakdown lipids and lecithin and both contribute to tissue invasion by their cytotoxic effects (Van Delden, 2004).

Protease IV is a serine protease implicated in the degradation of complement components as well as fibrinogen, plasmin and plasminogen (Engel *et al.,* 1998a) and plays an important role during corneal infections (Engel *et al.,* 1997; Engel *et al.,* 1998b).

P. aeruginosa produces two lipases, LipA and LipC, which were shown to enhance the induction of inflammatory mediators by phospholipase C (Konig *et al.,* 1996). It has therefore been hypothesized that the simultaneous production of phospholipase C and lipases might be dangerous to the host by inducing significant inflammation (Konig *et al.,* 1996; Van Delden, 2004).

Another exoproteins, including exoenzyme S, exoenzyme T, exoenzyme Y and exotoxin U require close contact with host cells and are secreted via the type III secretion pathway, using a complex secretion and translocation machinery to inject the effector proteins directly into the cytoplasm of target cells (Frank, 1997; Yahr *et al.,* 1997). Exoenzyme S, which is produced by about 40 % of clinical isolates (Sokurenko *et al.,* 2001), does not contribute to initial colonization but it has been postulated that it is responsible for tissue destruction and for bacterial dissemination (Nicas *et al.,* 1985a; Nicas *et al.,* 1985b). Exoenzyme S is a bifunctional cytotoxin, which disrupts actin filaments in eukaryotic cells with its amino terminus, whereas its carboxyl terminus comprises an ADP-ribosyltransferase domain (Coburn *et al.,* 1989). Exoenzyme T together with exoenzyme S plays a role in the ribosylation of ADP (Nicas and Iglewski, 1984; Barbieri, 2000), thus inhibiting wound healing, exocytosis and cell cycle progression (Krall *et al.,* 2000). The role of exoenzyme Y in the pathogenesis of *P. aeruginosa* infections remains an enigma. Exotoxin U is expressed in most isolates from corneal infections, in 40 % of isolates from acute respiratory tract infections in non-CF and 10 % of CF isolates (Finck-Barbancon *et al.,* 1998; Hauser *et al.,* 1998; Dacheux *et al.,* 2000). The secretion of ExoU clearly results in cytotoxicity in mammalian cells and current research suggests that it may have a phospholipase activity (Rabin and Hauser, 2003), thus contributing to degradation of eukaryotic cell membranes and subsequent cell death (Sato *et al.,* 2003).

Pyocyanin is a blue redox active phenazine pigment that generates reactive oxygen species, thus exposing host cells to oxidative stress (Britigan *et al.,* 1999; Muller, 2002). It induces apoptosis and inhibits generation of superoxide by neutrophils and inhibits proliferation of lymphocytes (Usher *et al.,* 2002).

Two *P. aeruginosa* siderophores, pyoverdine and pyochelin play a role in the uptake of iron and regulation of virulence factors and were shown to be therefore required for full expression of virulence of *P. aeruginosa* in animal models (Takase *et al.,* 2000; Lamont *et al.,* 2002).

Hydrogen cyanide is a poison involved in the blockade of cytochrome oxidase and subsequent inhibition of mitochondrial respiration and was shown to be responsible for rapid paralytic killing of the nematodes *C. elegans*.

Exopolysaccharides and alginate play an essential role in the formation of biofilms. Alginate produced *P. aeruginosa* strains were proven to be more resistant to antibiotics and disinfectants (Govan and Deretic, 1996).

Due to its major role in the control of extracellular virulence factor production, the quorum sensing circuit of *P. aeruginosa* could be also considered a virulence determinant (Van Delden, 2004).

1.3. Quorum sensing: the power of cooperation in the world of *Pseudomonas*

For many years, researchers thought of bacteria as individual cells designed to proliferate under various conditions but unable to interact with each other and to collectively respond to environmental stimuli, as it is typical for multicellular organisms. This view began to change few decades ago with the discovery of the cooperative regulation of luminescence in the Gram-negative marine bacterium *Vibrio fischeri* (Nealson *et al.*, 1970) and regulation of the genetic competence in the Gram-positive bacterium *Streptococcus pneumoniae* (Tomasz, 1965). These bacteria were shown to coordinate their behaviour via the secretion of specific signalling molecules in a population density-dependent manner. During growth the bacteria secrete these molecules, which accumulate in the surrounding environment as the population density increases until a critical threshold concentration is reached, which then triggers expression of certain sets of genes (Figure 1.1). This type of cell-to-cell communication was termed “quorum sensing” in order to emphasize the fact that a sufficient number of bacteria, the bacterial “quorum”, is needed to induce or repress expression of target genes (Fuqua *et al.*, 1994). The signalling molecules utilized by quorum sensing systems are often acylated homoserine lactones (AHL) in the case of Gram-negative bacteria (Figure 1.1), small peptides in the case of Gram-positive bacteria or autoinducer-2 (AI-2), which has been found in both Gram-negative as well as Gram-positive bacteria and therefore considered to be a universal bacterial language, the “bacterial esperanto” (Winans, 2002).

Since the discovery of population density dependent regulation of bioluminescence in marine bacteria, cell-to-cell communication has been proven to play an important role in the life of various bacterial species. Work of the past few years provided evidence that bacteria not only form well-organised communities but also exchange information with the other members of the community in order to coordinate their activities. This allows a group of bacteria to launch a unified, coordinated response to environmental stimuli and to accomplish tasks which would be difficult, if not impossible, to achieve for a single bacterial cell. Furthermore, evidence has accumulated that some bacterial signal molecules are used not only as population density sensors in one species but also for communication between bacteria of

different species or genera occupying the same ecological niche and even to interact with their eukaryotic hosts. Beside bioluminescence and genetic competence, quorum sensing is also involved in the regulation of a wide variety of different physiological processes including antibiotic biosynthesis, swarming, swimming and twitching motility, plasmid conjugal transfer, biofilm development or the production of bacterial virulence factors in plant, animal or human pathogens (for recent reviews see Miller and Bassler, 2001; Whitehead *et al.*, 2001; Camara *et al.,* 2002; Fuqua and Greenberg, 2002; Lazdunski *et al.,* 2004; Pappas *et al.,* 2004).

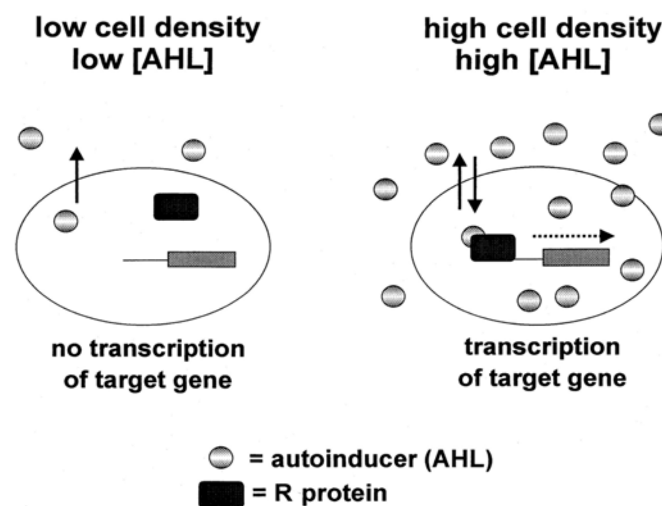


Fig. 1.1. The quorum sensing in Gram-negative bacteria. Quorum sensing in Gram-negative bacteria involves two regulatory components: the transcriptional activator protein (R protein) and the autoinducer molecule produced by autoinducer synthase. Accumulation of autoinducer occurs in a population density dependent manner until a threshold concentration is reached. Subsequently the autoinducer binds to and activates the R protein, which in turn induces or represses the expression of target genes (de Kievit and Iglewski, 2000).

One of the most extensively studied AHL-dependent cell-to-cell communication system is the one of the opportunistic human pathogen *Pseudomonas aeruginosa*. This bacterium, well known for its resistance against a variety of antibiotics, causes serious infections in immunocompromised patients or individuals with cystic fibrosis and is one leading source of nosocomial infections (Tümmler *et al.*, 1991). Work of the past few years showed that the quorum sensing circuitry that is operating in *P. aeruginosa* is essential for the expression of virulence factors as well as for biofilm

formation (Table 1.1). It has been speculated that quorum sensing dependent regulation of pathogenic traits is a highly effective strategy to establish an infection, as it gives the pathogen enough time to grow and inhabit its niche without displaying itself to the immune system of the host. Virulence factors are expressed in concert only when sufficient number of invading bacteria has accumulated, thus minimizing the threat of eradication by host immune system and increasing greatly the chance for successful infection (de Kievit and Iglewski, 2000; Greenberg, 2003).

Table 1.1. Functions controlled by the quorum sensing circuitry in *P. aeruginosa*.

Phenotypic traits under the control of quorum sensing in *P. aeruginosa*
Acyl homoserine lactone synthesis
Adhesins (lectins) biosynthesis
Biofilm formation
Exotoxin A biosynthesis
Hydrogen cyanide synthesis
Neuraminidase
Synthesis of the oxidative-stress responsive enzymes (catalase, superoxide dismutase)
Protease biosynthesis
Proteins of the type III secretion system
Pyocyanin synthesis
PQS synthesis
Rhamnolipid synthesis
Swarming motility

1.3.1. Paradigm of quorum sensing: bioluminescence in *V. fischeri*

Quorum sensing was first described approximately 35 years ago in the early 1970s in the marine luminescent bacterium *V. fischeri* (Nealson *et al.*, 1970). This bacterium lives in symbiosis with certain marine fish and squid species and provides them with light in exchange for nutrients (Graf and Ruby, 1998). The best-characterized model system of such a symbiotic relationship is the one between *V. fischeri* and the squid *Euprymna scolopes* (Ruby, 1996). *E. scolopes* posses a specialized light organ,

which is colonized from the early stages of development by *V. fischeri* (Montgomery and McFall-Ngai, 1994). *V. fischeri* can be found at low population densities as a free-living organism in seawater (10^2 cells ml^{-1}). However, only when the bacteria are living within the light organ of the squid, where the population density is very high (10^{10} – 10^{11} cells ml^{-1}) the cells emit detectable amounts of light (Boettcher and Ruby, 1995) (Figure 1.2). It was shown that during growth, *V. fischeri* synthesizes an extracellular factor, which was originally named autoinducer because expression of bioluminescence was found to be positively feedback-regulated, and whose structure was later identified as *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C_6-HSL). This molecule, which is synthesized from S-adenosylmethionine (SAM) and carrier protein (acyl-ACP) via LuxI, was shown to freely diffuse through the bacterial cell membrane (Eberhard *et al.,* 1981; Kaplan and Greenberg, 1985; Moré *et al.,* 1996). After reaching a critical threshold concentration, molecules of the autoinducer bind to their cognate receptor protein, LuxR, which in turn activates the expression of genes responsible for the luminous phenotype (the *lux* regulon) (Figure 1.2).

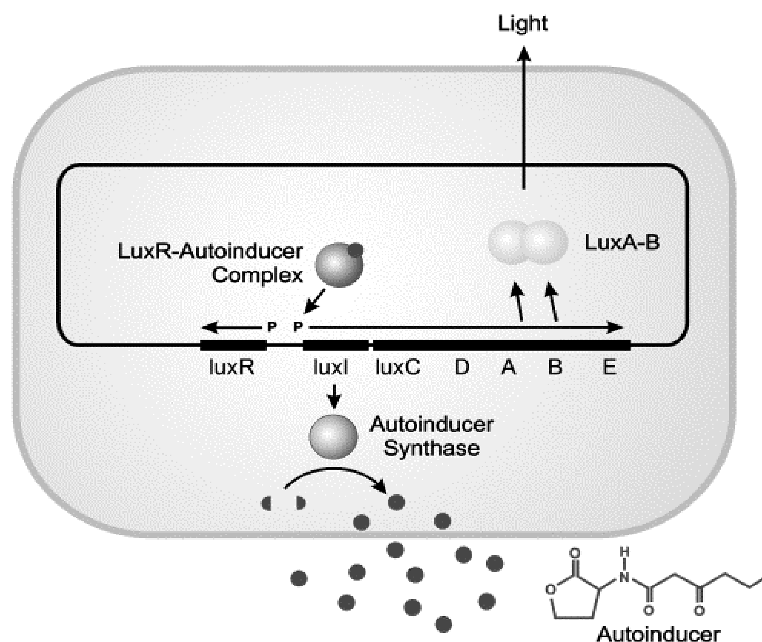


Fig. 1.2. Quorum sensing paradigm: The LuxR-LuxI quorum sensing system of *V. fischeri*. Autoinducer synthase, encoded by *luxI*, synthesizes *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C_6-HSL), which diffuses across the cell envelope and accumulates intracellularly only at high population density. After reaching a critical threshold concentration, it binds to the LuxR receptor protein and LuxR-3-oxo-C_6-HSL complex in turn activates transcription of the *luxICDABE* operon which is responsible for bioluminescence (Pappas *et al.,* 2004).

The *lux* regulon comprises eight genes, organised into two divergently transcribed transcription units, which are separated by an intergenic regulatory region (Engebrecht *et al.,* 1983; Engebrecht and Silverman, 1984). The leftward transcription unit consists of *luxR*, which encodes the autoinducer-binding LuxR. The binding of 3-oxo-C_6-HSL to LuxR results in a complex that activates transcription of the rightward *lux* operon. This operon contains the *luxI* gene, encoding the 3-oxo-C_6-HSL synthase (LuxI), followed by five genes crucial for light production (*luxCDABE*). The LuxR protein is modular in structure with the C-terminal domain necessary for DNA binding and the N-terminal region required for autoinducer binding. It is believed that binding of 3-oxo-C_6-HSL to the N-terminal domain of the LuxR induces conformational changes, which then allow the C-terminal region of the protein to bind to a 20 bp inverted repeat sequence, designated the *lux* box, in the *lux* intergenic region, which in turn activates the expression of the *luxICDABE* operon (Stevens *et al.,* 1994; England and Greenberg, 1999; Fuqua *et al.,* 2001). Due to the fact that LuxI itself is encoded by this operon a positive-feedback regulatory loop is created once the quorum sensing is triggered.

1.3.2. Hierarchical control of quorum sensing in *P. aeruginosa*

AHL-dependent cell-to-cell communication systems similar to the one of *V. fischeri* (Figure 1.2) have been identified recently in various bacterial species, including *P. aeruginosa*. The analysis of the fully sequenced *P. aeruginosa* genome revealed that more than 9% of the assigned open reading frames (ORFs) encode known or putative transcriptional regulators and two-component systems (Stover *et al.,* 2000). It is hypothesized that such a large amount of regulators gives this bacterium an opportunity to adapt to a wide variety of different environments and thus represents a key feature for the understanding of its enormous metabolic versatility.

When investigating the regulation of quorum sensing in *P. aeruginosa*, two pairs of *V. fischeri* LuxR and LuxI homologues have been identified, which are the major components of two quorum sensing systems operating in this organism: the *las* system, which consists of the transcriptional activator LasR and the AHL synthase LasI, which directs the synthesis of *N*-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C_{12}-HSL) and the *rhl* system, which consists of the transcriptional regulator RhlR and

the AHL synthase RhlI (Figure 1.3), which directs the synthesis of *N*-butanoyl-homoserine lactone (C_4-HSL) (Passador *et al.,* 1993; Latifi *et al.,* 1995; Pearson *et al.,* 1995).

While *P. aeruginosa* cells are freely permeable to C_4-HSL, active transport via the MexAB-OprM multidrug efflux pump is involved in the secretion of 3-oxo-C_{12}-HSL (Pearson *et al.,* 1999). The *las* system was shown to be involved in the regulation of various virulence factors, as well as *lasI* itself, thereby creating a positive regulatory feedback loop. Likewise, the rhl system affects the expression of a broad spectrum of genes. Some of the quorum sensing regulated genes are under control of both quorum sensing systems, and others are regulated specifically by either the *las* or the *rhl* system. However, the two systems do not operate independently as the LasR-3-oxo-C_{12}-HSL complex is also positively regulating transcription of RhlR and RhlI (Latifi *et al.,* 1996; Pesci *et al.,* 1997). A recent study on the transcriptional regulation of *rhlR* revealed that expression of this gene is not only dependent on LasR but also on Vfr (see below) and RhlR itself, indicating that the gene is subject to negative autoregulation (Medina *et al.*, 2003a). Furthermore, RhlR was shown to form a homodimer that can be dissociated into monomers by 3-oxo-C_{12}-HSL (Ventre *et al.,* 2003). In conclusion, these data provide strong evidence that the quorum sensing systems in *P. aeruginosa* are hierarchically arranged with the *las* system being on top of the signalling cascade.

In a recent study the transcriptional regulation of the promoter region of the *rhlAB* operon, which encodes the enzyme rhamnosyltransferase 1, an enzyme involved in the biosynthesis of the surfactant rhamnolipid, was studied in detail (Medina *et al.,* 2003b). This study showed that RhlR binds to specific sequences upstream of *rhlAB* independently of the presence or absence of C_4-HSL. However, in the former case transcription is activated, whereas in the latter case RhlR represses transcription. Such dual activator-repressor activities of LuxR-type regulators appear to be important for fine tuning of quorum sensing in *P. aeruginosa*.

1.3.3. Additional layers of quorum sensing regulation

The *P. aeruginosa* quinolone signal 2-heptyl-3-hydroxy-4-quinolone (PQS), produced maximally during the late stationary phase of growth adds a further level of complexity to the quorum sensing network, as it provides a link between the *las* and

rhl systems by modulating expression of *rhlRI* and *lasRI* (McKnight *et al.,* 2000). The direct precursor of PQS, 4-hydroxy-2-heptylquinoline (HHQ), is first released from and then taken up again by the cells, before it is eventually converted into PQS by the action of PqsH, whose expression is controlled by the *las* system (Gallagher *et al.,* 2002; Déziel *et al.,* 2004). Recent work has shown that the PQS signal molecule is able to overcome the cell-density-dependency of quorum sensing and can also be produced in the absence of LasR (Diggle *et al.,* 2003). The transcription of genes required for PQS synthesis is not only positively regulated by the *las* quorum sensing system but also under negative control of the *rhl* system. As a consequence, PQS production is dependent on the ratio of 3-oxo-C_{12}-HSL and C_4-HSL, suggesting a delicate balance between the two quorum sensing systems (McGrath *et al.,* 2004).
The quorum sensing cascade of *P. aeruginosa* is subject to regulation by a number of additional regulatory factors (Figure 1.3). One important factor is the two-component response regulator *gacA*, a highly conserved gene primarily responsible for the control of virulence factors and antibiotics production in fluorescent pseudomonads, but also shown to be involved in the regulation of C_4-HSL production in *P. aeruginosa*. A model has been proposed that places GacA upstream of LasR and RhlR (Reimmann *et al.,* 1997). Likewise, the global transcriptional regulator AlgR2 (AlgQ) was suggested to play a role in modulating quorum sensing in *P. aeruginosa*. Although originally implicated in alginate production, recent studies have shown that AlgR2 specifically binds to the LasR and RhlR promoter regions thereby downregulating the entire quorum sensing cascade (Ledgham *et al.,* 2003a). Furthermore, an important role in the quorum sensing circuitry was suggested for the RsaL repressor, as it competes with the LasR-3-oxo-C_{12}-HSL complex for the binding to the *lasI* promoter, thus negatively regulating *lasI* transcription (de Kievit *et al.,* 1999; Fagerlind *et al.,* 2004). Polyphosphate kinase, encoded by *ppk*, was shown to have a positive impact on 3-oxo-C_{12}-HSL and C_4-HSL synthesis and subsequently on the overall virulence of *P. aeruginosa* (Rashid *et al.,* 2000). Recent work identified a gene *mvaT*, which, when mutated, led to increased and premature expression of quorum sensing regulated genes, indicating that MvaT is required for the appropriate timing of expression of the quorum sensing regulon (Diggle *et al.,* 2002). Moreover, quorum sensing is regulated at the posttranscriptional level by the RsmA/RsmZ system (Pessi *et al.,* 2001; Heurlier *et al.,* 2004) as well as by DksA (Jude *et al.,* 2003). The quorum sensing signalling network of virulence is linked with the cAMP-

signalling network of virulence (Wolfgang *et al.,* 2003) via the transcriptional regulator Vfr (Albus *et al.,* 1997), a member of the cAMP receptor protein family. Vfr is important for the regulation of exotoxin A and protease, which is due in part to its influence on the expression of *lasR/lasI*. In addition, Vfr positively regulates the expression of over a hundred genes (Wolfgang *et al.,* 2003), including those encoding the type III secretion system that delivers toxic effector proteins directly into host cells. In *P. aeruginosa* cAMP is primarily synthesized by CyaB, a class III membrane-associated adenylate cyclase, which together with Vfr have been shown to act as a master regulator of virulence gene expression in *P. aeruginosa* (Wolfgang *et al.,* 2003; Smith *et al.,* 2004).
Bioinformatic analyses of the *P. aeruginosa* PAO1 genome revealed that, in addition to the two AHL signal receptors LasR and RhlR, the *P. aeruginosa* genome encodes additional LuxR homologue, termed QscR. The LuxR homologue, QscR, whose synthesis is regulated by GacA, has been shown to modulate the timing of quorum sensing gene expression by repressing transcription of *lasI* (Chugani *et al.,* 2001). In the absence of AHLs, QscR forms multimers interacting with itself and heterodimers with LasR or RhlR, thus offering an another possibility of fine tuning the quorum sensing circuitry in concert with other regulatory proteins (Lendgham *et al.,* 2003b). The alternative sigma factor RpoN was shown to negatively affect quorum sensing in *P. aeruginosa,* as mutation of *rpoN* was found to elevate levels of both signal molecules (Heurlier *et al.,* 2003; Thompson *et al.,* 2003). Furthermore, the stationary sigma factor RpoS was demonstrated to be involved in the modulation of expression of a large number of quorum sensing genes as well as the type III secretion system and anti-host effector proteins (Hogardt *et al.,* 2004; Schuster *et al.,* 2004). All the regulatory systems mentioned add additional levels of complexity to quorum sensing circuitry of *P. aeruginosa* (Figure 1.3).

According to genome-wide microarray-based transcriptome analyses, approximately 5 % of all *P. aeruginosa* genes are quorum sensing regulated. Comparing the expression profiles of wild type strains with those of respective QS mutants by the aid of DNA microarrays not only confirmed the quorum sensing dependent expression of many genes previously described as quorum sensing regulated but also allowed the identification of a large number of novel genes. Quorum sensing in *P. aeruginosa* was shown to modulate expression of a broad spectrum of extracellular proteins,

secondary metabolites, regulatory proteins as well as many proteins belonging to the class of conserved hypotheticals of unknown function (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003).

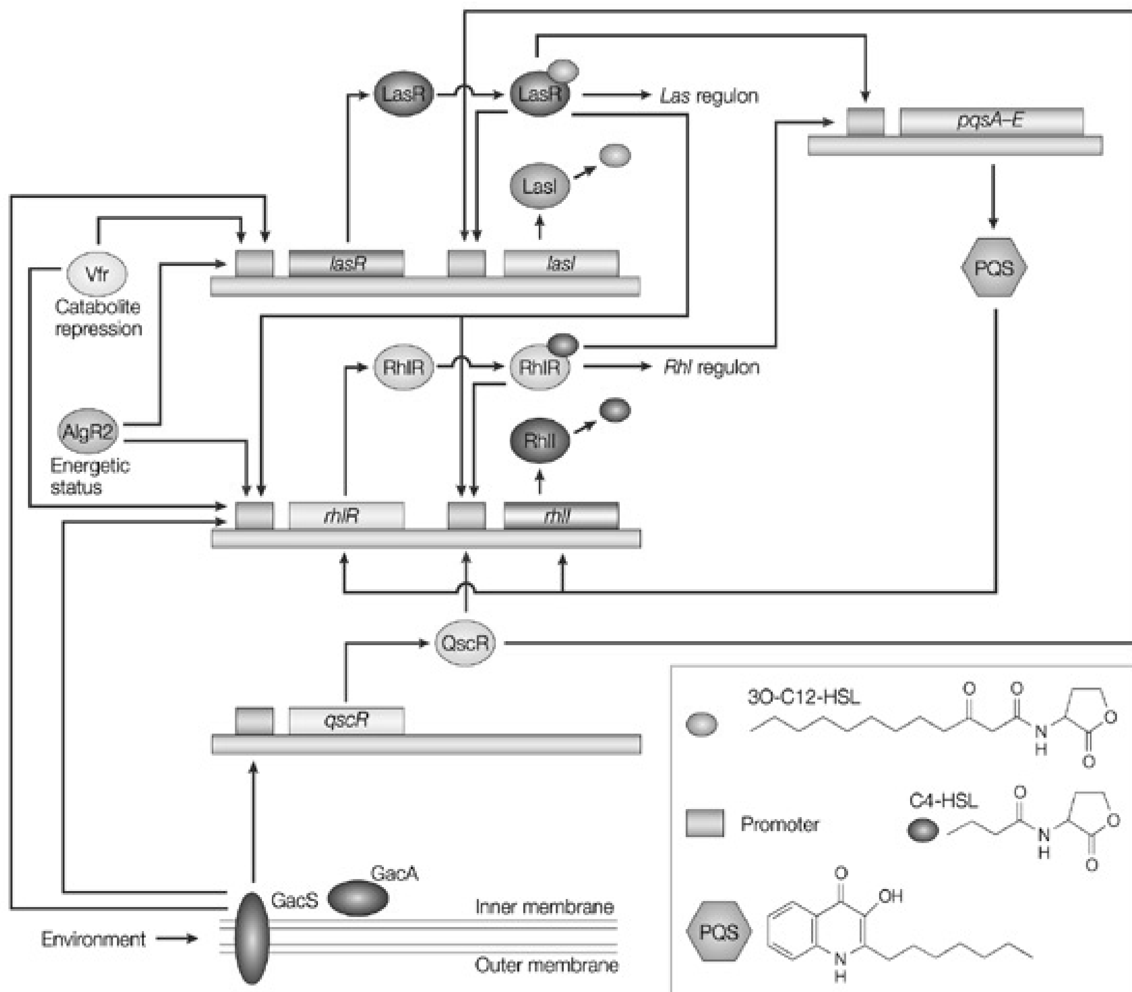


Fig. 1.3. The quorum sensing network in *P. aeruginosa*. For each circuit in the cell the interactions between the different QS systems are indicated by arrows. Signals from the environment, the intracellular metabolic status and other regulators, such as RpoS, RsmA and MvaT, also interface with this cellular circuitry (Lazdunski *et al.,* 2004).

1.3.4. Role of quorum sensing in host-pathogen interactions

Quorum sensing was found to modulate the expression of a wide variety of bacterial virulence genes (Passador *et al.,* 1993). The multiple extracellular pathogenic traits regulated by quorum sensing in *P. aeruginosa* include elastases, alkaline protease, exoenzyme S, neuraminidase, hemolysin, lectins, pyocyanin, rhamnolipids, hydrogen cyanide or oxidative stress-responsive enzymes catalase and superoxide dismutase (Passador *et al.,* 1993; Latifi *et al.,* 1995; Pearson *et al.,* 1997; Hasset *et al.,* 1999; Pessi and Haas, 2000; Winzer *et al.,* 2000) (Table 1.1).

All these extracellular virulence factors are crucial for the competence of *P. aeruginosa* to establish and maintain the infection. Mutants defective in quorum sensing are typically compromised in their ability to establish a successful infection. The importance of quorum sensing for the pathogenicity of *P. aeruginosa* has been demonstrated in a number of animal models. Mutants defective in quorum sensing were substantially less pathogenic than their parental strains in the burned mouse model, the mouse agar bead model or in the neonatal mouse model of pneumonia (Tang *et al.,* 1996; Rumbaugh *et al.,* 1999; Wu *et al.,* 2000a). Quorum sensing mutants also showed reduced virulence in a number of non-mammalian infection models, including *Caenorhabditis elegans*, *Dictyostelium discoideum* and *Arabidopsis thaliana* (Rahme *et al.,* 1995; Tan *et al.,* 1999; Cosson *et al.,* 2002).

Direct involvement of quorum sensing in the host infection process was also confirmed by experiments analyzing sputum samples from CF patients colonized with *P. aeruginosa* (Middleton *et al.,* 2002). CF sputum was shown to contain mRNA for the major regulators of quorum sensing in sufficient amounts to drive heterologous expression of AHL-dependent fusion reporter genes (Singh *et al.,* 2000; Erickson *et al.,* 2002). Similar experiments, exploiting mice infected with *P. aeruginosa* together with an *E. coli* AHL reporter strain, confirmed *in vivo* production of AHLs in the lungs of the host (Wu *et al.,* 2000b).

The quorum sensing system of *P. aeruginosa* contributes to its pathogenesis not only by regulating expression of virulence factors, but also by inducing inflammation. Dermal injections in mice stimulated the production of proinflammatory cytokines and arachidonic acid metabolites (Smith *et al.,* 2002). 3-oxo-C_{12}-HSL exerts numerous immunomodulatory activities on lymphocytes, macrophages and antibody production,

influences the balance between Th1 and Th2 cells (Telford *et al.,* 1999) and strongly promotes the production of interleukin 8 (Smith *et al.,* 2001). On the other hand, if the human host is infected with *P. aeruginosa*, his innate defence is able to inactivate quorum sensing signal molecules. Airway epithelium and body fluids can inactivate 3-oxo-C_{12}-HSL but not C_4-HSL (Chun *et al.,* 2004).

1.3.5. Involvement of quorum sensing in the biofilm formation

Since van Leeuwenhoek’s discovery that bacteria tend to attach and form organised communities or biofilms on surfaces, this field of microbiology has attracted much attention. Biofilms can be characterised as assemblages of surface associated microorganism enclosed in a slime-like extracellular polymeric substance matrix. The ability of *P. aeruginosa* to form biofilms has severe implications for infected patients, as cells grown in biofilms are much more resistant against host defense systems and exhibit increased resistance against a variety of antibiotics (Greenberg, 2003).
Intriguingly, *P. aeruginosa* biofilms are often not just flat layers of bacterial cells but rather complicated structures consisting of tower-and mushroom-shaped microcolonies. An involvement of the quorum sensing circuitry in the regulation of biofilm formation was originally reported by Davis *et al.,* 1998 (Figure 1.4). In this study it was shown that a *lasI* mutant of *P. aeruginosa* only forms flat and undifferentiated biofilms when compared with the wild type, which formed characteristic microcolonies separated by water channels. Importantly, the *lasI* mutant biofilm exhibited greater sensitivity to the biocide dodecyl sulphate than did the wild type biofilm. On the basis of this observation is has been suggested that the *las* system is required for the development of a typical biofilm architecture (Figure 1.4). In other studies, using slightly changed experimental settings, no differences between the biofilms of the wild type and those formed by signal negative mutants were observed (Stoodley *et al.,* 1999; Heydorn *et al.,* 2002). Interestingly, Purevdorj *et al.* (2002) reported minor structural differences between wild type and mutant biofilms, but these differences were only apparent when particular hydrodynamic conditions were used for growing the biofilms. Finally, in a very recent study on the effects of the two quorum sensing systems on biofilm structures using advanced image analysis tools evidence was presented that in fact both quorum sensing

systems of *P. aeruginosa* participate in the regulation of biofilm development but that the differences were only apparent in rather old biofilms, i.e. after 5 to 7 days of incubation (Hentzer *et al*., 2004). In conclusion, these data suggest that the experimental settings have a major impact on the structural development of *P. aeruginosa* biofilms and that under certain conditions yet unidentified quorum sensing regulated factors are required for biofilm maturation.

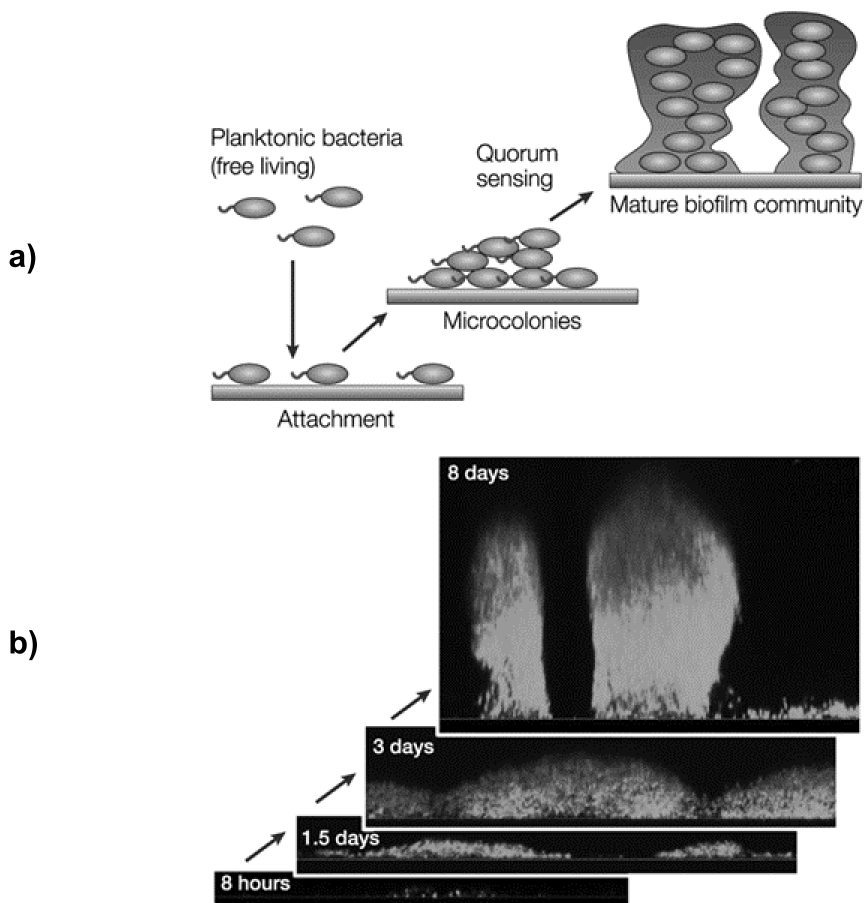


Fig. 1.4. Quorum sensing and biofilms. Development of *P. aeruginosa* biofilms involves a programmed pathway, with quorum sensing playing an important role in the last step of conversion of microcolonies on a surface into a mature biofilms with cells enclosed in a slime-like extracellular polymeric substance matrix (Fuqua and Greenberg, 2002).

a) The steps involved in biofilm development.
b) Confocal-microscope images of a *P. aeruginosa* biofilm developing over time on a microscope slide. The cells are producing the green fluorescent protein. The mushroom and tower-like structures that appear by 8 days are 100 μm high.

1.3.6. Exploiting quorum sensing for antimicrobial therapy

Many studies warn before the threat of emerging bacterial strains, which are partially or completely resistant against a broad spectrum of classical antibiotics. The rapidly increasing frequency of the occurrence of multidrug resistant strains is demanding novel therapeutic approaches. Given that that the quorum sensing circuitry in *P. aeruginosa* plays an important role in controlling pathogenicity as well as biofilm formation, it represents a highly attractive target for the development of novel antimicrobial agents (Passador *et al.,* 1993; Davies *et al.,* 1998; Hartman and Wise, 1998). It has been suggested that the use of AHL antagonists that specifically inhibit expression of pathogenic traits without affecting growth of the bacterium has the advantage of minimizing the possibility of selecting resistant mutants. Although such mutants may eventually arise, they would not have a selective growth advantage and thus would not out-compete the parental strain (Hentzer and Givskov, 2003).

Disruption of quorum sensing may be accomplished in several ways: (i) blockade of AHL synthesis, (ii) AHL signal molecule degradation, and (iii) inhibition of AHL receptor activation. Although previous work has provided evidence that S-adenosyl methionine analogs can inhibit quorum sensing in *P. aeruginosa* because of blockage of AHL synthesis (Parsek *et al.,* 1999), no specific inhibitors, i.e. compounds that would not also affect bacterial growth, have yet been derived on this basis. Dong *et al*., 2001 isolated the first enzyme (AiiA) from *Bacillus* sp. that inactivates AHLs by hydrolysing the lactone bond of the molecules, a process that was named quorum quenching. Work of the past few years identified a large number of additional genes from various organisms that encode enzymes exhibiting AHL-inactivating activities. More importantly, it was also shown that heterologous expression of these enzymes in *P. aeruginosa* not only reduces production of virulence factors but also attenuates the pathogenicity of the organism (Reimmann *et al*., 2002; Lin *et al*., 2003). However, whether AiiA or a related enzyme is applicable for treatment of human infections remains to be seen.

At present, the most promising strategy for quorum sensing disruption appears to be the blockage of the AHL receptor protein. In 1996 Givskov *et al.* demonstrated that the macroalgae *Delisea pulchra* produces compounds, commonly known as halogenated furanones, which have the ability to interfere with AHL-regulated processes. While the exact mode of action of the furanones remains to be elucidated,

it was shown that these compounds not only block cell-to-cell communication in a *P. aeruginosa* biofilm but, upon prolonged incubation, also cause the sloughing of the biofilm (Hentzer *et al.,* 2002). More recently, DNA microarrays were used to provide unambiguous evidence that a certain furanone compound specifically interferes with the expression of quorum sensing regulated genes (Hentzer *et al.,* 2003). More importantly, this study also showed that this compound can clear *P. aeruginosa* lung infections in a mouse model, indicating that furanone compounds indeed are very promising candidates for the development of novel agents for treatment of pseudomonads infections (Hentzer and Givskov, 2003). Evidence has accumulated over the past few years that inhibition of quorum sensing is a commonly used strategy of eukaryotic organisms for battling undesired bacteria. In fact, production of AHL antagonists was demonstrated for different higher plants (Teplitski *et al*., 2000) as well as the animal *Flustra foliacea* (Peters *et al*., 2003).

1.4. Hidden and dangerous: Intracellular pathogens

To establish and maintain a successful infection, microbial pathogens have evolved a variety of strategies, including intracellular lifestyle, which allow them to invade the host, avoid or resist the innate immune response, damage the cells, and multiply in specific and normally sterile regions (Cossart and Sansonetti, 2003). Some bacteria are obligate intracellular parasites that can only replicate inside their host cells. Other bacteria can replicate extracellularly, but choose an intracellular lifestyle to obtain a favourable niche within the host (Gruenheid and Finlay, 2003).

During phagocytosis by phagocytes, bacteria play a passive role. In contrary, some invasive bacteria as for instance *Yersinia pseudotuberculosis, Listeria monocytogenes, Shigella* or *Salmonella* can actively induce their own uptake by phagocytosis in normally nonphagocytic cells and then to establish a protected niche within which they survive and replicate, or disseminate from cell to cell by means of an actin-based motility process (Isberg and Barnes, 2001; Cossart *et al.,* 2003; Galan, 2001; Sansonetti, 2001; Yoshida *et al.,* 2002; Finlay and Cossart, 1997). Once inside a host cell, an intracellular pathogen must use a strategy to avoid or withstand or suppress the maturation of its vacuole into a phagolysosome. Some pathogens as for instance *Coxiella burnetii* have adapted to resist and thrive in the harsh phagolysosomal environment, others (*Listeria, Shigella, Rickettsia*) lyse their vacuole and escape to the cytoplasm and the members of the last group (*Mycobacterium tuberculosis, Salmonella*) are able to actively modify the vacuole to suit their needs (Méresse *et al.,* 1999; Gruenheid and Finlay, 2003). *M. tuberculosis* was shown to interfere with intracellular trafficking events, thus preventing the fusion of a phagosome containing the internalised bacterium with the host cell's lysosomal system (Russel, 2003; Stewart *et al.,* 2003).

Furthermore, the apoptosis, cell cycle-and inflammation-related signalling pathways were also shown to be manipulated by intracellular pathogens in order to take over the fate of their host cells (Hilbi *et al.,* 1998; Hersh *et al.,* 1999; Orth *et al.,* 1999; Muller *et al.,* 1999; Tran Van Nhieu *et al.,* 2003). However, the ultimate success of an infection in all cases depends on the messages that the two players-the bacterium and the cell send to each other (Cossart and Sansonetti, 2003).

1.4.1. Intracellular survival in PMN

Macrophages and polymorphonuclear neutrophils (PMNs) are “guards” that seek and destroy invading pathogens. The PMN is a small leukocyte, about 10 µm in diameter, which is well-known as a first line of defence against invading pathogens (König *et al.*, 1992). PMNs can be found in the bloodstream or attached to the epithelium and are capable of active moving towards the site of inflammation (Lala *et al.,* 1992; Smith, 2000). Moreover, PMNs possess an extensive array of antibacterial weaponry (Elsbach, 1998; Gudmundson and Agerberth, 1999). Elimination of invading microorganisms is caused by phagocytosis, a process whereby the pathogens are engulfed into a plasma-derived phagosome (Scott *et al.,* 2003). Following formation, the phagosome sequentially fuses with a series of endomembrane compartments and thereby acquires microbicidal and degradative properties (Beron *et al.,* 1995; Aderem and Underhill, 1999; Tjelle *et al.,* 2000). Prior to phagocytosis of target microorganism, the PMN begins to consume oxygen, a process known as oxidative or respiratory burst. This oxidative burst is a consequence to the assembly of a membrane-associated NADPH oxidase from membrane components gp91^{phox} and gp22^{phox} and cytosolic components p67^{phox} and p47^{phox} with the participation of several auxiliary proteins, including the small GTP binding protein, rac2 (Rosen, 2004). The NADPH oxidase transfers electrons from NADPH to dissolved molecular oxygen thus allowing formation of superoxide anions and reactive antimicrobial oxygen species in the phagolysosomes which contributes greatly to intensification of the antimicrobial features of these compartments.

Intracellular survival in host defence cells is the typical lifestyle of a few obligatory pathogens; however, few pathogenic bacteria have developed successful mechanisms to modify host cell metabolism, that enable them to survive and replicate within potentially lethal PMNs. Among the most interesting bacterial mechanisms are the type III and type IV secretion systems, which represent a hollow tube structures penetrating the eukaryotic cell membranes and serve as a conduit for the injection of bacterial exoproducts into the host cytosol (Hueck, 1998; Cornelis, 2002; Fischer *et al.,* 2002; Sexton and Vogel, 2002; Nagai and Roy, 2003). The injected effector molecules, variable from species to species and even among strains

of the same species, modify the metabolism of the host cell in the fashion that presumably benefits the survival and replication of the microorganism (Rosen, 2004). Human pathogenic *Yersinia* species (*Y. pestis, Y. enterocolitica, Y. pseudotuberculosis)* evade phagocytosis by adhesion to the surface of PMNs or opsonization and by subsequent injection of the Yop (Yersinia outer proteins) into the cytosol of PMNs, which results in disorganization of actin polymerisation that suppress active phagocytosis (Grosdent *et al.,* 2002). In contrary, an obligate intracellular pathogen, *Anaplasma phagocytophilum* needs to be phagocyted by PMNs, but is able to actively suppress oxidative burst by PMNs thus not allowing them to generate antimicrobial oxygen species, dioxygen and hydrogen peroxide (Wang *et al.,* 2002). Furthermore, *Anaplasma* was shown to be able to facilitate its intracellular survival in PMNs by suppression of the fusion of phagosomes with lysosomes and reduction of PMN apoptosis (Gokce *et al.,* 1999; Scaife *et al.,* 2001). *Salmonella typhimurium* exploits one of its pathogenicity islands, SPI-2 (*Salmonella* pathogenicity island 2) which encodes type III secretion system and which, when in close contact with phagocytes, prevents assembly or misdirects the localization of the phagocyte NADPH oxidase to the phagosome (Vazquez-Torres *et al.,* 2000; Gallois *et al.,* 2001; Vazquez-Torres and Fang, 2001). Interestingly, *Escherichia coli* is able to mount a defensive response to the reactive oxygen species generated by PMNs by increasing synthesis of antioxidant enzymes which are under the control of an oxidant-sensing transcription factor, OxyR (Staudinger *et al.,* 2002).

PMN-mediated phagocytosis is also known as the host's most proficient antipseudomonal weapon (Döring *et al.*, 1995). Even though, around 20% of analyzed *P. aeruginosa* isolates from the lungs of CF patients were shown to be able to overcome this defense mechanism by increased cytotoxicity towards PMNs, which leads to oncosis and lysis of PMNs. (Dacheux *et al.*, 2000). Investigation of this phenomenon has led to the identification of a novel regulatory system for type III secretion and subsequent cytotoxicity towards PMNs (Dacheux *et al.*, 2002). *P. aeruginosa* strain TB (TBCF10839) capable of survival and replication in PMNs irrespective of whether they were isolated from patients with CF or healthy donors, that combats PMNs by other, type III-independent mechanisms has been detected in the late 1980s (Tümmler, 1987).

1.4.2. Intracellular survival of *P. aeruginosa* TB in PMN

TB (TBCF10839 isolate) is a highly virulent strain of *P. aeruginosa,* which was isolated in 1983 from the sputum of a CF patient who had suffered from an acute and chronic infection with *P. aeruginosa.* The patient was severely ill and this *P. aeruginosa* strain had been eradicated from his airways only by the high-dose antipseudomonal chemotherapy (Tümmler, 1987). TBCF10839 was the most virulent isolate from strain TB isolates that were frequently identified in CF patients and burn patients at the Medizinische Hochschule Hannover in the 1980s (Tümmler *et al.*, 1991; Kiewitz and Tümmler, 2000).

While examining the survival rate of different *P. aeruginosa* strains in freshly isolated PMNs, the reason for the high virulence of strain TB (TBCF10839) has been uncovered. Whereas the genetic reference strain PAO1 was efficiently phagocytosed and lysed under standard conditions, the cfu of TBCF10839 initially declined within the first 30 minutes but continuously increased thereafter indicating cell growth (Miethke, 1985). Electron microscopy revealed that after 30 minutes only cell debris was visible from *P. aeruginosa* PAO1 in the phagolysosomes (Figure 1.5).

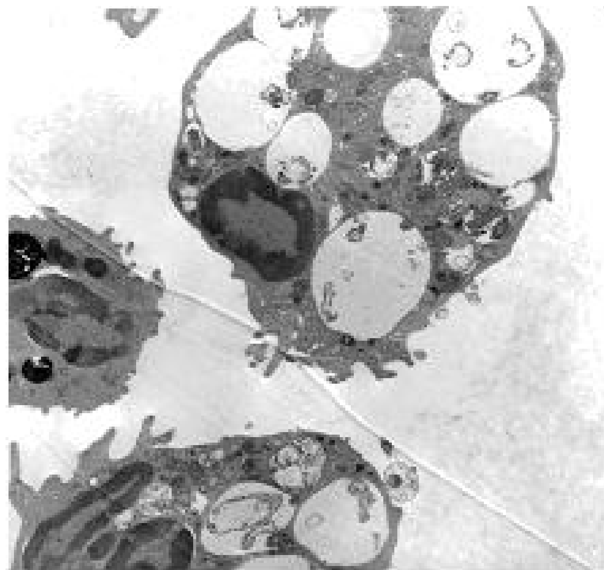


Fig. 1.5. Electron micrograph of *P. aeruginosa* strain PAO1 incubated with granulocytes. Bacteria were added at a multiplicity of infection (MOI) 20 and incubated at 37 ^{o}C for 30 min.

In contrast, *P. aeruginosa* strain TB (TBCF10839) in phagolysosomes was still intact, thus confirming its increased survival ability in PMNs as observed by Miethke, 1985 (Figure 1.6).

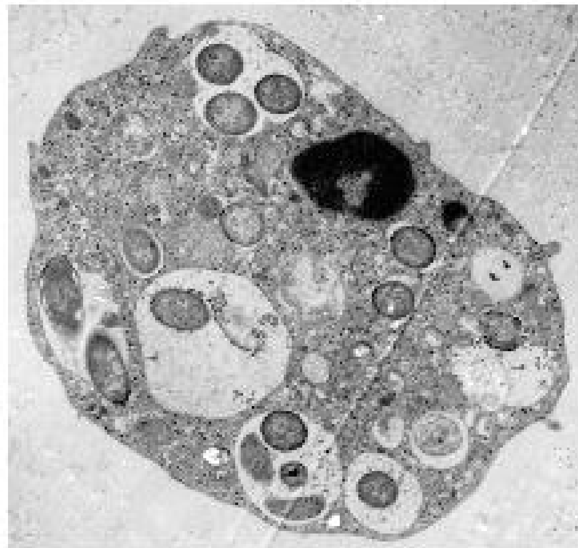


Fig. 1.6. Electron micrograph of *P. aeruginosa* strain TBCF10839 incubated with granulocytes. Bacteria were added at a multiplicity of infection (MOI) 20 and incubated at 37 ^{o}C for 30 min.

Interestingly, by 60 minutes and thereafter the TBCF10839 bacteria were shown to multiply in the phagolysosomes (Wiehlmann, 2001).

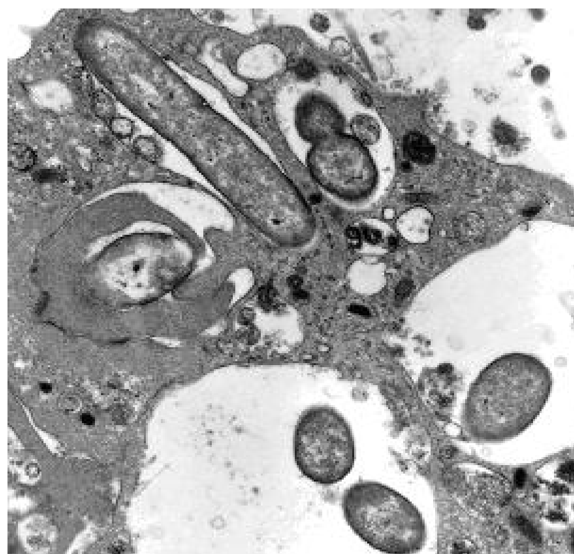


Fig. 1.7. Electron micrograph of *P. aeruginosa* strain TBCF10839 incubated with granulocytes. Bacteria were added at a multiplicity of infection (MOI) 20 and incubated at 37 ^{o}C for 60 min.

Live *P. aeruginosa* TBCF10839 bacteria as well as debris from another bacterial cells were shown to be present in the same vacuole, thus revealing that the uptake of the bacteria by the PMN does not prevent the subsequent maturation of phagosomes resulting in their fusion with lysosomes (Figure 1.7). Moreover, the lysis of the phagolysosomal membrane was observed at higher magnification, thus allowing the bacteria to escape to the cytosol of the PMN. Therefore, the TBCF10839 bacteria were shown to reside not only in the phagolysosomes of the PMNs but also in the extracellular space and in the cytosolic compartment. These experiments showed that the resistance of *P. aeruginosa* TBCF10839 to the harsh conditions in the PMN phagolysosomes and thus its survival ability in PMNs is much higher than that of the common *P. aeruginosa* strains.

The enhanced resistance of *P. aeruginosa* TBCF10839 towards PMNs is not limited to those of CF patients. PMNs from 40 tested healthy donors consistently showed all the same phenotype of bacterial growth in the phagocytosis assay under standard conditions of a tenfold excess of bacteria over PMNs. This shows that the opportunistic pathogen *P. aeruginosa* can convert into a highly virulent microorganism (Wiehlmann *et al.*, 2002).

Generally, the intracellular growth in PMNs is very rare amongst bacteria and has yet not been reported for *P. aeruginosa.* Interestingly, as shown above, *P. aeruginosa* TBCF10839 not only survives but also replicates in PMNs. Moreover, *P. aeruginosa* TBCF10839 does not need a lag phase for replication in PMNs that is typical for other intracellular pathogens to adapt to harsh conditions in phagolysosomes (Philpott *et al.*, 2001). All known evidence indicates that some novel mechanisms operate in the *P. aeruginosa* TBCF10839 allowing this bacterium to thrive in PMNs.

1.5. DNA microarray technology

We are witnessing a remarkable change in the scale of molecular microbiological research and we are entering an era of “big science”. In the past decade we have moved from a time when entire research papers were based on the sequencing of a single gene or operon to a single paper describing the sequence of the whole genome. The completion of microbial genomes is continuing fastly (http://www.tigr.org/tdb/mdb/mdbcomplete.html) and the availability of this level of genetic information has spawned the terms “functional genomics”, “transcriptomics” (Velculescu *et al.,* 1997) and “proteomics” (Wasinger *et al.,* 1995), which describe the large-scale application of mass mutagenesis, gene expression profiling and global protein analysis. Assessment of transcription at the genomic scale has been achieved with DNA microarrays, which are glass slides containing an ordered mosaic of the entire genome as a collection of either oligonucleotides (high-density oligonucleotide microarrays) or polymerase chain reaction (PCR) products representing individual genes (commonly referred as cDNA microarrays or spotted microarrays). In the spotted microarray, presynthesized single-stranded or double-stranded DNA is bound or 'printed' onto glass slides. The DNA can be generated from cloned, synthesized or PCR-amplified material. Because of the technical simplicity of this approach, spotted microarrays can be produced in house as well as purchased from commercial providers. High-density oligonucleotide arrays are constructed by synthesizing short (25-mer) oligonucleotides *in situ* on glass wafers using a photolithographic manufacturing process and are thus available only from commercial vendors (Lipshutz *et al.*, 1999).

Since the first report of DNA microarray technology in 1995 (Schena *et al.,* 1995) the potential of DNA microarrays has certainly captured the imagination of biologists worldwide. The major advantage of microarrays is their ability to measure simultaneously the presence of tens of thousands of different nucleic acid sequences. The recent innovative robotic techniques facilitated the construction of microarrays containing up to 50 000 genes on a single slide (DeRisi *et al.,* 1996; Shalon *et al.,* 1996). This allows a single hybridisation to be performed against multiple replicates of a single bacterial genome, or against copies of several unrelated genomes on a single glass slide (Lucchini *et al.,* 2001). Thus, this technique permits the quantification of specific genes and their expression patterns in

a comprehensive genome-wide framework. Although expensive relative to other quantitative hybridization and amplification methods, the high-throughput capacity makes it a cost-effective technique for a variety of applications (Kato-Maeda *et al.,* 2001).

The application of microarray technology is only limited by our imagination. Thus microarrays have been already used with success to identify targets for novel drugs (Braxton and Bedilion, 1998; Anzick and Trent, 2002), to study genetic polymorphism of pathogenic bacteria (Gingeras *et al*., 1998; Troesch *et al*., 1999; Salama *et al*., 2000; Kato-Maeda *et al*., 2001; Fitzgerald *et al.*, 2003), as well as to study microbial evolution (Winzeler *et al.,* 1998; Gingeras *et al.,* 1998; Behr *et al.,* 1999) and to analyze gene expression *in vivo* (Lochkart *et al.,* 1996; Luo *et al.,* 1999; Wang *et al.,* 2000). Moreover, DNA microarrays can be also used to analyze the host-pathogen interactions (Manger and Relman, 2000; Schoolnik, 2002; Brooks *et al.*, 2003). A robust microarray database with microbial response profiles from a wide variety of well-characterized environmental conditions can allow more insightful interpretation of transcription patterns recorded in novel host environments. Finally the integration of host response data with bacterial response can provide a more complete understanding of the two-way conversation between host and pathogen (Relman, 2002).

One of the most impressive examples of the use of microarrays for bacterial research is implicated with the investigation of the whole regulons. The definition of important regulons by the use of appropriate regulatory mutants provides the framework for a better understanding of complex cellular responses (Lucchini *et al.,* 2001).

1.6. Objectives

During the last decades we have witnessed an explosion of information about the organisation of many bacterial genomes, including that of the opportunistic human pathogen *P. aeruginosa* (Stover *et al.,* 2000). Thus, one of the greatest challenges of the future as well as of the presented thesis is the functional analysis of putative ORFs identified in this genome sequencing project and their integration into regulatory networks and metabolic pathways.

Besides the availability of the complete genome sequence, the construction of the signature tagged mutagenesis (STM) library of *P. aeruginosa* strain TB by a previous Ph.D. student in the group (Wiehlmann, 2001) provided an important basis for completion of the presented work. When screening this STM library comprising minitransposon Tn*5* non-auxotrophic isogenic mutants, four putative regulatory virulence genes were identified: *vqsR*, *gltR*, *47D7* and *icsF*. These four genes should be integrated into regulatory circuits and pathways in the presented thesis using combined approach of up-to-date technologies of functional genomics, genetics *in silico* and bioassays, thus helping to pave the way to comprehensive understanding of the complex phenotypes of *P. aeruginosa* pathogenicity.

To achieve this objective, the following tasks will be accomplished:

1. Extensive *in silico* analysis of the investigated putative ORFs will be performed in the first step, which should provide valuable hints about the function of respective genes.
2. Examined ORFs with no homologues in the sequenced *P. aeruginosa* strain PAO1 will be sequenced.
3. The investigated ORFs will be complemented *in trans* to ensure that the observed phenotypes of mutants are caused by the transposon inactivation of the respective genes and not by any other secondary genetic event.
4. The mutants will be investigated for the ability to produce quorum sensing autoinducers and for the phenotypic traits that are known to be under the control of quorum sensing, as it is for instance the ability to secrete many extracellular virulence factors.
5. The mutants in the investigated genes will be tested for their ability to survive intracellularly in PMNs.

6. The nematode *Caenorhabditis elegans* infection model will be used to examine the global impact of the investigated genes on pathogenicity.
7. Differences in gene expression will be examined by Northern blot growing bacteria under various stress conditions.
8. DNA microarrays comprising the whole genome of the sequenced *P. aeruginosa* strain PAO1 (Affymetrix) will be exploited to examine the impact of the investigated genes on the global gene expression pattern.

2. Materials and methods

2.1. Materials

2.1.1. Consumables and equipment

Balance BP3100 S	
BP210 S	Sartorius
Bio-Rad Pulser	
Centrifuge	Hettich Universal
Eppendorf centrifuge 5415C	Eppendorf
Eppendorf centrifuge 5417R	Eppendorf
Eppendorf tubes (0.5 ml, 1.5 ml, 2 ml)	Sarstedt
Filter Cellulose ester HA 0.45 μM pore size	Millipore
Filter Cellulose acetate 0.2 & 5 μM pore size	Sartorius
Filter paper GB003	Schleicher and Schuell
Heating block DR-block DB-3	Techne
Hybridization oven 400 HY	Bachofer
Hybridization oven	Biometra
Incubator	Heraeus
Minifold I Vacuum blotter	Schleicher and Schuell
Pasteur pipette	Sarstedt
Petri plates 9 cm Ø	Sarstedt
Pipette tips (10 μl, 200 μl, 1 ml)	Sarstedt
pH meter 766 Calimatic	Knick
Plastic tubes (15 ml, 50 ml)	Greiner
Polaroid film 667	Polaroid
Spectrophotometer U3000	Hitachi
Thermocycler	Landgraf
Thermomixer	Eppendorf
TLC plate	Merck
UV-Transilluminator	Bachofer
UV Stratalinker 1800	Stratagene
Vacuum concentrator	Bachofer
Voltage supply power pack 300	Bio-Rad
X-ray film	Kodak, AGFA

2.1.2. Chemicals and enzymes

Affymetrix	Enzo BioArray Terminal Labelling Kit, Oligonucleotide B2, *P. aeruginosa* GeneChip
Amersham Pharmacia Biotech	Deoxynucleotides, Hybond N+ nylon membrane, OnePhorAll-buffer, RNase-free DNase I, TEMED
Ambion	SUPERaseIn (RNase Inhibitor)
Becton Dickinson	Columbia blood agar
Blood bank MHH	AB serum
Biozym	SeaKem GTG Agarose
Die & Bernsten (DK)	Formalin buffer
Difco	Agar, Bacto-Peptone, Beef extract
Gibco BRL	Agar, Agarose, DMEM medium, FCS, Fetal calf serum, Lymphocyte separation medium, PBS Tablets, RPMI 1640 medium
In ViTec	*Taq*-Polymerase & buffers
Invitrogen	Bromophenol blue, Bovine serum albumin, Gentamicin, MOPS buffer, Random Primers, RNA-standard, Serva Blue G-250, SuperScript II Reverse Transcriptase, Xylene cyanol FF
Merck	Formaldehyde 35 %, Hydrogen peroxide 30 %, Liquemin (Heparin), Uranyl acetate
Molecular Probes	Streptavidine-Phycoerythrine, SYBRGreen
MWG-Biotech	Oligodeoxynucleotides, Primers
New England Biolabs	DNA-ladder standards, Restriction enzymes + buffers, T4-DNA-Ligase
Pierce Chemical	Streptavidine
Promega	Herring-Sperm DNA, RNase Inhibitor
Qiagen	Plasmid Spin Kit, Qia-Mini, Midi, Maxi and Giga Prep plasmid kits, QIAquick Gel Extraction Kit, RNeasy Kit, Rnase A
Roche Molecular Biochemicals	Alkaline Phosphatase, Anti-Dioxigenin AP fab fragment, DNA Labelling and Detection Kit, Klenow Polymerase I, RNase-free DNase I, Terminal Transferase + Buffers, Blocking reagent
Roth	Acrylamide solution (Rotiphorese-Gel 40), Ethidium bromide, Phenol (Rotiphenol) solutions, DEPC
Serva	Gentamicin
Sigma	Chelex-100 (iminodiacetic acid), Ethidium bromide, Proteinase K, Propidium iodide, Tween 20, H_2SO_4, Gold star polymerase, Elastin-Congo red
Tropix	CDP-Star, DEA
Vector Laboratories	Biotin anti-Streptavidine

2.1.3. Media and solutions

2.1.3.1. Media

ABC minimal medium:

A:	$(NH_4)_2SO_4$	20 g/l (0.15 M)
	$Na_2HPO_4 \cdot 2H_2O$	60 g/l (0.33 M)
	KH_2PO_4	30 g/l (0.22 M)
	NaCl	29 g/l (0.5 M)
B:	$MgCl_2 \cdot 6H_2O$	0.4 g/l (2 mM)
	$CaCl_2 \cdot 2H_2O$	0.014 g/l (0.1 mM)
	$FeCl_3 \cdot 6H_2O$	0.008 g/l (0.003 mM)
C:	Sodium citrate	10 mM

pH 5.5

B was filter-sterilized, while A and C were sterilized by autoclaving. Subsequently all components were mixed together.

Columbia blood agar (Becton Dickinson):

This complex agar, which is used frequently in microbiology, was exploited for the analysis of hemolytic activity of the investigated *P. aeruginosa* strains.

King's A medium:

Pepton	20 g/l
KOH	20 g/l
H_2SO_4	5.5 ml
$MgCl_2$ x 6 H_2O	3.3 g/l
Glycerin	10 g/l

pH 7.2

LB Medium:

Peptone	15 g/l
Yeast extract	5 g/l
NaCl	10 g/l (0.17 M)

LB Agar:

LB medium	
Agar	15 g/l

LB T-selective:

LB medium	
Tetracycline	50 μg/ml or 200 μg/ml

M9-Medium (10X):

Na_2HPO_4	68.14 g/l (0.48 M)
KH_2PO_4	30 g/l (0.22 M)
NaCl	5 g/l (85 mM)
NH_4Cl	10 g/l (0.18 M)

M9-Agar:

The M9 medium (10x) and water agar (15 g/l water) were autoclaved separately. Casein (0.75 % w/v) was dissolved in a 50 ml of M9 (10x) medium by heating, cooled to the temperature of about 60 ^{o}C and subsequently added to the 450 ml of melted water agar.

Vogel-Bonner minimal medium:

A (10x):	Citrate x H_2O	21 g/l
	$NaNH_2PO_4$ x $4H_2O$	58.6 g/l
	K_2HPO_4 x 3 H_2O	84.4 g/l
	pH 7.2	
B (5x):	Kalium-D-Gluconate	250 g/l
C (50x):	$MgSO_4$ x 7 H_2O	41 g/l

The solution B was sterile-filtrated, while A and C were autoclaved. The ready-to-use Vogel-Bonner medium was obtained via mixing all three components A, B and C and addition of required volume of the didestilled H_2O.

SOB:

A:	Bactotryptone	20 g/l
	Yeast Extract	5 g/l
	NaCl	0.58 g/l (10 mM)
	KCl	0.185 g/l (2.5 mM)
	pH 7.0	
B:	$MgCl_2$	1 g/l (10 mM)
C:	$MgSO_4$	1.2 g/l (10 mM)

The filter-sterilized stock solutions B and C were added to the autoclaved A mixture.

SOC:

SOB	
Glucose	3.6 g/l (20 mM)

2.1.3.2. Solutions

TBE-Buffer (10X):

Tris	108 g/l (0.9 M)
Boric Acid	55 g/l (0.9 M)
EDTA	7.7 g/l (0.02 M)
pH 8.3-8.5	

Loading Buffer (6X):

Ficoll 400	15 % v/v
Bromophenol Blue	0.25 % w/v
Xylene cyanol	0.25 % w/v
EDTA	146 g/l (0.5 M)
pH 8.0	

PBS (10X):

NaCl	80 g/l (1.37 M)
KCl	2 g/l (27 mM)
$Na_2HPO_4 \cdot 7H_2O$	11.5 g/l (4.3 mM)
KH_2PO_4	2 g/l (1.4 mM)
pH 7.3	

TB Buffer:

PIPES	3 g/l (10 mM)
$CaCl_2$	1.6 g/l (15 mM)
KCl	18.6 g/l (250 mM)
$MnCl_2$	(9.58 g/l (50 mM)
pH 6.7	

Sterilized by filtration and stored at 4 ^{o}C.

TE Buffer:

Tris-HCl	1.2 g/l (10 mM)
EDTA	0.38 g/l (1 mM)
pH 8.0	

Bradford solution:

Coomassie brilliant blue	70 mg
Ethanol	50 ml/l (96 % w/v)
H_3PO_4	100 ml/l (85 % w/v)

After the preparation, the solution was incubated at room temperature for 24 hours, filtrated and kept in a dark flask.

Lysis Buffer:

Tris-acetate	4.84 g/l (40 mM)
Sodium acetate	20 mM
EDTA	0.38 g/l (1 mM)
SDS	1 % w/v
pH 7.8	

Plasmid DNA isolation:

Solution I:	Tris-Cl	6 g/l (50 mM)
	EDTA	3.8 g/l (10 mM)
	DNase free RNase A	100 μg/ml
	pH 8.0	
Solution II:	NaOH	16 g/l (0.4 M)
	SDS	1 % w/v
Solution III:	Potassium acetate	294 g/l (3 M)
	Acetic acid	115 ml/l (2 M)

Fixation solution:

PBS	1x
Paraformaldehyde	1 % v/v
$CaCl_2$	0.11 g/l (1 mM)
pH 7.2–7.4	

Colour solution for gel filtration:

Dextran Blue	0.8 % w/v (2x 10^6 g/mol)
Phenol red	0.5 % w/v (376 g/mol)

RNA Lysis buffer:

SDS	2 % w/v
Sodium acetate	3 mM
EDTA	0.1 % w/v
pH 5.5	

DNase buffer (10 x):

Sodium acetate	500 mM
$MgCl_2 \cdot 6H_2O$	20.3 g/l (100 mM)
$CaCl_2 \cdot 2H_2O$	2.94 g/l (20 mM)
pH 6.5	

MOPS buffer (10 x):

MOPS	41.8 g/l (200 mM)
Sodium acetate	100 mM
EDTA	2.9 g/l (10 mM)
pH 7.0	

RNA loading buffer:

Glycerol	50 % v/v
EDTA	0.29 g/l (1 mM)
Bromophenol blue	0.25 % w/v
pH 6.0	

SSC buffer (20 x):

NaCl	175 g/l (3 M)
Sodium citrate	0.3 M
pH 7.0	

Northern hybridization solutions:

Pre-hybridization buffer:

Formamide	50 % v/v
Denhardt's solution	5x
SSC	5x
SDS	1 % w/v
Herring sperm DNA	100 µg/ml

Prior to adding to hybridisation solution the Herring sperm DNA was subjected to fragmentation by ultrasound sonication and denaturation for 10 min at 95 ^{o}C.

Denhardt's solution:

Ficoll (Type 400)	2 % v/v
Polyvinylpyrrolidine (PVP-40)	2 % v/v
Bovine serum albumin (BSA)	2 % v/v

Buffer I:

NaCl	175 g/l (3 M)
Tris-HCl	12 g/l (0.1 M)
Tween 20	0.3 % v/v
pH 8.0	

Buffer II:

Buffer I	
Blocking reagent	0.5 % w/v

Buffer III:

Tris-HCl	12 g/l (100 mM)
NaCl	5.8 g/l (100 mM)
$MgCl_2$	4.8 g/l (50 mM)
pH 9.5	

Solutions for GeneChip experiments:

cDNA Reaction mixture:

1st Strand buffer	5 x
DTT	10 mM
dNTPs	0.5 mM
SUPERaseIn	0.5 U/μl
SuperScript II	25 U/μl

MES hybridization buffer:

MES	100 mM
NaCl	58 g/l (1 M)
EDTA	5.84 (20 mM)
Tween 20	0.01 % v/v

Light-protected and stored at 2−8 ^{o}C.

Non-stringent buffer:

SSPE	6 x
Tween 20	0.01 % v/v

Steril-filtered.

Stringent wash buffer:

MES	100 mM
NaCl	5.8 g/l (0.1 M)
Tween 20	0.01 % v/v

Steril-filtered, light-protected and stored at 2−8 ^{o}C.

Primary stain solution (Streptavidin solution mix):

MES	100 mM
NaCl	58 g/l (1 M)
Streptavidin	10 μg/ml
BSA	2 mg/ml
Tween 20	0.05 % v/v

Secondary stain solution (Biotin Anti-streptavidin solution mix):

MES	100 mM
NaCl	58 g/l (1 M)
Biotin Anti-streptavidin	5 μg/ml
Goat IgG	0.1 mg/ml
BSA	2 mg/ml
Tween 20	0.05 % v/v

Tertiary stain solution (Streptavidin-Phycoerythrin solution mix):

MES	100 mM
NaCl	58 g/l (1 M)
Streptavidin-Phycoerythrin	10 μg/ml
BSA	2 mg/ml
Tween 20	0.05 % v/v

Non radioactive detection:

Hybridization wash buffer:

$NaH_2PO_4 \cdot 2H_2O$	6.3 g/l (40 mM)
SDS	1 % w/v
EDTA	0.38 g/l (1 mM)
pH 7.2	

Buffer I:

Tris-HCl	12 g/l (100 mM)
NaCl	8.7 g/l (150 mM)
pH 7.5	

Buffer II:

Buffer I	
Blocking reagent	0.5 % w/v

Antibody solution:

1:5000 dilution of Anti-Digoxigenin AP F_{ab}-alkaline phosphatase conjugate in buffer II.

Buffer III:

Tris-HCl	12 g/l (100 mM)
NaCl	5.8 g/l (100 mM)
$MgCl_2$	4.8 g/l (50 mM)
pH 9.5	

Wash solution:

NaOH	8 g/l (0.2 M)
SDS	0.1 % w/v

Sucrose solution:

Sucrose	171 g/l (0.5 M)
Tris-HCl	12 g/l (0.1 M)
pH 8.0	

TET buffer:

Tris-HCl	1.2 g/l (10 mM)
EDTA	1.9 g/l (5 mM)
Triton-X100	0.1 % v/v
pH 8.0	

2.1.4. Infection models, bacterial strains and plasmids

2.1.4.1. Infection models

Caenorhabditis elegans

Bristol N2 (wild type), provided by the *Caenorhabditis* Genetics Centre (University of Minnesota, St Paul's, MN, USA) was used throughout this study.

2.1.4.2. Bacterial strains

***Pseudomonas aeruginosa* strains**

TB:

Cystic fibrosis airways isolate — Tümmler *et al.* (1991)

892:

Clonal variant of the strain TB — Tümmler *et al.* (1991)

PAO1:

Wound isolate, genetic reference strain — Holloway *et al.* (1994)

TB*vqsR:*

Tn*5*::*vqsR* mutant of TB — Wiehlmann *et al.* (2001)

TB*icsF:*

Tn*5*::*icsF* mutant of TB — Wiehlmann *et al.* (2001)

TB*gltR:*

Tn*5*::*gltR* mutant of TB — Wiehlmann *et al.* (2001)

TB*47D7:*

Tn*5*::*47D7* mutant of TB Wiehlmann *et al.* (2001)

TB*vqsR*(pME6010*vqsR*):

Tn*5*::*vqsR* mutant of TB complemented with pME6010(*vqsR*)
Juhas *et al.* (2004)

TB*icsF*(pME6010*icsF*):

Tn*5*::*icsF* mutant of TB complemented with pME6010(*icsF*)
This study

TB*gltR*(pME6010*gltR*):

Tn*5*::*gltR* mutant of TB complemented with pME6010(*gltR*)
This study

TB*47D7*(*pME6010(47D7*):

Tn*5*::*47D7* mutant of TB complemented with pME6010(*47D7*)
This study

***Pseudomonas putida* strain**

F117:

AHL negative derivate of *P. putida* IsoF, *ppuI*$^-$ Steidle *et al*. (2001)

***Escherichia coli* strains**

MT102:

araD139 (*ara-leu*)*7697 lac thi hsdR* Leo Eberl, lab. collection

JM105:

F' *traD36 lacI*^{q} (*lacZ*)M15 *proA*$^+$*B*$^+$*/thi rpsL*(Str^r) *endA sbcB15 sbcC hsdR4*(r_K m_K) (*lac-proAB*) Yanisch-Perron *et al.* (1985)

One Shot® TOP10 Chemically competent *E. coli*

F' *mcrA* (*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ* M15 *lac*X74 *deo*R *rec*A1 *ara*D139 (*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R)*end*A1 *nup*G

Invitrogen Life technologies

2.1.4.3. Plasmids

pJBA89:

AHL monitor plasmid; pUC18Not-*luxR*-P_{luxI}-RBSII-*gfp* (ASV) -T_0-T_1; Ap^r

Andersen *et al.* (2001)

pKR-C12:

AHL monitor plasmid; pBR1MCS-5 carrying P_{lasB}-*gfp*(ASV) P_{lac}- *lasR*; Gm^r

Steidle *et al*. (2001)

pSB403:

broad-host-range AHL monitor plasmid; Tc^r Winson *et al.* (1998)

pME6010:

Shuttle vector replicable in Gram-negative bacteria; Tc^r

Heeb *et al.* (2000)

pME6010*vqsR:*

pME6010 carrying the *vqsR* gene This study

pME6010*icsF:*

pME6010 carrying the *icsF* gene This study

pME6010*gltR:*

pME6010 carrying the *gltR* gene This study

pME6010*47D7:*

pME6010 carrying the *47D7* gene This study

2.2. Methods

2.2.1. Microbiological methods

2.2.1.1. General bacterial growth conditions

All bacterial cultures and strains were cultivated at 37 ^{o}C in Luria broth (LB) medium unless specified otherwise. Strains carrying pME6010, pME6010*vqsR,* pME6010*icsF,* pME6010*gltR* or pME6010*47D7* were grown in LB medium containing 50 µg/ml tetracycline. Alternatively, a single bacterial colony was inoculated in a 15 ml Falcon tube containing 4-5 ml of LB medium with or without antibiotic and incubated at 250 rpm on a rotatory shaker for approximately 16 h at 37 ^{o}C. Alternatively, to achieve higher cell yields, 1 ml of bacteria were inoculated in Erlenmeyer flasks containing 400 ml LB medium and incubated at 250 rpm on a rotatory shaker for approximately 16 h at 37 ^{o}C.

2.2.1.2. Bacterial growth for RNA isolation (LB medium)

In this condition, as well as in all other used for RNA isolation, bacteria were grown in 125 ml Capsenberg flasks to achieve the same aeration rate for all bacterial samples.

1. Bacterial cultures were grown in 15 ml of LB medium in 125 ml Capsenberg flasks at 300 rpm on a rotatory shaker at 37 ^{o}C up to the optical density of 1.5 for early exponential phase or alternatively of up to 3.5 for late exponential phase at 600 nm.
2. Afterwards, the bacteria were harvested by centrifugation at 3800 *g* for 2 min and subjected to RNA isolation.

2.2.1.3. Bacterial growth for RNA isolation (ABC minimal medium)

1. Bacterial cultures were grown in 15 ml of ABC minimal medium in 125 ml Capsenberg flasks at 250 rpm on a rotatory shaker at 30 ^{o}C up to an optical density of 1.0 at 600 nm.
2. Afterwards, the bacteria were harvested by centrifugation at 3800 *g* for 2 min and subjected to RNA isolation.

2.2.1.4. Bacterial growth for RNA isolation (H_2O_2)

1. Bacterial cultures were grown in 15 ml of LB medium in 125 ml Capsenberg flasks at 300 rpm on a rotatory shaker at 37 ^oC up to the optical density 5.0 at 600 nm (stationary phase). In order to measure OD this highly dense bacterial culture was diluted 1:10 with LB.
2. 3 x 10^{10} cells from this stationary phase culture were resuspended in 5 ml of LB and added into a 6 cm long dialysis tube (14 kDa cut-off, 25 mm width).
3. The dialysis tube containing bacteria was incubated in 1L Erlenmeyer flasks containing 10 mM hydrogen peroxide in 600 ml of LB medium at 200 rpm on a rotary shaker at 37 ^oC for 2 h. Erlenmeyer flasks containing only pure LB medium without the addition of hydrogen peroxide served as control.
4. Afterwards, the bacteria were quickly recovered from the dialysis tube, harvested by centrifugation at 3800 *g* for 2 min and subjected to RNA isolation.

2.2.1.5. Bacterial growth for RNA isolation (Serum)

1. Bacterial cultures were grown in 15 ml of LB medium in 125 ml Capsenberg flasks at 300 rpm on a rotatory shaker at 37 ^oC up to the optical density 5.0 at 600 nm (stationary phase). In order to measure OD this highly dense bacterial culture was diluted 1:10 with LB.
2. 2 x 10^{10} cells from this stationary phase culture were resuspended in 2 ml of RPMI1640 medium and added into a 3 cm long dialysis tube (300 kDa cut-off).
3. The dialysis tube containing bacteria was incubated in 125 ml Capsenberg flasks containing 15 ml of 10 % v/v human blood serum in RPMI1640 medium at 200 rpm on a rotatory shaker at 37 ^oC for 2 h.
4. Afterwards, bacteria were quickly recovered from the dialysis tube, harvested by centrifugation at 3800 *g* for 2 min and subjected to RNA isolation.

2.2.1.6 Bacterial growth for RNA isolation (PMNs)

1. Bacterial cultures were grown in 15 ml of LB medium in 125 ml Capsenberg flasks at 300 rpm on a rotatory shaker at 37 ^oC up to the optical density 5.0 at 600 nm

(stationary phase). In order to measure OD this highly dense bacterial culture was diluted 1:10 with LB.

2. 2 x 10^{10} cells from this stationary phase culture were resuspended in 2 ml of RPMI1640 medium and added into a 3 cm long dialysis tube (300 kDa cut-off).
3. Simultaneously, PMNs were isolated from 30 ml of freshly drawn blood from a healthy donor. One aliquot of isolated PMNs was kept intact, whereas the second aliquot was lysed and subsequently both parts were resuspended in 15 ml of RPMI1640 medium containing 10 % v/v human blood serum (see chapter 2.6.1).
4. Then the dialysis tube containing bacteria was incubated in 125 ml Capsenberg flasks containing 15 ml of PMNs mixture at 200 rpm on a rotatory shaker at 37 ^{o}C for 2 h.
5. Afterwards, bacteria were quickly recovered from the dialysis tube, harvested by centrifugation at 3800 *g* for 2 min and subjected to RNA isolation.

2.2.1.7. Bacterial cell density determination

The optical density (OD) for the examined bacterial strains was measured spectrophotometrically at 600 nm (OD_{600}). The correlation between optical density and colony forming units (cfu) was adjusted according to Wiehlmann (2001):

Pseudomonas aeruginosa strains:	OD 0.6 ≈	1 x 10^9 cfu/ml
Escherichia coli strains:	OD 1.0 ≈	0,8 x 10^9 cfu/ml

2.2.1.8. Maintenance of bacterial cultures

Bacterial cultures were typically maintained on LB agar plates and stored for a limited period of 2-4 weeks at 4 ^{o}C. For long-term storage all bacterial cultures were kept in LB medium containing 15 % glycerol v/v and stored at −80 ^{o}C.

2.2.2. Isolation of DNA

2.2.2.1. Isolation of genomic DNA

The genomic DNA from *P. aeruginosa* was isolated according to the protocol by Chen and Kuo (1993).

1. The bacteria were harvested from 1.5 ml of overnight grown culture in LB medium by centrifugation at 14. 000 *g* for 3 min.
2. The pellet of harvested bacterial cells was resuspended in 300 μl of Lysis buffer.
3. 100 μl of 5 M NaCl were added to the suspension and the whole volume was mixed thoroughly.
4. Cell debris was separated by centrifugation at 14. 000 *g* for 60 min at 4 ^{o}C and the supernatant was transferred into a fresh eppendorf tube.
5. RNA was removed by incubation of the supernatant with 3 μl of RNase (10 mg/ml) at 37 ^{o}C for 30 min.
6. Proteins were removed by mixing of the supernatant with equal volumes of phenol, phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) and separation of the phases by centrifugation at 14. 000 *g* for 15 min.
7. DNA was precipitated by addition of an equal volume of isopropanol and subsequent centrifugation at 14.000 *g* for 15 min.
8. Finally the pellet of the genomic DNA was washed with 70 % v/v ethanol, dried and resuspended in 50 μl of TE buffer.

2.2.2.2. Isolation of plasmid DNA

To isolate ultra-pure plasmid DNA for sequencing, Qiagen Mini-Midi-Maxi and Giga Prep kits were used according to the manufacturer's protocol. The plasmid DNA from the transformed *E. coli* was isolated by the modified alkaline lysis method (Birnboim & Doly, 1979).

1. The bacteria were harvested from 3 ml of the overnight grown culture in LB medium by centrifugation at 5.000 *g* for 5 min.
2. The pellet of harvested bacterial cells was resuspended in 300 μl of solution I.

3. Then 300 μl solution II were added to the suspension, the whole volume was mixed thoroughly by inverting the eppendorf tube and incubated for 5 min at room temperature.
4. Afterwards, 300 μl solution III was added, mixed thoroughly and incubated on ice for 15 min.
5. The precipitate was centrifuged at 10.000 *g* for 10 min at 4 ^{o}C and the supernatant was transferred into a fresh eppendorf tube.
6. Proteins and lipids were removed by mixing of the supernatant with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and separation of the phases by centrifugation at 14000 *g* for 2 min at 4 ^{o}C. The aqueous phase was mixed with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 10.000 *g* for 10 min at 4 ^{o}C.
7. Plasmid DNA was precipitated from the solution by addition of an equal volume of isopropanol and subsequent centrifugation at 14.000 *g* for 15 min at room temperature.
8. Finally, the pellet of the plasmid DNA was washed with 70 % v/v ethanol, dried and resuspended in 50 μl of TE buffer.

2.2.3. Separation of DNA by agarose gel electrophoresis

1. The 0.8-1.5 % w/v agarose gel was prepared by solubilizing agarose in a proper volume of TBE buffer. 1x TBE buffer was used as a running buffer.
2. The DNA was mixed with 1/5th volume of loading buffer and loaded on an agarose gel. Lambda phage DNA digested with *Bst*EII restriction endonuclease was used as molecular size standard.
3. 8.5 V/cm field strength was applied and the gel was run until the front of the dye reached approximately 2/3rd of the gel length. Alternatively for overnight run, only 2.0 V/cm field strength was applied.
4. Afterwards, the gel was stained with 0.5 μg/ml ethidium bromide for 20-30 min and subsequently destained twice in water for 2x 20-30 min.
5. Finally the DNA was visualized and photographed on a UV transilluminator at 312 nm.

2.2.4. Quantification of DNA

If DNA is free of contaminants, spectrophotometric measurement of UV absorbance is used as a common method to quantify its amounts. The absorption was measured at 260 nm in a 1 cm thick quartz cuvette (Ausubel *et al.*, 1988; Sambrook *et al.*, 1989). Double distilled water was used as a control. The ratio of OD_{260} / OD_{280} provides the estimation of DNA purity and for the ultra-pure DNA equals 1.8.

Optical density (OD) of 1.0 at 260 nm corresponds to:

50 μg/ml of double stranded DNA

33 μg/ml of single stranded DNA

Therefore the concentration of the DNA can be easily calculated:

Concentration of DNA (μg/ml) = OD_{260} X 50 X dilution factor

2.2.5. Restriction digestion of DNA

The DNA was digested with the required restriction enzymes according to the manufacturer's protocols. Usually 10 U of the restriction enzyme were used per μg of DNA and digested for 3-24 h depending on the enzyme and the length of the DNA sequence at recommended temperature. Digestion with two different enzymes was performed in a buffer in which both restriction enzymes had their optimal intensity. Restriction enzymes were inactivated by heating or phenol-chloroform extraction.

Restriction digestion of the target sequences was pre-designed *in silico* with the webcutter 2.0 software (http://www.firstmarket.com/cutter/cut2.html).

2.2.6. Polymerase chain reaction

Polymerase chain reaction (PCR) is a simple and efficient method that exploits knowledge of the principles of DNA replication for amplification of target DNA sequences. When using this technique, the DNA segment between two regions of known sequence is amplified by the polymerase if the specific primers and template are added to the reaction (Mullis *et al.,* 1986). In this work, PCR was used for the amplification of target sequences of *P. aeruginosa* genes for the preparation of probes for Northern hybridisation and for the amplification of whole ORFs of the analysed *P. aeruginosa* regulatory genes.

2.2.6.1. Construction of the primers for PCR

1. The primer was constructed to be solely complementary to the target DNA sequence so that it could not hybridise to any other sequence in the genome.
2. Care was taken that the primer was not self-complementary to avoid formation of hairpin structures.
3. The melting temperature (T_m), calculated as $T_m = 4\ ^oC x\ \Sigma(GC) + 2\ ^oC x\ \Sigma(AT)$ for each primer constructed was higher than 60 ^oC to avoid non-specific amplification.
4. The length of the primers was approximately 20-25 base pairs.
5. Primers ended with at least two G-C bases to ensure stable hybridisation.
6. Primer 3 software was usually used to construct primers (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).
7. Two different restriction sites (one in each primer from the respective primer pair) were incorporated into the primers used for the PCR of the whole ORFs used for complementation.

2.2.6.2. Reaction mixtures used for PCR

Standard reaction mixtures used for PCR:

0.5 μl	Template DNA (50- 100 ng/μl)
2.5 μl	Primer 1 (5 μM)
2.5 μl	Primer 2 (5 μM)
2.5 μl	10x Buffer (InViTek)
1.25 μl	DMSO
0.75 μl	$MgCl_2$ (50 mM)
2.5 μl	dNTPs (8 mM)
0.2 μl	*Taq*-DNA Polymerase (5 U/μl)
12.3 μl	Double distilled water

Gold star polymerase was used for the amplification of long ORFs.

2.2.6.3. Program used for PCR

The following program was usually used for the DNA amplification:

Denaturation	at 98 ^{o}C for 10 min
Amplification (30-40 cycles)	
Denaturation	at 98 ^{o}C for 30 s
Primer extension	at Tm for for 60 s
DNA synthesis	at 72 ^{o}C for 60 s
Incubation	at 72 ^{o}C for 10 min
Maintenance	at 4 ^{o}C

For the amplification of long ORFs, the program was slightly modified:

Denaturation	at 98 ^{o}C for 10 min
Amplification (30-40 cycles)	
Denaturation	at 98 ^{o}C for 90 s
Primer extension	at Tm for for 60 s
DNA synthesis	at 72 ^{o}C for 120- 140 s
Incubation	at 72 ^{o}C for 10 min
Maintenance	at 4 ^{o}C

2.3. Genetic complementation

A library of oligonucleotide-tagged mini-Tn*5* transposon mutants of *P. aeruginosa* strain TB was constructed by a modified signature tagged mutagenesis protocol (Wiehlmann *et al.,* 2002) and screened for increased susceptibility to human serum and PMNs or for loss of quorum sensing. Four virulence-attenuated mutants with predicted strong impact on the expression of other genes were selected for the future experiments. Subsequently these pre-selected regulatory genes were complemented *in trans* to ensure that the observed striking phenotypes were caused by the transposon inactivation of the respective genes and not by any other secondary genetic event.

2.3.1. Preparation of the ORFs for ligation

1. The open reading frames (ORFs) intended to be cloned into a suitable vector were first amplified by PCR (see chapter 2.2.6).
2. The concentration of the PCR product was estimated with 5 μl by agarose gel electrophoresis (see chapter 2.2.3).
3. Optional: The PCR product was purified using Qiaquick GelExtraction Kit (Qiagen) or PCR purification Kit (Qiagen) according to the manufacturer's instruction to remove polymerase enzyme which might block the restriction sites.
4. The ORFs were subjected to restriction digestion (see chapter 2.2.5). 20 μl of the PCR amplificate of the respective gene, together with 2x 2 μl of both restriction enzymes, 4 μl of the recommended buffer and 12 μl of double distilled water were incubated at 37 ^{o}C for 3 hours.
5. The digested ORFs were purified using Qiaquick GelExtraction Kit (Qiagen) or PCR purification Kit (Qiagen) according to the manufacturer's instruction to remove restriction enzymes, which might block the ligation sites of the DNA.

2.3.2. Preparation of the vector for ligation

Plasmid pME6010 was used as a vector for the complementation. This shuttle vector was chosen due to its ability to replicate in both *Esccherichia coli* and *P. aeruginosa* (Heeb *et al.,* 2000). pME6010 maintains the tetracycline resistance genes so the

successful complementants could be easily detected. The multi cloning site (MCS) of pME6010 is following the kanamycin promoter which was used to induce the expression of the cloned genes (Figure 2.1).

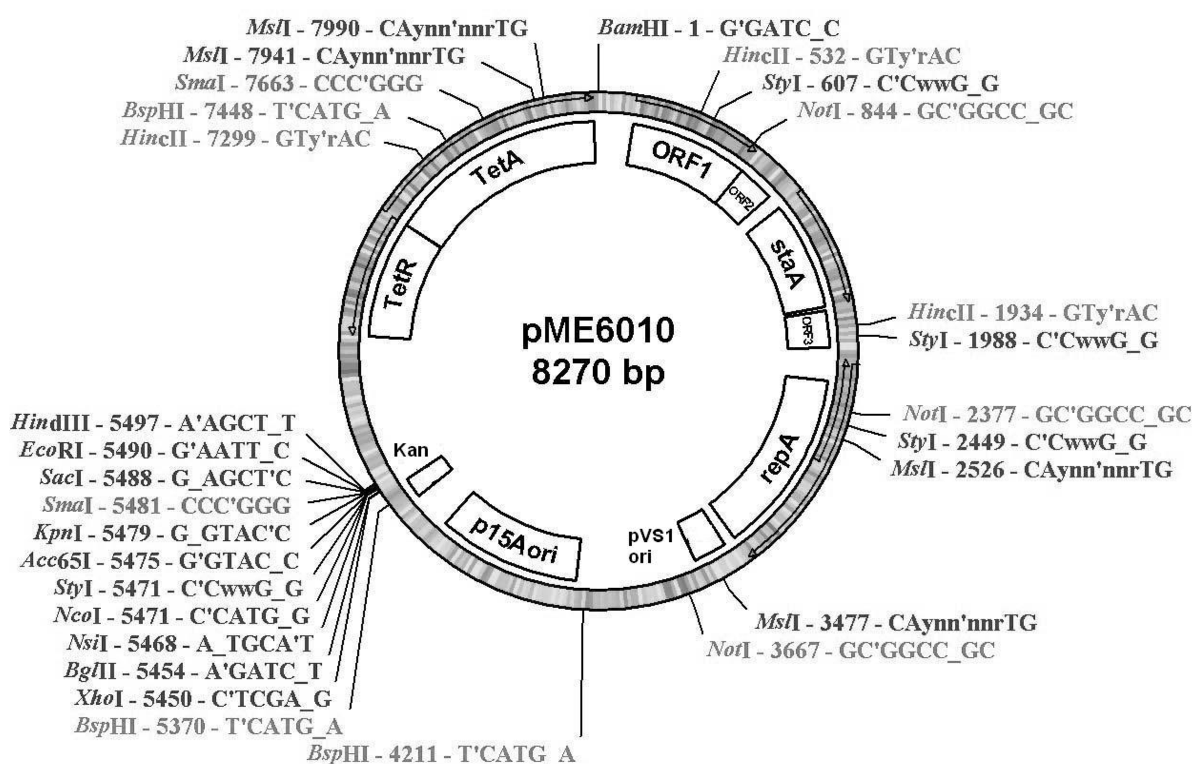


Fig. 2.1. The restriction map of the pME6010. This plasmid was used as a vector for the complementation of investigated genes (http://www.acaclone.com).

1. The pME6010 was isolated from the *E. coli* culture (see chapter 2.2.2.2) and concentrated 10 times by isopropanol concentration: the plasmid was precipitated from the solution by addition of the equal volume of isopropanol and subsequent centrifugation at 14.000 *g* for 15 min at room temperature, washed with 70 % ethanol v/v, dried and resuspended in double distilled water or TE buffer.
2. The plasmid was subjected to restriction digestion (see chapter 2.2.5). 20 µl of the 10 times concentrated vector with 2x 2.5 µl of both restriction enzymes, 5 µl of the recommended buffer and 20 µl of double distilled water was incubated at 37 ^{o}C for at least 16 hours.

3. The restriction enzymes were removed from the plasmid by phenol/ chloroform/ isoamylalkohol extraction: Suspension from the step 2. was mixed with equal volumes of phenol and chloroform:isoamylalkohol (24:1) and the phases were separated by centrifugation at 14. 000 *g* for 10 min. The aqueous phase with pME6010 plasmid was transferred into a fresh eppendorf tube.
4. The plasmid was dephosphorylated by addition of 4 μl of alkaline phosphatase and 6 μl of alkaline phosphatase buffer to the 50 μl of the suspension and subsequent incubation at 37 ^{o}C for 1 hour.
5. Optional: second phenol/ chloroform/ isoamylalkohol extraction of the plasmid (see step 3).
6. Optional: second dephosphorylation of the vector (see step 4).
7. Optional: third phenol/ chloroform/ isoamylalkohol extraction of the plasmid (see step 3).
8. The plasmid was purified using Qiaquick GelExtraction Kit according to the manufacturer's instruction.

2.3.3. Ligation

Different vector: insert molar ratios were tested to obtain maximal ligation.

Standard ligation mixtures:

1 μl	T4 ligase
1 μl	T4 ligase buffer
1- 4 μl	Insert
1- 6 μl	Vector
1- 4 μl	Double distilled water

Optional: The successfulness of the ligation was checked with 2 μl of the ligation mixture by agarose gel electrophoresis (see chapter 2.2.3). Lower agarose concentrations (0.5 % - 1 %) were used to separate the fragments with higher molecular weights (Figure 2.2).

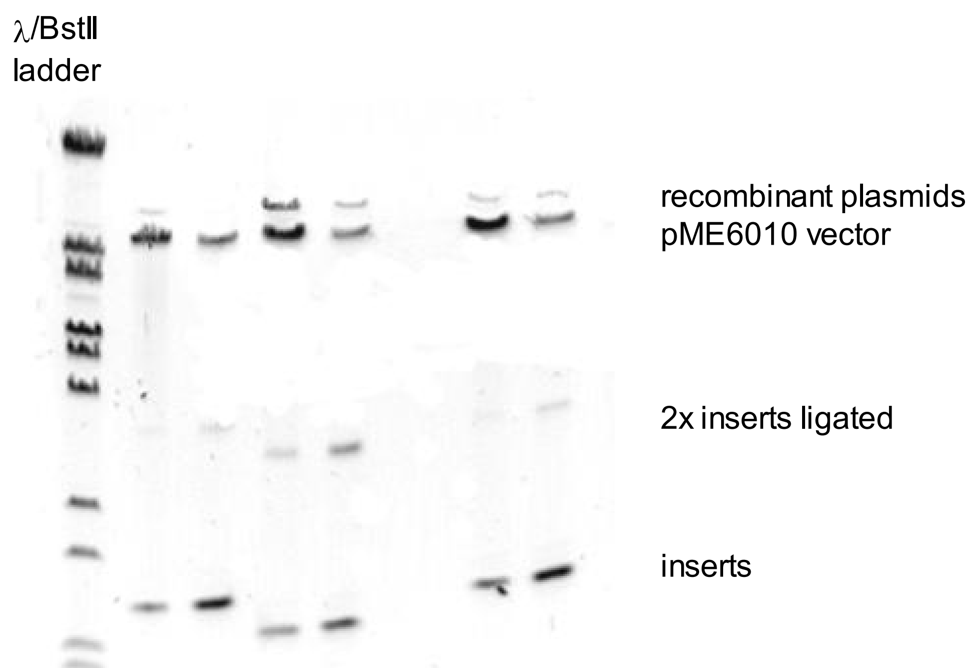


Fig. 2.2. Testing successfulness of the ligation by agarose gel electrophoresis. Different vector: insert molar ratios were examined to obtain maximal quantities of the recombinant plasmid.

2.3.4. Transformation of *E. coli*

One Shot® TOP10 Chemically competent *E. coli* (Invitrogen) were used for the transformation.

1. 3 μl of the ligation product were added to 10 μl of *E. coli* cells, mixed gently and incubated for 30 min on ice.
2. The tubes were incubated at 42 ^{o}C for exactly 30 s and subsequently placed on ice.
3. 100 μl of the pre-warmed S.O.C medium was added to the tube and incubated at 250 rpm at 37 ^{o}C for 1 hour.
4. The mixture was spread on LB plate containing Tc, the plate was inverted and incubated at 37 ^{o}C overnight.
5. Colonies were analysed for the presence of the constructed recombinant plasmid by plasmid isolation (see chapter 2.2.2.2).

2.3.5. Electroporation of *P. aeruginosa*

A modified protocol for the electroporation of freshly plated *P. aeruginosa* cells was used (Enderle and Farwell, 1998).

1. *P. aeruginosa* strains to be complemented were cultivated in 4 ml of LB medium at 250 rpm at 37 ^{o}C overnight and pelleted by centrifugation at 13. 000 *g* for 3 min.
2. To achieve successful electroporation, the amount of exopolysaccharides in the culture needs to be as low as possible so they were removed from the pellet with repeated washing with double distilled water and centrifugation at 13. 000 *g* for 3 min.
3. Bacterial cells were resuspended in 200 μl of double distilled water.
4. 90 μl of this suspension was mixed with 1-5 μl of the constructed recombinant plasmid isolated from *E. coli* using Qiagen Mini Prep kit according to the manufacturer's protocol.
5. The mixture was poured in the electroporation cuvette and the *P. aeruginosa* cells were electroporated with the Bio-Rad pulser (400 Ω, 25 μFD, 2.5 V, time constant 5 ms).
6. 0.5 ml of the LB medium was added in the cuvette and the whole volume was transferred into the fresh eppendorf tube.
7. Bacteria were incubated at 300 rpm at 37 ^{o}C for 3 hours.
8. Afterwards, 50-200 μl of the bacterial culture were spread on LB plate containing Tc, the plate was inverted and incubated at 37 ^{o}C overnight.
9. *P. aeruginosa* colonies were analysed for the presence of the recombinant plasmid by plasmid isolation (see chapter 2.2.2.2).

2.4. RNA-working techniques

2.4.1. RNA storage and handling

Special care was taken when working with RNA to avoid degradation by RNases.

1. All solutions used in the RNA-working techniques were prepared from double distilled water and treated with 0.05 % v/v Diethylpyrocarbonate (DEPC). After addition of DEPC, solutions were incubated overnight at 37 ^{o}C and subsequently autoclaved to inactivate DEPC. Those solutions containing Tris or Acetate which can cause undesired cross-reactions with DEPC were prepared directly from the already autoclaved DEPC treated double distilled water.
2. The majority of the devices used in the RNA-working techniques was autoclaved at 121 ^{o}C for 60 min. Glass wares were sterilized at 250 ^{o}C for 5 h. Metal devices such as scalpels or scissors were cleaned with 70 % v/v ethanol and flame-sterilized.
3. Heat-sensitive devices were first cleaned with sterile double distilled water and afterwards with 70 % v/v ethanol.
4. Gel chambers for electrophoresis of RNA samples were first filled with 3 % v/v H_2O_2 and afterwards with 70 % v/v ethanol.
5. For long-term storage, RNA was dissolved in DEPC treated water or in RNase-free buffer and stored in the –80 ^{o}C freezer. During work, RNA samples were kept on ice and treated with RNase inhibitors when required.
6. Fresh stocks of plasticware, such as eppendorf tubes or pipette tips were used every time.

2.4.2. Quantification of RNA

If RNA is free of contaminants, spectrophotometric measurement of UV absorbance is used as a common method to quantify its amounts. The absorption was measured at 260 nm in a 1 cm thick quartz cuvette (Ausubel *et al.*, 1988; Sambrook *et al.*, 1989). Double distilled water was used as a control. The ratio of OD_{260} / OD_{280} provides the estimation of RNA purity and for the ultra-pure RNA equals 2.0.

Optical density (OD) of 1.0 at 260 nm corresponds to: 40 μg/ml of RNA

Therefore the concentration of the RNA can be easily calculated:

Concentration of RNA (μg/ml) = OD_{260} X 40 X dilution factor

2.4.3. RNA isolation

The protocol used for the isolation of total RNA was based on the modified hot phenol method (Oelmüller *et al.,* 1990; Tao *et al.*, 1999). All steps were performed at 4 ^{o}C

1. Approximately 3 x 10^{10} of bacterial cells were harvested by centrifugation at 3800 *g* for 2-4 min at 4 ^{o}C.
2. Harvested cells were quickly resuspended in 0.5 ml distilled water and lysed at 65 ^{o}C in 7.5 ml of preheated 5 ml phenol/ 2.5 ml RNA lysis buffer mixture (pH 5.5) by vigorous shaking for 10 min.
3. The cell lysate was centrifuged at 3800 *g* for 20 min and the supernatant was purified by subsequent phenol/chloroform (3-5 of phenol:chloroform:isoamyl alcohol mixture, 25:24:1 v/v) and chloroform (chloroform:isoamyl alcohol, 24:1 v/v) extractions.
4. Nucleic acids were precipitated overnight at −20 ^{o}C in 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol and centrifuged at 3800 *g* for 30 min.
5. The pellet was washed with 5 ml of 70 % v/v ethanol and resuspended in 175 µl of DEPC-treated water.
6. DNA was digested by the addition of 40 U DNase I and 20 U SUPERaseIn in DNase I buffer in a total volume of 200 µl for 30 min at 37 ^{o}C.
7. RNA purification with RNeasy columns (Qiagen) was performed according to the manufacturer’s instructions and was followed by quantification of its total yield by the UV absorption spectrometry at 260 nm.
8. RNA integrity was monitored by formaldehyde agarose gel electrophoresis.

2.4.4. Formaldehyde agarose gel electrophoresis

Only denatured RNA can be separated as a function of its length by gel electrophoresis. To keep RNA in denaturated state during electrophoresis, the gel solution as well as the running buffer were treated with formaldehyde. (Ausubel *et al.*, 1988; modified). To monitor RNA integrity electrophoresis was performed in mini gel chambers (5 x 7 cm) with the gel volume of 40 ml. If the RNA was transferred to a nylon membrane for hybridisation afterwards, large blot gel chambers (8 x 11 cm) with the gel volume of 220 ml were used. The chambers were first cleaned with 3 %

v/v H_2O_2 and subsequently with 70% v/v ethanol. Only RNase free agarose (SeaKemGTG, Biozym) was used to prepare gels: 1.2 % w/v and 2 % w/v for mini gels and blot gels, respectively. Due to the toxicity of formaldehyde, electrophoresis was running in a fume hood.

Table 2.1. Sample preparation for formaldehyde gel electrophoresis of RNA.

Mini-gel	Blot-gel	Components
2 µl	10 µg	RNA
	x µl	Double distilled water
	0.5 µl	Ethidium bromide (10 mg/ml)
2 µl	10 µl	Formaldehyde (37 %, w/v)
5 µl	25 µl	Formamide
2 µl	9.5 µl	RNA-loading buffer
1 µl	5 µl	MOPS-buffer (10 x)
12 µl	60 µl	

1. The agarose was autoclaved for 30 min at 121 ^oC in 1x MOPS buffer and cooled down to approximately 50 ^oC.
2. 2.4 ml or 13.3 ml of formaldehyde (0.7 M final concentration) were added to mini or blot gel, respectively.
3. The RNA samples were denatured at 65 ^oC for 10 min, cooled down on ice and centrifuged at 14.000 *g* for 30 s at 4 ^oC.
4. Samples (Table 2.1), together with the 5 µg of RNA molecular weight standard were loaded on the formaldehyde agarose gel with 1x MOPS buffer used as running buffer after the pre-electrophoresis running for 5 min at 60 V.
5. Constant field strength 5 V/cm was applied for mini gels, thus running for approximately 1 h and 3 V/cm for blot gels, thus running for approximately 5 h. The electrophoresis of the blot gels was finished when the bromphenol blue dye had moved to the two-thirds of the gel length.
6. The mini gels were stained for 15 min with 10 µg/ml ethidium bromide, destained twice for at least 15 min and photographed under the UV transilluminator.
7. After the electrophoresis, the blot gel was photographed under UV transilluminator with a molecular weight standard using 16S and 23S ribosomal standards as a reference for the RNA integrity checking (Figure 2.3).

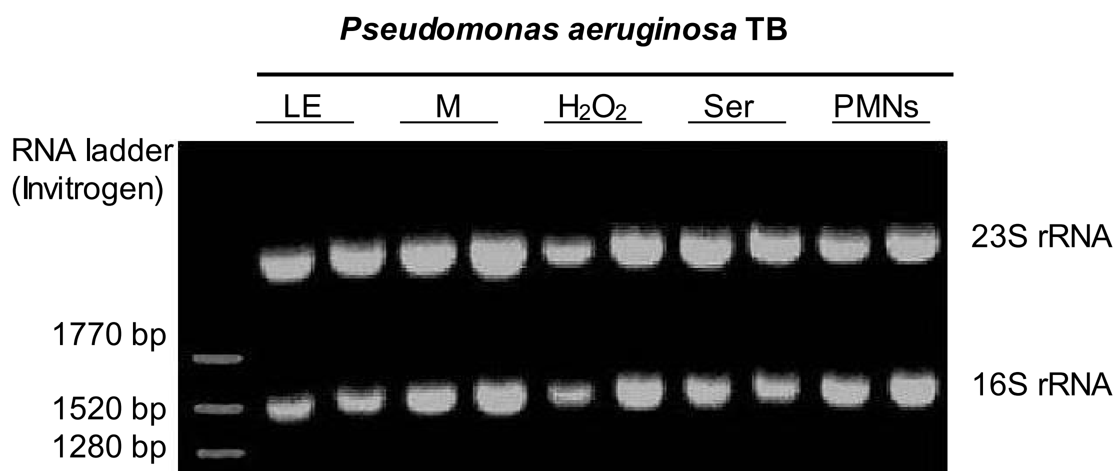


Fig. 2.3. Formaldehyde agarose gel electrophoresis of RNA isolated under different growth conditions. The molecular weights of 23S and 16S rRNA are 2985 & 1536 bp respectively (Stover *et al.*, 2000). Isolated RNA was subsequently transferred to a positively charged nylon membrane for hybridisation. LE: late exponential phase, M: ABC minimal medium, H_2O_2: presence of 10 mM H_2O_2, Ser: presence of 10 % serum, PMNs: presence of PMNs.

2.4.5. Northern blotting

To detect the intensity of the gene expression via RNA-DNA hybridisation procedure, total RNA, completely denatured and separated by formaldehyde agarose gel electrophoresis was transferred on a positively charged nylon membrane (Hybond N^+) (Ausubel *et al.*, 1988). Capillary blotting is the most frequently used procedure of the transfer and immobilization of the RNA fragments. In this approach, the formaldehyde agarose gel is held on the porous paper (Whatman filter paper), which is emerged into a reservoir containing transfer buffer. This procedure is also called the sandwich method, because the membrane is localized between the gel and filter papers, which soaks buffer through the gel. The RNA is transferred from the gel and immobilised on the membrane by the flow of transfer buffer.

1. The blot gel was washed with the double distilled water for 20 min and subsequently with 20x SSC buffer for 20 min prior to blotting apparatus assembly.

2. A Blotting apparatus: The gel was placed upside down on the Whatman filter paper bridge. The positively charged nylon membrane was kept between the gel,

another Whatman filter paper and the pile of papers. Nylon membrane, Whatman filter paper and the pile of papers used were the same size as the gel. The iron plate (500 g) was placed on the top of the apparatus and the 20x SSC buffer was added in the bath to serve as a transfer buffer.

3. Blotting was performed at room temperature for at least 20 h.
4. Afterwards the position of the wells on the gel was marked on the membrane and the membrane was removed and washed with 2x SSC buffer for 1 min to remove residual gel traces.
5. The membrane was placed in the filter paper and aluminium foil and dried at room temperature for 10 min and subsequently at 65 ^{o}C for 20 min.
6. Finally the membrane was crosslinked from both sides with UV 254 nm and used immediately for hybridisation or stored at −20 ^{o}C for future use.

2.4.6. Construction of the DNA probes for Northern blotting

The DNA probes for Northern blot hybridisation were constructed by PCR amplification. The primers for PCR amplification were constructed so that they resulted in the amplification of approximately 500-600 bp long DNA fragments from the ORFs of investigated genes.

The PCR reaction mixture containing the amplified sequence was separated by agarose gel electrophoresis and the target sequence was recovered from the gel by the method described by Qiagen (Hilden, Germany). All the centrifugation steps were carried out at 10.000 *g* at room temperature.

1. At least 45 μl of the PCR mixture were loaded in the each well of the gel and the DNA was separated by agarose gel electrophoresis.
2. Afterwards, the gel was stained with ethidium bromide, destained and the DNA fragments were visualized under the UV transilluminator.
3. The desired DNA fragment was excised from the gel with the scalpel and placed in the eppendorf tube.
4. Three volumes of solubilization buffer QG (Qiagen) were added to 1 volume of the excised gel fragment and the eppendorf tubes were incubated at 50 ^{o}C for 10 min or until the gel slice completely dissolved.
5. One volume of isopropanol was added to the mixture and the contents of the tube was mixed by inverting the tube for several times.

6. The whole volume of this mixture was afterwards applied to a MiniElute column (Qiagen) and centrifuged for 1 min. Maximum 800 μl can be applied on the column at once so if I had higher volumes of the mixture, the flow-through was discarded and the same column was re-used for the same sample.
7. The column was washed with 500 μl of solubilization buffer QG and subsequently with 750 μl of wash buffer PE (Qiagen).
8. Then the column was centrifuged for an additional 1 min to remove residual wash buffer PE.
9. 10 μl of elution buffer EB (Qiagen) were applied in the centre of the membrane of the column and finally the DNA was eluted by centrifugation of the column for 1 min.

2.4.7. Random primer labelling and purification of the probes

Random primer labelling (Feinberg and Vogelstein, 1983) exploits the ability of the *E.coli* DNA polymerase I-Klenow fragment to synthesize a new DNA strand complementary to the template DNA strand starting from a free 3' hydroxyl end in order to label both strands simultaneously. The hexanucleotides, which bind with statistical efficiency to the DNA and thus serve as starting primers for the Klenow fragment of the DNA polymerase I, are exploited in the random primer labelling. Digoxigenin-labelled nucleotides added in the reaction are then incorporated into the DNA strands. This procedure ensures uniform labelling of the DNA probes and is widely used due to its high efficiency requiring only small amount of the template DNA. The DNA was labelled by using the kit supplied by Roche Molecular Biochemicals.

1. Prior to starting labelling the DNA fragments were denaturated by boiling at 95 ^{o}C for 10 min and cooled down on ice.
2. To label the DNA, the following components were added to the reaction:

15 μl	Denatured DNA
2 μl	DIG DNA labelling mix (10x)
2 μl	Hexanucleotide mix (10x)
1 μl	Klenow enzyme

3. The reaction mixture was incubated overnight at 37 ^{o}C.
4. To purify the labelled DNA, a Sephadex G50 column was prepared in a 1 ml syringe and equilibrated with TE buffer and subsequently centrifuged at 1000 *g* for 45 s to remove the residual buffer.
5. 72 µl of TE buffer and 8 µl of the colour solution for gel filtration were added to the labelled DNA mixture and loaded on the Sephadex column. The eppendorf tube was placed at the bottom of the column.
6. The column was centrifuged at 1000 *g* for 30 s and the labelled and purified DNA was recovered in the eppendorf tube at the bottom of the column.
7. The DNA was used immediately for Northern blotting or stored at −20 ^{o}C for the future use.

2.4.8. RNA-DNA hybridisation

1. Northern hybridisation was performed in a hybridisaton oven (Biometra) in a big glass tubes (Biometra) or alternatively in 50 ml plastic Falcon tubes. The membrane was placed into the tube with the RNA adhered side facing inward of the tube. The prehybridization buffer was preheated to 42 ^{o}C prior to the procedure.
2. 25 ml or 10 ml of the prehybridisation buffer for the glass and for the 50 ml plastic tubes, respectively were added in the tube and prehybridised 42 ^{o}C for 2 h.
3. The Dig labelled DNA probe for Northern blotting was denatured by boiling at 95 ^{o}C for 10 min in a 10 ml of prehybridization buffer and subsequently added into tube containing membrane.
4. The hybridisation was carried out for at least 16 hours at 42 ^{o}C.
5. After hybridization, the probe was saved for future use at −20 ^{o}C and the hybridisation signals on the membrane were detected.

2.4.9. Detection of Northern blot hybridisation signals

After hybridization, the hybridisation signals on the membrane were detected by immunological reaction with an anti-fluorescein alkaline phosphatase conjugate (Engler-Blum, 1990). The DIG labelled DNA probe exhibit fluorescence signals with the help of anti-Dioxigenin-dUTP antibodies. The Alkaline Phosphatase catalyzes a

chemo-luminescence reaction with CDP Star$^{(TM)}$ and emitted light (480nm) can be detected by exposing of the membrane to X-ray film.

1. To remove non-specifically bound probe, the membrane was washed for 5 min with 6 x SSC + 3 % w/v SDS at room temperature, for 20 min with 2 x SSC + 3 % w/v SDS at 42 ^{o}C and for 20 min with 0.2 x SSC + 3 % w/v SDS at 42 ^{o}C subsequently.
2. After these washes, the membrane was removed from the tube and equilibrated in buffer I for 5 min.
3. To remove non-specific bound probe, the membrane was incubated on a shaker in 170 ml of buffer II containing 0.5 % w/v of Blocking reagent for 30 min.
4. Then, the membrane was incubated in 30 ml of buffer II containing 0.5 % w/v of Blocking reagent and 1:10.000 dilution of Anti-fluorescein-AP conjugate for 30 min.
5. To remove the unbound conjugate, the membrane was washed 3 times for 15 min in buffer I.
6. The membrane was equilibrated in a filtered buffer III for 2 min and subsequently incubated with 1: 500 v/v diluted CDP star in 10 ml of filtered buffer III for 5 min.
7. X-OMAT or Bio-MAX (Kodak) films were used to detect chemoluminescence. The maximum signal intensity was usually detected after approximately 30 min of exposition of the X-ray film to luminescent signals, but generally the time of exposure was adjusted depending upon the signal intensity (Bronstein *et al.*, 1989).

2.4.10. Regeneration of the membranes

The hybridised Northern blot membranes can be re-used after washing by SSC and SDS solutions, even though the quality of this regeneration is not ideal and therefore it is recommended to use fresh membrane for each hybridisation.

1. The hybridised membranes were washed with double distilled water for 5 min.
2. Then the membranes were incubated in 5 x SSC for 20 min and subsequently washed with nearly boiling (95 ^{o}C) 0.1 % SDS solution for 5 min.

2.5. *P. aeruginosa* microarrays (Affymetrix)

2.5.1. *P. aeruginosa* GeneChip

The *P. aeruginosa* microarray (GeneChip) from Affymetrix used in this work was designed by the Cystic Fibrosis Foundation Therapeutics Inc. (CFFTI, USA). The construction of the chip was based on the information from the *P. aeruginosa* genome-sequencing project (Stover *et al.*, 2000) financed by CFFTI. There are probes for 5549 open reading frames on the *P. aeruginosa* GeneChip (Affymetrix), and additional probes for one out of four rRNA operons (23S, 16S, 5S rRNA), 18 tRNA genes and for 199 selected intergenic regions exceeding 600 base pairs from the completely sequenced and annotated genome of *P. aeruginosa* PAO1 (Stover *et al.*, 2000). Moreover, probes from 117 open reading frames of other *P. aeruginosa* strains are also present on the GeneChip. All in all, 5986 different *P. aeruginosa* genome sequences can be analyzed on the GeneChip.

As a control, the GeneChip also contains 14 genes from *Bacillus subtilis, Saccharomyces cerevisiae* and *Arabidopsis thaliana*. The probes present on this GeneChip are based on the sequences from the GENE BANK database available at http://www.ncbi.nlm.nih.gov. The detailed information for the construction of the GeneChip can be also obtained from the NETAFFX database available at http://www.affymetrix.com/analysis/index.affx.

Sequences on the GeneChip from Affymetrix which can be examined are represented by a varying number of 25-mer oligonucleotides correspondingly to their lengths (Lipshutz *et al.*, 1999). Each protein-coding gene on the *P. aeruginosa* Genechip represent 8-13 of such oligonucleotides, while tRNA genes are covered with 8-16 and intergenic regions with 13-122 oligonucleotides.

Exploiting special photo-lithographic technology in combination with solid phase combinatorial chemistry each oligonucleotide is synthesized as a probe cell *in situ* on a glass surface of 20 µm x 20 µm (Fodor *et al.*, 1991). The probe cell complementary with the analysed sequence (perfect match oligo) is synthesized right beside the oligonucleotide, which has incorporated a non-complementing base pair in the position 13 (mismatch oligo). Perfect match and mismatch oligo constitute the probe pair. The signal intensity of the mismatch oligo represents the background signal of the specific hybridisation, therefore it is subtracted from the signal intensity of the perfect match oligo in the following step of an *in silico* hybridisation analysis. All probe pairs on the GeneChip representing specific sequence are called the probe set

(Figure 2.4). The probe pairs of the same probe set are distributed randomly on the 1.28 cm x 1.28 cm surface of the Gene Chip, thus minimizing the negative effect of the possible local irregularities (non-uniform hybridization and staining or mechanical damages, e.g. scratches) caused during hybridization.

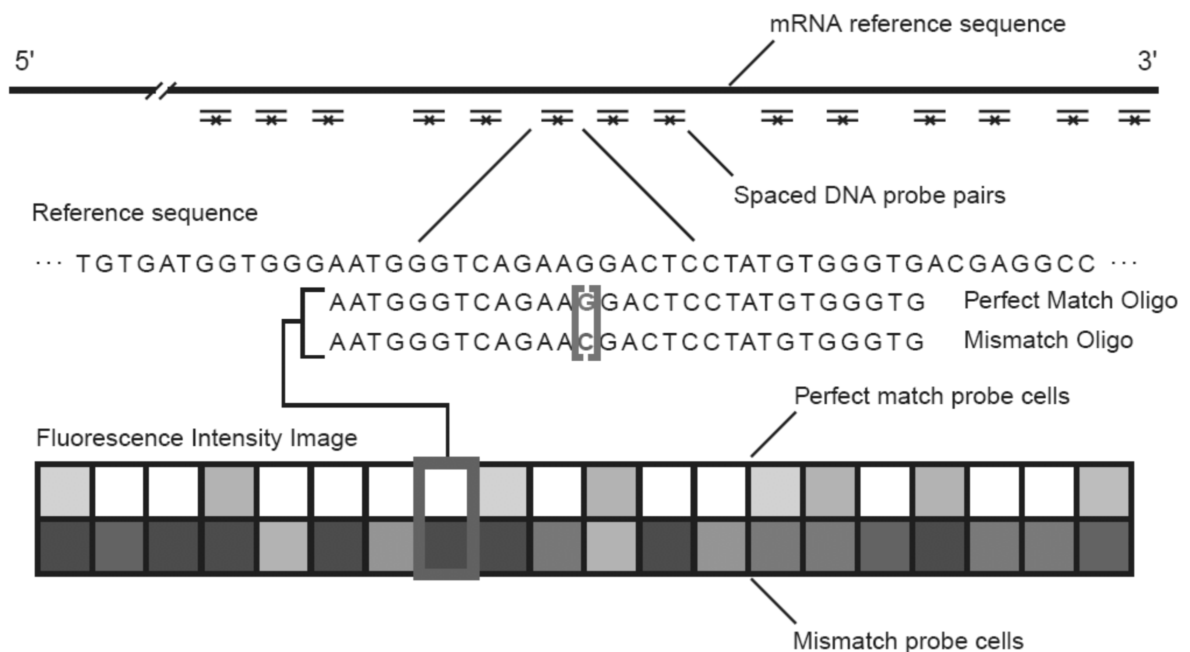


Fig. 2.4. The construction of the probe set on the GeneChip.

A single gene on the GeneChip is represented by the probe set of 25-mer oligonucleotides. Each probe pair constitutes an oligonucleotide perfectly complementing to the analysed sequence (perfect match oligo) and an oligonucleotide with base pair exchange on the position 13 (mismatch oligo) (Lipshutz *et al.*, 1999).

2.5.2. Generation and fragmentation of cDNA

RNA from three independent isolations was pooled to minimize the influence of handling. cDNA synthesis, fragmentation and labelling with Biotin-ddUTP were performed as described in the protocol provided by Affymetrix. Briefly, 10 µg of RNA was mixed together with 750 ng random primers Invitrogen and 8.67 pM from 10 nonpseudomonal control transcripts (kindly provided by S. Lory and co-workers, Harvard) (Table 2.2) in a total volume of 30 µl.

Table 2.2. Nonpseudomonal control transcripts (S. Lory, Harvard).

Gene	Bacterial strain	Length (bp)	Final conc. (pM)
YEL002C/WBP1	*Saccharomyces cerevisiae*	1300	1
YEL018W	*Saccharomyces cerevisiae*	840	2
YEL024W/RIP1	*Saccharomyces cerevisiae*	650	10
YER148W/SPT15	*Saccharomyces cerevisiae*	720	5
dapB	*Bacillus subtilis*	740	1
lysA	*Bacillus subtilis*	1320	5
pheB	*Bacillus subtilis*	440	10
thrC	*Bacillus subtilis*	1100	2
trpD	*Bacillus subtilis*	1000	2
GAPDH	*Arabidopsis thaliana*	1000	5

This reaction mixture was incubated for 10 min at 70 °C and 10 min at 25 °C and subsequently chilled to 4 °C. The cDNA mixture was added to the reaction mixture containing RNA. cDNA was generated by incubating this reaction mixture for 10 min at 25 °C, for 60 min at 37 °C, for 60 min at 42 °C, and subsequently the enzyme was inactivated by incubating for 10 min at 70 °C.

RNA was hydrolyzed by the addition of 20 µl 1M NaOH and incubation for 30 min at 65 °C, followed by neutralization of the reaction by 20 µl of 1M HCl. The generated cDNA was purified with a Qiaquick column from Qiagen and quantified by UV absorption at 260 nm.

The 3-5 µg of cDNA was fragmented with 0.5 U DNaseI μg^{-1} cDNA in One Phor-All buffer in a total volume of 50 µl Amersham Pharmacia Biotech for 10 min at 37 °C and the reaction was stopped by 10 min incubation at 98 °C. Gel electrophoresis of the 5 µl of fragmented product and staining with 2 % SYBRGreen (Molecular probes) for 50 min was performed to ensure that the majority of cDNA fragments was within the required 50-200 bp range (Figure 2.5).

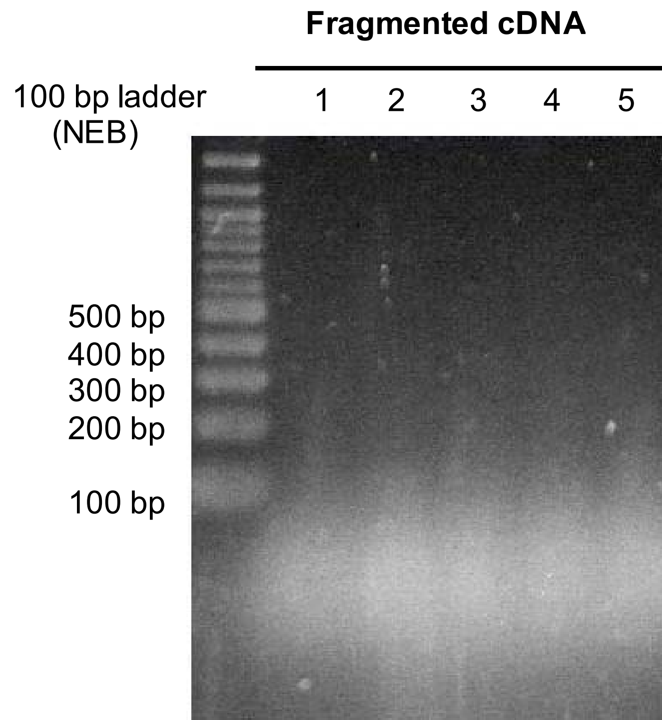


Fig. 2.5. Representative SYBR-Green stained agarose gel with fragmented cDNA.
For the hybridisation of GeneChips, cDNA fragments with a size of approximately 50-200 bp are optimal, therefore the majority of the cDNA fragments after fragmentation has this required size.

2.5.3. Labelling of fragmented cDNA with biotin

Enzo BioArray^{Tm} Terminal Labelling Kit with Biotin-ddUTP was used for terminal labelling of generated cDNA fragments. For this purpose, 3-5 µg of fragmentation product was mixed with 5x Reaction Buffer, 10x $CoCl_2$ solution, 1 µl Biotin-ddUTP and 2 µl terminal deoxynucleotide transferase in a total volume of 60 µl and incubated at 37 ^oC for 60 min. Subsequently, the reaction was stopped by addition of 2 µl of 0.5 M EDTA. Such labelled fragmented cDNA was ready for immediate hybridisation onto GeneChips or could be stored for future use at – 20 ^oC.

2.5.4. Hybridisation of biotin-labelled cDNA to GeneChips

GeneChips are normally stored at 4 ^oC, therefore it is necessary to equilibrate them at room temperature before starting the hybridisation process. The hybridization

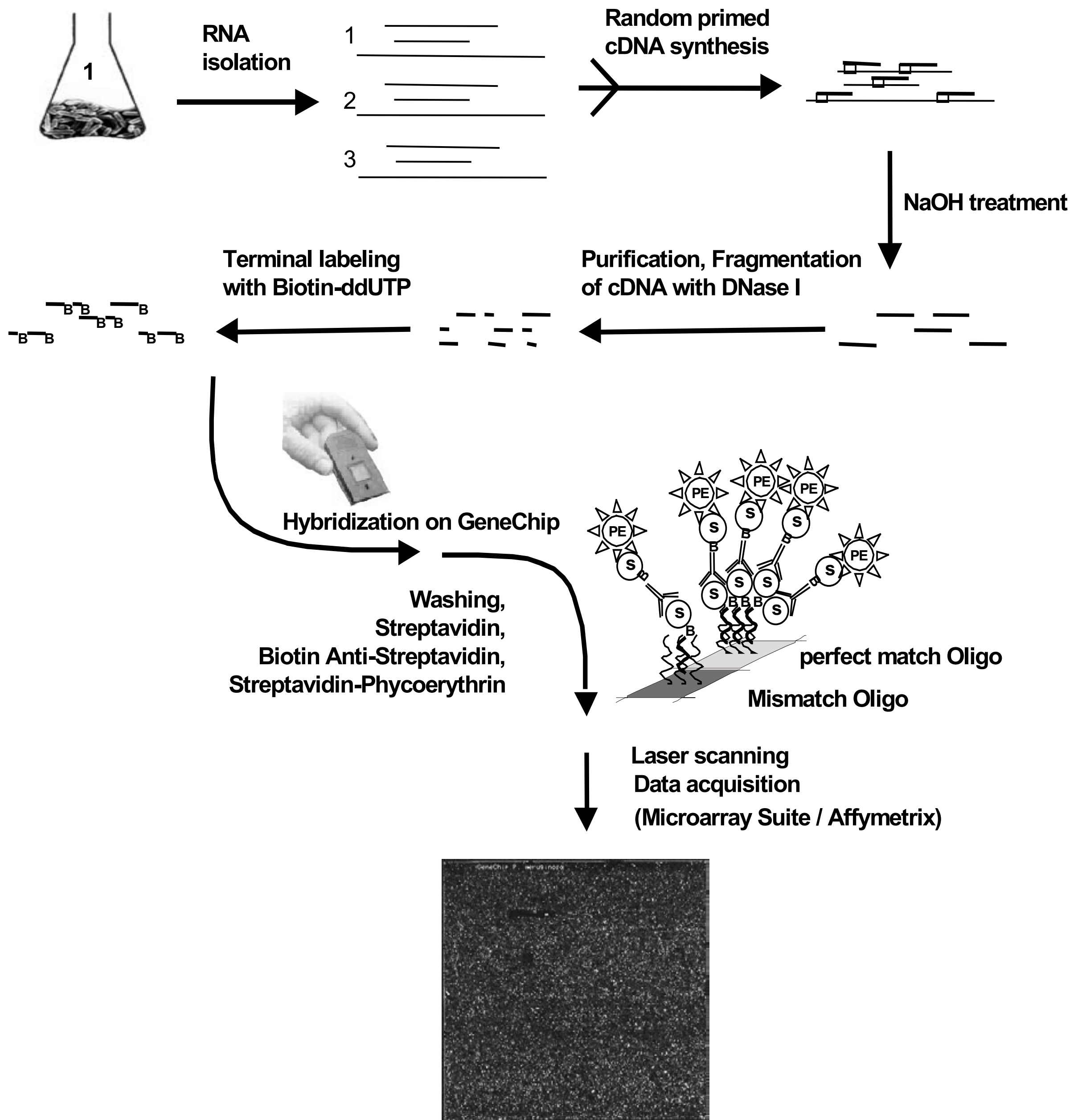


Fig. 2.6. Overview of all steps of the GeneChip experiment (von Götz, 2003).

solution contained 51 µl of fragmented and biotin-labelled cDNA in a MES hybridization buffer, 50 pM of B2 control oligonucleotide, 0.1 mg/ml of herring sperm DNA, 0.5 mg/ml of acetylated bovine serum albumin, and 7 % v/v of DMSO in a total

volume of 130 µl. This hybridization mixture was loaded onto a GeneChip and incubated at 50 ^{o}C for 16 h at 60 rpm in an Affymetrix GeneChip hybridization oven 640. After hybridisation, this mixture was removed and the GeneChips were put into the Affymetrix Fluidics station 400 for washing.
Subsequently the microarrays were subjected to fluorescent labelling by antibody-mediated signal amplification using Streptavidin-Phycoerythrin. The GeneChips were washed 20 times with nonstringent buffer at 25 ^{o}C and afterwards 60 times with stringent buffer at 50 ^{o}C. Subsequently, the hybridised and washed cDNA was labelled with streptavidin mixture (primary stain solution) for 10 min at 25 ^{o}C and washed again 40 times with nonstringent buffer at 30 ^{o}C. Afterwards, microarrays were stained with biotin anti-streptavidin antibodies (secondary stain solution) at 25 ^{o}C for 10 min and subsequently with streptavidin-phycoerythrin antibodies (tertiary stain solution) at 25 ^{o}C for 10 min. Finally, the excess of fluorescent label was removed by 60 washings with nonstringent buffer at 30 ^{o}C.
The schematic representation of all the individual steps performed during GeneChip experiments are shown in Figure 2.6.

2.5.5. GeneChip analyses

All information for analyses of GeneChips can be found online at http://www.affymetrix.com (Affymetrix, 2001a, 2001b, 2001c, 2001d).

2.5.5.1. Data mining from the GeneChips

The GeneChips were scanned with the Affymetrix Microarray Suite 5.0 Software (MAS 5.0) controlled HP Affymetrix GeneChip scanner. All necessary information for the experiment, including details about the hybridisation protocol were automatically saved in the .exp file. The GeneChip scanner is based on confocal microscopy and uses argon-ion laser to excite the fluorescence of phycoerythrin at 570 nm with 3 µm resolution. 64 pixels were taken per single probe cell and saved as .dat file. The corresponding gene sequence was found to each probe cell according to its coordinates on the GeneChip and signal intensity was assigned to it. MAS 5.0 analysed the dat file and derived a single intensity value for each probe cell on the array. These data were generated automatically and saved as .cel file (Figure 2.7).

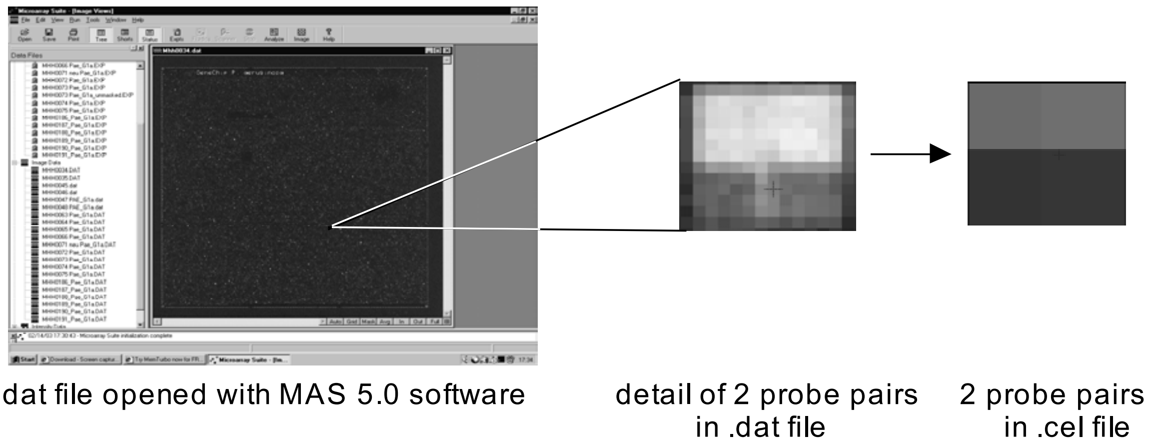


Fig. 2.7. Example of the two probe pairs converted from .dat file to .cel file.
Single intensity value is generated for each probe cell on the array from the 64 pixels in .dat file and saved as .cel file (Affymetrix, 2001a; von Götz, 2003).

2.5.5.2. Background subtraction and normalisation

The important step in analysis is to correct for background caused due to unspecific binding of the streptavidin-phycoerythrin across the entire array. To achieve this, the whole array is divided into 16 equally spaced zones and an average background is assigned to the centre of each zone, which corresponds to 2 percent of the zone. Subsequently, the weighting factor for each probe cell is computed from its relative distance to neighbouring zone centres. Finally the background signal is computed for each probe cell by applying the weighting factor to the zone average (Figure 2.8).

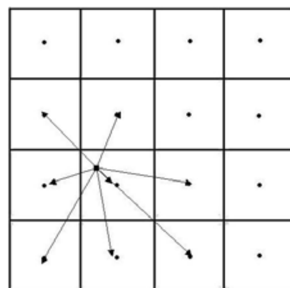


Fig. 2.8. Array zones for computing background signal. The arrow lengths indicate the relative weights.

Our study required comparison of a number of GeneChips in order to detect alterations in the expression of individual genes in different mutants or growth conditions. Small discrepancies between arrays due to variables such as the amount and quality of labelling of hybridised cDNA, staining or probe array lot could cause a serious problem, if the results were not subjected to the so called linear normalisation afterwards. This mathematical technique allows comparison analysis of the experimental results from different GeneChips via multiplication of all signal intensities on the GeneChip by a certain number. This number is specific to each array and when multiplied with the average signal intensity of the examined GeneChips results in the permanently adjusted scaling factor (150).

2.5.5.3. Four-comparison survival method

RNA from three independent preparations was pooled together and hybridized onto each chip to minimize the errors caused due to different handling. Experiments were carried out in duplicate, thus a total of two GeneChips for each mutant and each wild type were compared by four-comparison survival method (Chen *et al.*, 2000; Bakay *et al.*, 2002) (Figure 2.9). Only genes regulated in all four independent comparisons were considered to be differentially expressed.

2.5.5.4. Expression analysis

The data were imported into the Microsoft Acces database and selected by Wilcoxon rank test for genes with significant changes in their expression and at least two-fold differential regulation in all the four comparisons. The arithmetic mean and the standard deviation (SD) of the 4 comparisons were calculated. Finally, a Bonferroni correction for multiple testing (the total number of 5900 ORFs on the GeneChip) was applied as an independent rigorous criterion for significantly changed signal intensities. When exploiting this method, firstly the ratio of calibrated hybridization signals per gene S_i from both GeneChips of the mutant or wild type grown under identical condition was verified to follow a Gaussian distribution and subsequently the variance σ was calculated. The analysed genes were considered to be significantly differentially expressed only in the case, if the ratio $S(i)_{mutant}$ / $S(i)_{wild\ type}$ or $S(i)_{wild\ typel}$ /

$S(i)_{mutant}$ exceeds the threshold (1 + u σ). The factor u defines that the upper boundary of the normalized Gaussian integral $\Phi(u)$ where $\Phi(u) = x^n$ matches the Bonferroni-corrected 95 % confidence interval:

$$(1-\alpha) = x^n \qquad (n = 5900, \alpha = 0.025, 0.975 << x < 1.0)$$

Significantly differentially expressed ORFs were classified using latest internet annotation from the web site of the *Pseudomonas* Genome Project (http://www.pseudomonas.com).

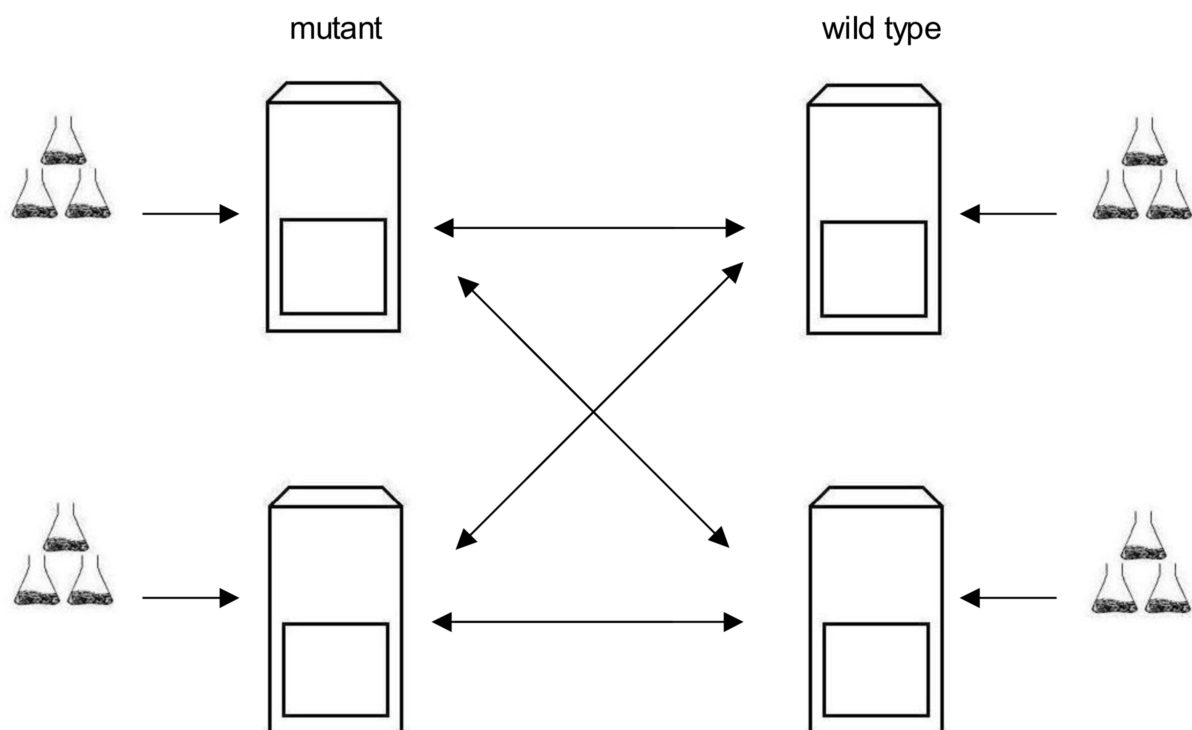


Fig. 2.9. Four-comparison survival method as a tool to identify differentially expressed genes (Chen *et al.*, 2000; Bakay *et al.*, 2002).

2.6. Phenotypic analyses

2.6.1. Intracellular survival in PMNs

Fresh blood from a healthy donor was used as a source of PMNs. Human blood serum was also added to the tube when examining the intracellular survival ability of tested strains because PMNs require a functional complement system for opsonization and recognition of bacteria. Due to decreasing activity of isolated PMNs, special care was taken to complete the whole experiment within 6 hours. In addition, to avoid unwanted apoptosis and escape of the intracellular bacteria, the incubation period of bacteria with PMNs was no longer than 2 and half hours.
The schematic representation of this phagocytosis assay is shown in Figure 2.10.

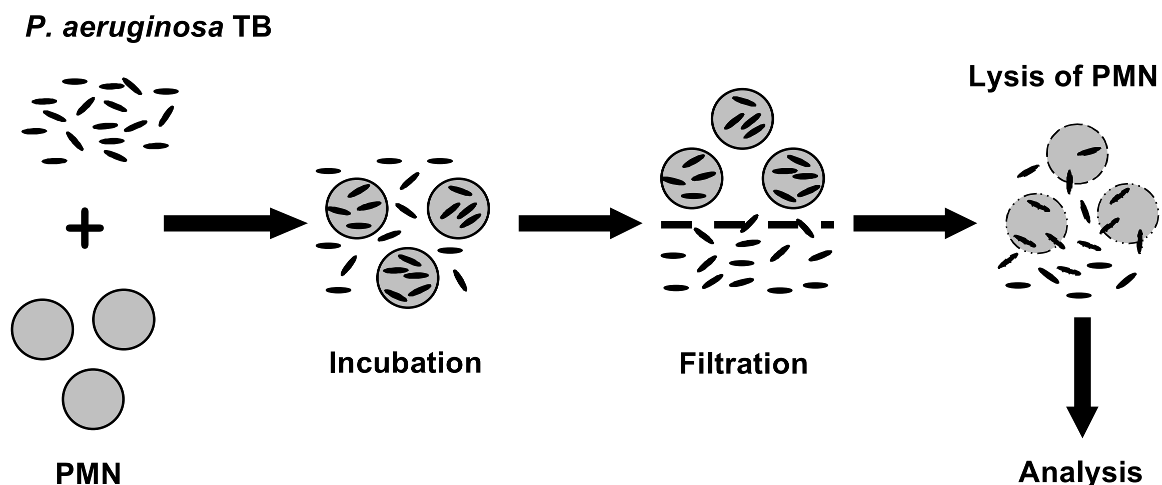


Fig. 2.10. Overview of the individual steps of the intracellular survival assay.

2.6.1.1. Isolation and determination of the concentration of PMNs

1. 10 ml of blood with 100 µl of liquemin was added in the glass tube containing 5 ml of 10 % hydroxyethyl starch (HES) and incubated for 45 min at room temperature to separate the sedimented erythrocytes from the blood plasma.
2. Using a Pasteur pipette the supernatant was transferred in the 15 ml plastic Falcon tube.

3. Using a Pasteur pipette 2 ml of the lymphocyte separation medium were added to the bottom of the plasma in the tube and the tube was centrifuged at 3000 *g* for 15 min.
4. The pellet of granulocytes was resuspended in 1 ml of RPMI1640 medium and placed on ice.
5. The granulocyte concentration was determined by counting in a Neubauer chamber. The number of PMNs was counted in 10 different squares to minimize the probability of errors: One granulocyte counted in the large square represents a concentration of 2.5 x 10^5 granulocytes in 1 ml and approximately 10^7 PMNs were obtained from 1 ml of blood.

2.6.1.2. Intracellular survival assay

1. Approximately 10^7 of the freshly isolated PMNs from 10 ml of blood, together with a 10 fold excess (10^8) of bacteria were added in the RPMI1640 medium containing 10 % human blood AB-serum to final volume of 2 ml and cultivated at 200 rpm at 37°C for 2 hours. The same 2 ml mixture was also prepared in the second tube, but without addition of PMNs that served as a control.
2. After the incubation period the tube was placed on ice for 10 min and subsequently centrifuged at 400 *g* for 10 min to pellet the granulocytes with internalised bacteria.
3. The pellet was resuspended in 200 µl RPMI1640 medium, applied on the nitrocellulose filter with the size of pores 5 µM and washed with 5 ml of PBS.
4. The filter was transferred in the plastic tube containing 3 ml of double distilled water and vortexed for 5 min to lyse the PMNs and separate them from bacteria.
5. The mixture was transferred in the fresh plastic tube and centrifuged at 4000 *g* for 10 min.
6. The pelleted bacteria were resuspended in 100 µl PBS buffer and plated on LB agar. The plate was incubated at 37 ^{o}C overnight and the number of intracellularly survived bacteria was determined by counting the cfu.

2.6.2. Survival in serum

1. The examined bacterial strains were grown in 5 ml of LB medium in 15 ml Falcon tubes on a rotatory shaker at 37 ^{o}C overnight.
2. The optical density at 600 nm for all strains was measured.
3. Approximately 10^{8} of bacteria were added in 2 ml of RPMI1640 medium containing 10 % human blood AB-serum and cultivated at 200 rpm at 37°C for 2 hours.
4. The control mixture without serum was simultaneously prepared in the second tube.
5. After the incubation period the appropriate dilutions from the bacterial cultures were taken and plated on LB agar. The plate was incubated at 37 ^{o}C overnight and the number of bacteria which survived the serum stress was determined by counting the cfu.

2.6.3. Secretion of quorum sensing controlled exoproducts

2.6.3.1. Secretion of pyocyanin

For the secretion of pyocyanin by the investigated strains a special medium called King's medium A was used (King *at al.,* 1954). This medium favours the secretion of pyocyanin by *P. aeruginosa* and simultaneously inhibits the secretion of pyoverdin.

1. The examined bacterial strains were grown in 5 ml of LB medium in 15 ml Falcon tubes on a rotatory shaker at 37 ^{o}C overnight.
2. The optical density at 600 nm for all strains was measured.
3. Bacteria were inoculated in the 15-25 ml of King's medium in 125 ml Capsenberg flasks to optical density 0.05 and cultivated at 300 rpm on a rotatory shaker at 37 ^{o}C for 12 hours.
4. 2 ml of this bacterial culture were centrifuged at 14.000 *g* for 3 min at room temperature.
5. The pelleted bacterial cells were discarded, whereas the absorbance of the supernatant was measured at 695 nm.

2.6.3.2. Secretion of proteases

P. aeruginosa secretes a battery of extracellular virulence factors. The important virulence extracellular factors whose secretion is controlled by quorum sensing are proteases. The secretion of proteases was determined by growth of the investigated *P. aeruginosa* mutants on M9 agar plates supplemented with 0.75 % casein.

1. The examined bacterial strains were grown in 5 ml of LB medium in 15 ml Falcon tubes on a rotatory shaker at 37 ^{o}C overnight.
2. The optical density at 600 nm for all strains was adjusted to 1.0 and 2 µl thereof were inoculated on M9 agar plates.
3. The plates were incubated at 37 ^{o}C for at least 24 hours and then checked for the size of the transparent zone around bacteria indicating proteolytic lysis of the casein.

2.6.3.3. Secretion of elastase

For the examination of the elastase secretion the modified elastin-Congo red assay was used (Rust *et al.,* 1994). This assay exploits elastin covalently linked to a Congo Red dye as substrate. Secreted elastase digests this substrate, thus releasing the red dye which causes an easily measurable colorimetric reaction.

1. The examined bacterial strains were grown in 5 ml of LB medium in 15 ml Falcon tubes on a rotatory shaker at 37 ^{o}C overnight.
2. The optical density at 600 nm for all strains was measured.
3. Bacteria were inoculated in the 15-25 ml of LB medium in 125 ml Capsenberg flasks to optical density 0.05 and cultivated at 300 rpm on a rotatory shaker at 37 ^{o}C for 12 hours.
4. 5 ml of the 30 mM Tris buffer (pH 7.2) were added to 50 mg of elastin-Congo red and vortexed to avoid clumping of substrate.
5. 0.5 ml of the bacterial culture supernatant were added to the mixture and cultivated at 200 rpm on a rotatory shaker at 37 ^{o}C for 4-6 hours.
6. Undigested elastin-Congo red was pelleted by centrifugation at 1200 *g* for 10 min at room temperature and the absorbance of the supernatant was measured at 495 nm.

2.6.3.4. Secretion of hemolysins

Hemolytic activity is another important virulence trait known to be controlled by quorum sensing in *P. aeruginosa. P. aeruginosa* was shown to produce two different hemolysins: phospholipase C and rhamnolipids. In order to analyse the hemolytic activity the investigated *P. aeruginosa* strains were grown on Columbia blood agar (Becton Dickinson).

1. The examined bacterial strains were grown in 5 ml of LB medium in 15 ml Falcon tubes on a rotatory shaker at 37 ^{o}C overnight.
2. The optical density at 600 nm for all strains was adjusted to 1.0 and 2 µl thereof were inoculated on Columbia agar plates.
3. The plates were incubated at 37 ^{o}C for at least 48 hours and then checked for the size of the transparent zone around bacteria indicating hemolytic activity of the tested strains.

2.6.4. Determination of the protein concentration

The Bradford method was used to determine the concentration of proteins secreted by the analysed bacterial strains (Bradford, 1976; Schmidt *et al.,* 1963). This procedure exploits the binding of a dye, Coomassie brilliant blue to the proteins after the NaOH mediated lysis of the cells to determine the protein concentration.

1. 1 ml of the bacterial culture was mixed with the equal volume of 2M NaOH and incubated at 80 ^{o}C for 1 hour.
2. Lysed bacterial cells were placed on ice for 2 min, resuspended thoroughly and mixed with 40 times larger volume of the Bradford solution.
3. The suspension was incubated at room temperature for 40 min and the absorbance was measured at 595 nm.

2.6.5. Virulence towards *C. elegans*

Nematode killing assays were performed in collaboration with D. Jordan and I. Steinmetz from the Institute of Medical Microbiology and Hospital Epidemiology of the Hannover Medical School. All *C. elegans* strains were maintained under standard culturing conditions on nematode growth medium with *Esccherichia coli* OP50 as a food source (Stiernagle, 1999). Bristol N2 (wild type), provided by the *Caenorhabditis*

Genetics Centre (University of Minnesota, St Paul's, MN, USA) was used throughout this study. *C. elegans* killing experiments were performed in a liquid-medium-based system using 24-well plates. Nematodes were exposed to a suspension (OD_{650} 0.5) of bacterial cells for each strain tested.

2.7. Internet databases and software

2.7.1. Databases

1. Most frequently used *Pseudomonas* database comprising completely sequenced genome of *P. aeruginosa* strain PAO1-*PSEUDOMONAS* GENOME PROJECT (http://www.pseudomonas.com) (Benson *et al.,* 2002).

2. Reannotation *P. aeruginosa* genome database-PSEURECA (http://maine.ebi.ac.uk:8000/services/pseureca) (Weinel *et al.,* 2003).

3. Gene and protein database-GENBANK (http://www.ncbi.nlm.nih.gov) (Benson *et al.,* 2002).

4. Sequences and annotation of GeneChip probes-NETAFFX (http://www.affymetrix.com/analysis/index.affx).

5. Kyoto encyclopedia of genes and genomes-KEGG (http://www.genome.ad.jp/kegg) (Kanehisa *et al.,* 2002).

6. Database of protein families-PFAM (http://www.sanger.ac.uk/Software/Pfam/index.html) (Bateman *et al.,* 2002).

7. Database of the Institute for Genomic Research-TIGR (http://www.tigr.org).

2.7.2. Software

1. The bendability/ curvature propensity plot was calculated with the BEND.IT server (http://hydra.icgeb.trieste.it/~kristian/dna/bend_it.html), using Dnase I-based bendability parameters and consensus bendability scale as described by Brukner *et al.,* 1995.

2. The restriction map of the shuttle vector pME6010, which was used for complementation of analysed genes was created with the help of the freeware pDRAW32 (http://www.acaclone.com).

3. For the examination of homology between tested ORFs BLAST software was used (http://www.ncbi.nlm.nih.gov or http://blast.genome.ad.jp) (Altschul *et al.,* 1990; Altschul *et al.,* 1997).

4. Restriction digests of target sequences were pre-designed *in silico* with the WEBCUTTER 2.0 software (http://www.firstmarket.com/cutter/cut2.html).

5. PRIMER 3 software was usually used to facilitate the construction of primers (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

3. Results and discussion

3.1. Global regulation of quorum sensing and virulence by VqsR

3.1.1. Features of the *vqsR* (PA2591) gene

The sequence of *vqsR* (807 bp = 268 amino acids) corresponds to the ORF PA2591 of the *P. aeruginosa* PAO1 genome (http://www.pseudomonas.com) (Figure 3.1). According to the information stored in the PAO1 database, *vqsR* (PA2591) encodes a putative transcriptional regulator with 46% homology to DMSO reductase regulatory protein DorX of *Rhodobacter sphaeroides.* The *vqsR* is the first gene of an operon, follows a promoter region and contains a palindromic terminator sequence at the end of the ORF.

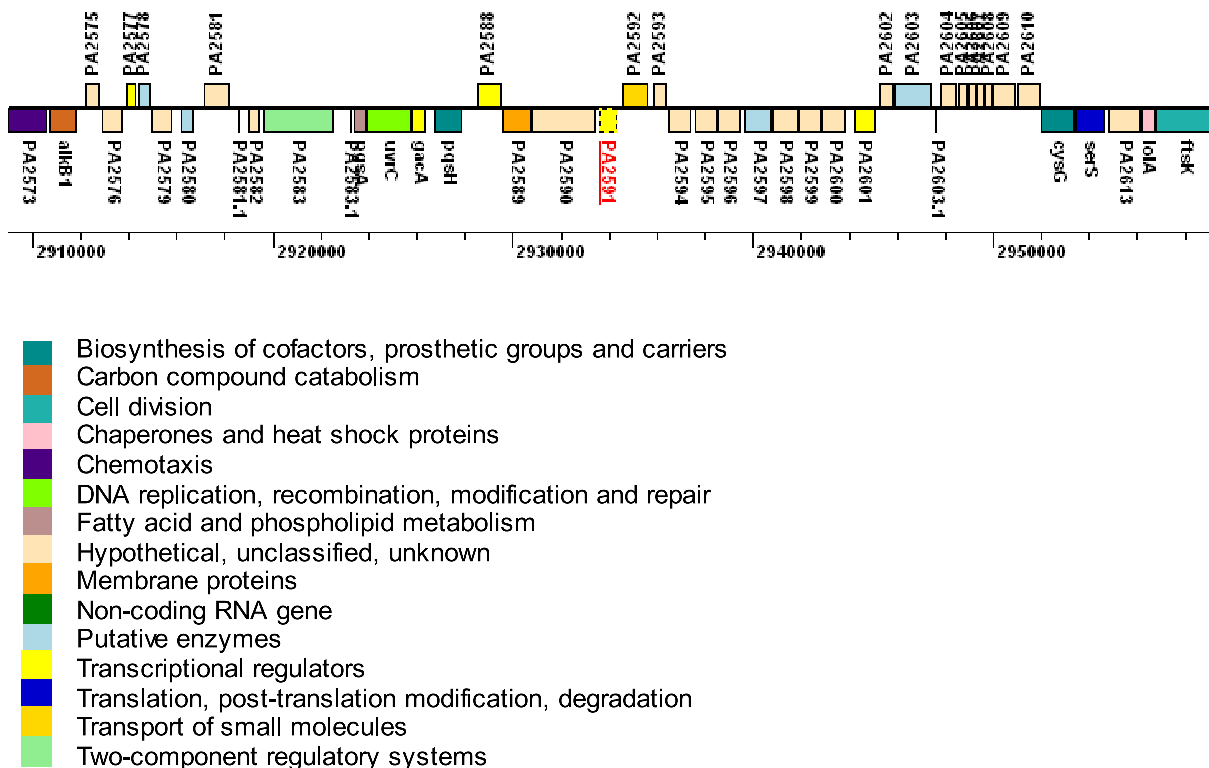


Fig. 3.1. The map showing the part of the *P. aeruginosa* chromosome area around the investigated *vqsR* (PA2591) gene (http://www.pseudomonas.com).

The G+C content of this gene (58.33 mol%) (Figure 3.2) is lower than the average G+C content (66.6 mol%) of the *P. aeruginosa* genome (Stover *et al.*, 2000), but its codon adaptation index (0.688) calculated from codon usage is similar to that typically found for *P. aeruginosa* transcriptional regulators (Kiewitz *et al.*, 2002). This suggests that despite its lower G+C content, *vqsR* was not acquired by phylogenetically recent horizontal transfer, but is rather a part of the core *P. aeruginosa* genome.

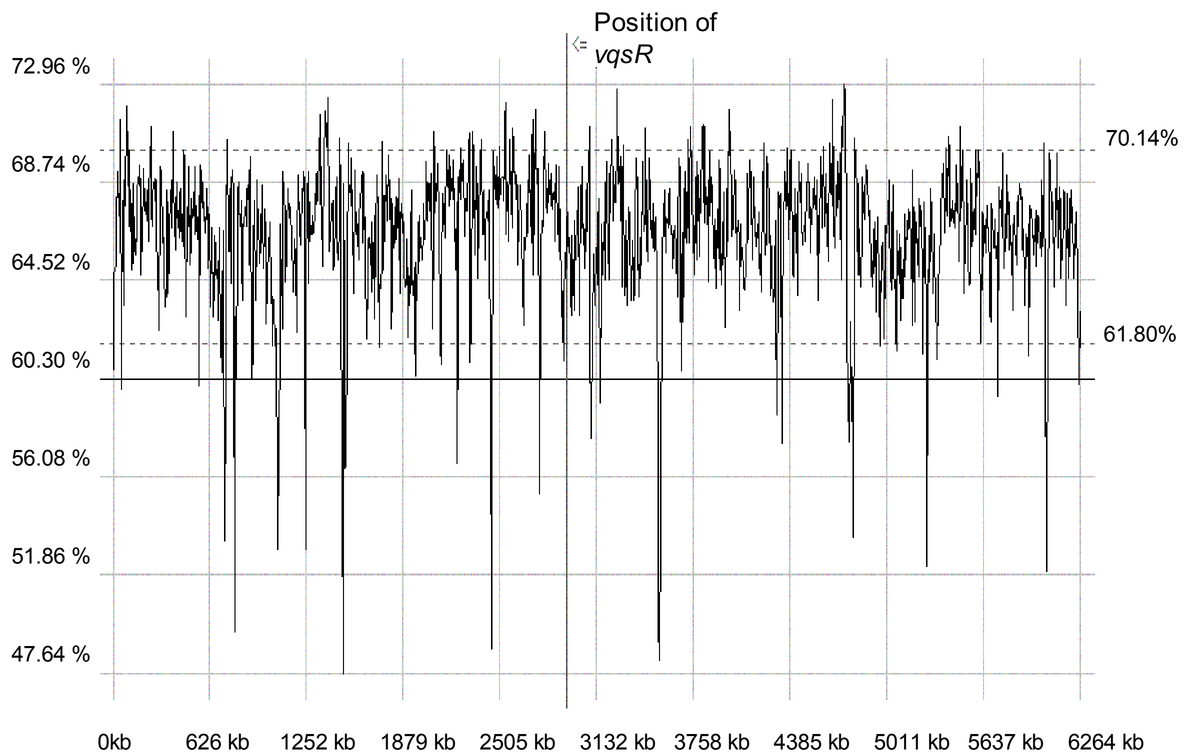


Fig. 3.2. The scheme showing the G+C content of the whole *P. aeruginosa* genome. The position of the *vqsR* (PA2591) gene is indicated (http://www.tigr.org).

VqsR possesses a *las* box (5'-AACTACCAGTTCTGGTAGGT-3') in its -157 to -138 upstream region (Wagner *et al*., 2003), which exhibits homology with the palindromic *lux* box DNA elements identified in *Vibrio fischeri*. *las* boxes are usually located upstream of LuxR-regulated genes and serve as binding sites for regulatory protein-autoinducer complexes. A bendability/curvature propensity plot revealed a low curvature DNA sequence in the upstream region of *vqsR* (Figure 3.3). Hence, binding of a protein-autoinducer complex could promote more extensive bending of the upstream sequence leading to the transcriptional activation of this gene.

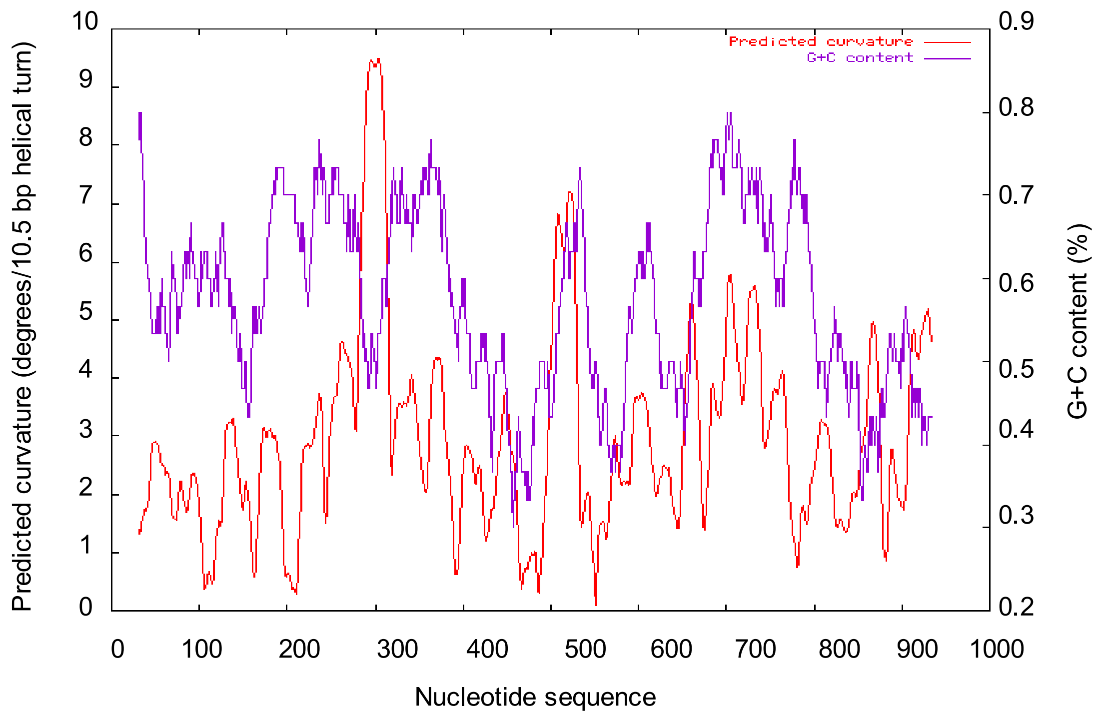


Fig. 3.3. Curvature propensity plot. Curvature propensity plot of the *vqsR* (PA2591) gene (20-826) and its upstream sequence (827-984) containing a *las* box (965-984) until the start of PA2592 gene (980), which is transcribed in the opposite direction. The upstream region of *vqsR* contains a relatively low curvature DNA sequence which suggests necessity of subsequent bending by a protein-autoinducer complex for transcriptional activation of this gene (http://hydra.icgeb.trieste.it/~kristian/dna/bend_it.html).

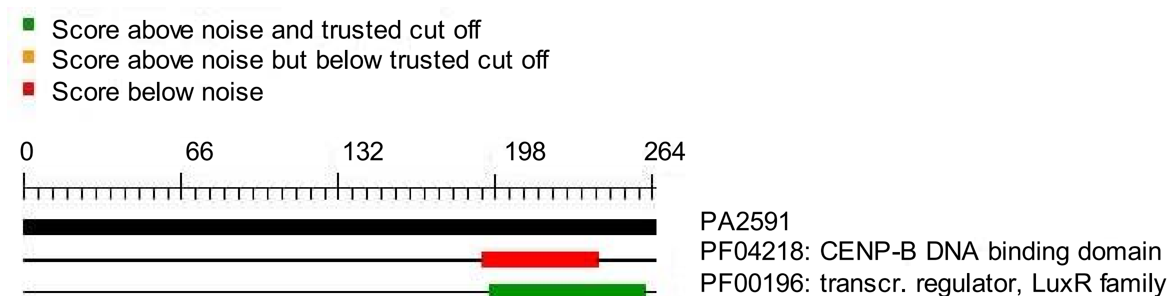


Fig. 3.4. The scheme showing the pfam domains of the *vqsR* (PA2591) gene (http://www.tigr.org). *vqsR* maintains a DNA binding domain in its carboxyl-terminus that is typical for the LuxR group of transcriptional regulators.

VqsR moreover harbours a DNA binding domain in its carboxyl-terminus that is typical for the LuxR group of transcriptional regulators (Fuqua *et al.,* 1996) (Figure 3.4). These *in silico* findings suggest that the *vqsR* gene plays an important role in the *P. aeruginosa* quorum sensing cascade.

3.1.2. Complementation of the *vqsR* gene

The *P. aeruginosa vqsR* mutant was generated by Tn*5* transposon mutagenesis as described previously (Wiehlmann *et al.,* 2002). This mutant was complemented *in trans* to ensure that all the subsequently observed striking phenotypes (see chapters 3.1.3 – 3.1.5) were caused by the transposon inactivation of the respective gene and not by any other secondary genetic event.

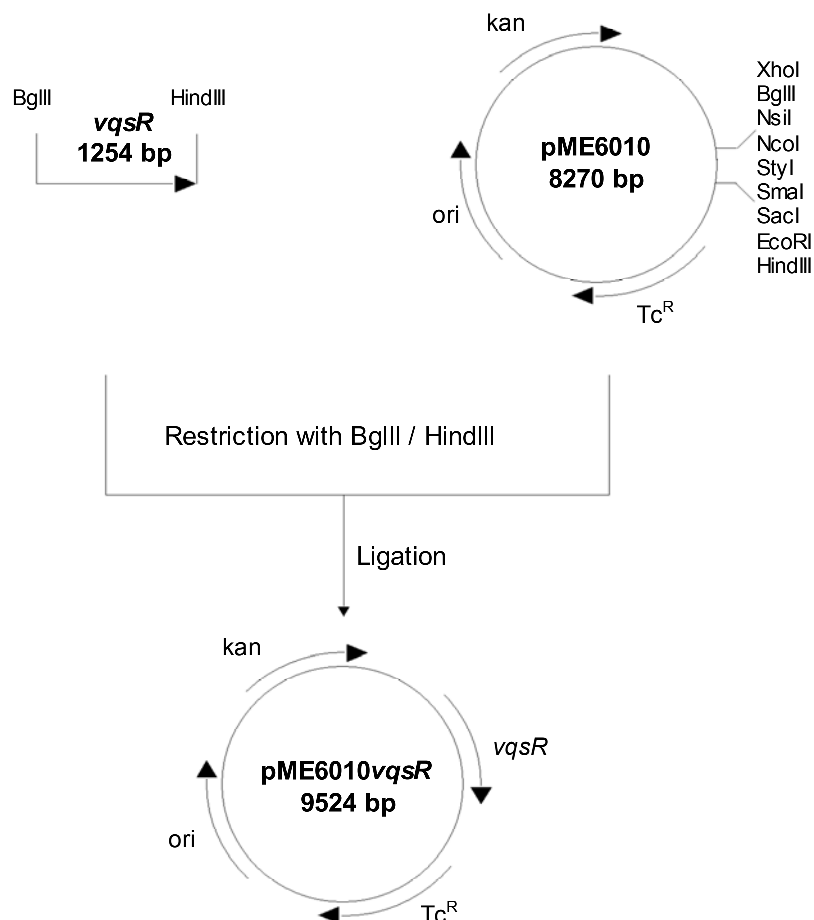


Fig. 3.5. Scheme of the cloning of the *vqsR* gene into pME6010 vector.
The PCR fragment with the length of 1254 bp comprising the analysed *vqsR* gene was cloned into the broad host range vector pME6010 maintaining the tetracycline resistance. The recombinant plasmid pME6010*vqsR* was used for complementation of the phenotype of the *P. aeruginosa vqsR* mutant.

The pME6010*vqsR* plasmid used for complementation *in trans* was constructed by cloning the 1254 bp of the PA2591 (*vqsR*) gene generated by PCR with primers: 5'-CTT GAA CAA GCT TTC GTC CTG CGC GTA-3', 5'-GAT TAT AGA TCT GTG GAT ATC GCA TTG CAC-3' into the *Bgl*II/HindIII- restricted pME6010 shuttle vector (Figure 3.5).

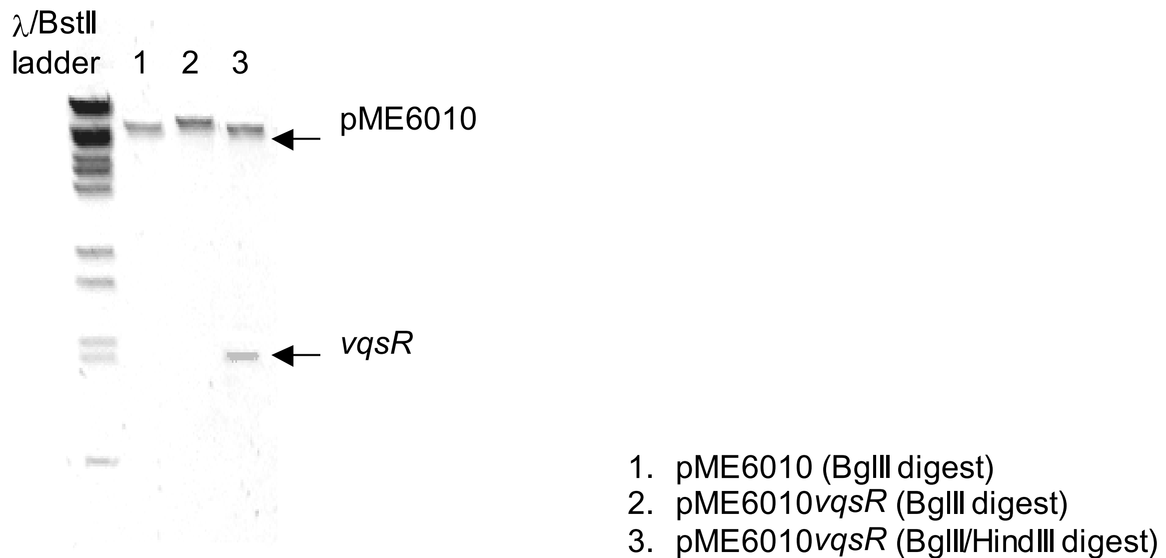


Fig. 3.6. The restriction digest of the recombinant plasmid pME6010*vqsR*. pME6010*vqsR* was isolated from the complemented *P. aeruginosa* TB*vqsR*(pME6010*vqsR*) strain.

This plasmid, based on the minimal pVS1 replicon, was demonstrated to express proteins in both *E. coli* and *P. aeruginosa* (Heeb *et al.,* 2000). The recombinant pME6010*vqsR* plasmid was introduced into *E. coli* OneShot®TOP10 chemically competent cells (Invitrogen) by transformation and subsequently into the *P. aeruginosa vqsR* mutant via electroporation (see chapter 2.3). The restriction digest of the recombinant plasmid pME6010*vqsR* isolated from *P. aeruginosa* confirmed genetic complementation of the *vqsR* mutation (Figure 3.6).

3.1.3. VqsR is essential for the production of autoinducer molecules

Quorum sensing in *P. aeruginosa* employs acylhomoserine lactones (AHLs) as autoinducer signalling molecules (Pearson *et al.,* 1994). In order to examine the effect of the investigated *vqsR* gene on AHL synthesis, we streaked the *P. aeruginosa* TB wild type strain and its Tn*5*::*vqsR* transposon mutant close to the GFP-based broad range AHL sensor *E. coli* JM105(pJBA89) (Andersen *et al.,* 2001). No AHL production was observed with the *P. aeruginosa* Tn*5*::*vqsR* transposon mutant.

P. aeruginosa possesses two different quorum sensing acylhomoserine lactones: *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butyryl homoserine lactone (C_4-HSL), which are the major components of *las* and *rhl* systems respectively (Wagner *et al*., 2003). Therefore, the cross-streak experiments were also performed with the biosensor *P. putida* F117 (pKR-C12), which only detects a narrow range of long chain AHLs, being most sensitive for 3-oxo-C_{12}-HSL (Steidle *et al.,* 2001). As with *E. coli* (pJBA89), the *vqsR* mutant did not provoke a positive signal (Figure 3.7.a and 3.7.b), supporting the view that VqsR plays a crucial role in *P. aeruginosa* quorum sensing cascade.

For more detailed analysis, thin-layer chromatography (TLC) was performed exploiting AHL biosensors *E. coli* MT102 (pSB403) and *Chromobacterium violaceum* CV026 (McClean *et al.,* 1997; Shaw *et al.,* 1997; Winson *et al.,* 1998; Geisenberger *et al.,* 2000). Using this highly sensitive technique, the *vqsR* mutant was confirmed to be significantly impaired in the production of AHLs, including both C_4-HSL (Figure 3.7.c) and 3-oxo-C_{12}-HSL (Figure 3.7.d).

Complementation of Tn*5*::*vqsR in trans* was performed in order to verify that the observed phenotype of the mutant was caused by the transposon mutation and not by any other secondary genetic event (see chapter 3.1.2). Complementation *in trans* restored the AHL molecules secretion ability of the *vqsR* mutant to levels comparable to the wild type (Figure 3.7.a, 3.7.b, 3.7.c and 3.7.d). Hence these experiments revealed the important role of VqsR in the initial steps of the quorum sensing cascade: in the production and secretion of quorum sensing autoinducers.

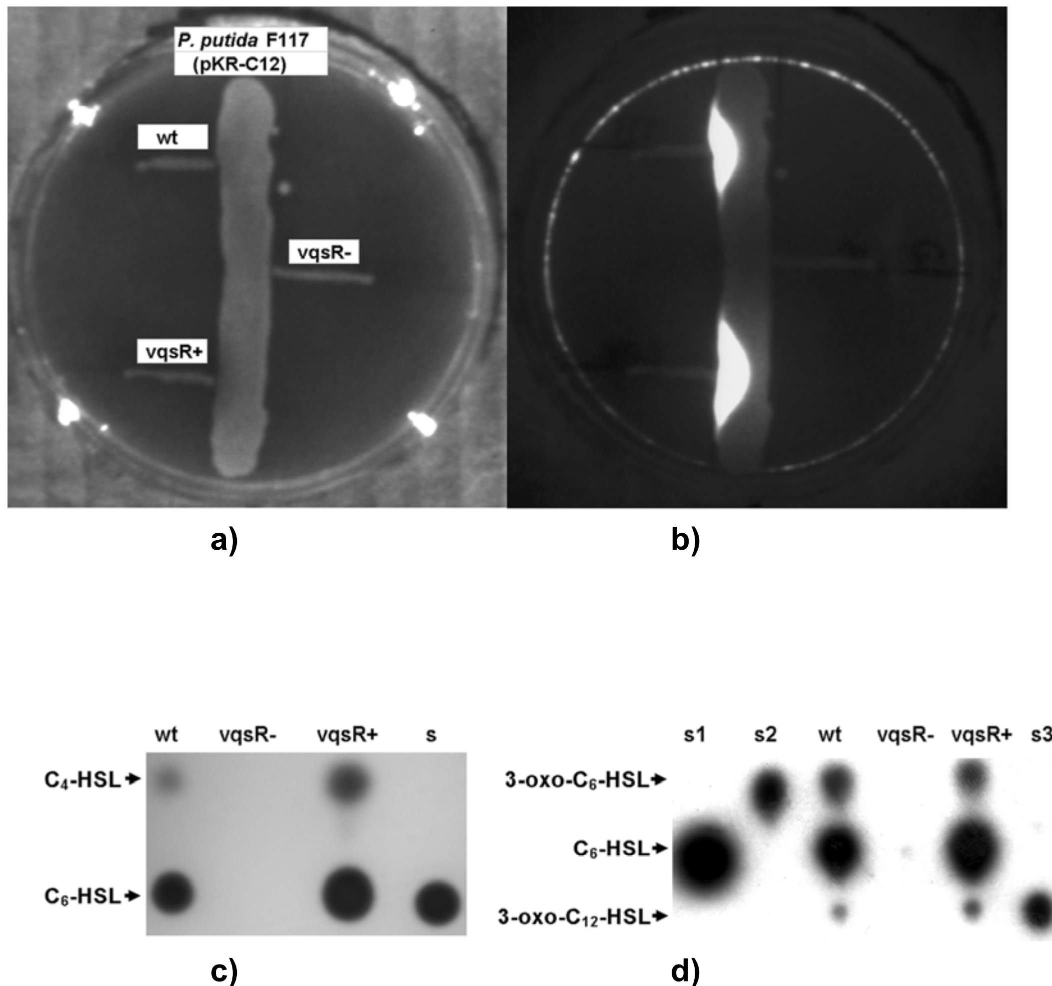


Fig. 3.7. Secretion of AHLs by *P. aeruginosa* TB wild type (wt), its Tn*5*::*vqsR* transposon mutant (vqsR-) and complemented mutant (vqsR+).

a) tested *P. aeruginosa* strains were cultivated with *P. putida* F117 harbouring plasmid pKR-C12, which contains a translational fusion of the *lasB* promoter to *gfp* and the *lasR* gene under control of a lac-type promoter and illuminated with normal light.

b) tested strains illuminated with blue light exciting green fluorescent protein. Notice the full restoration of the ability to produce autoinducer molecules in the Tn*5*::*vqsR* complemented mutant (vqsR+).

c) TLC analysis of AHLs secreted by tested *P. aeruginosa* strains using AHL biosensor *Ch. violaceum* CV026 which is able to detect C_4-HSL. Note the significantly diminished ability to secrete AHLs, including C_4-HSL (upper spot) in the *vqsR* mutant (vqsR-).

d) TLC analysis of AHLs secreted by tested *P. aeruginosa* strains using AHL biosensor *E. coli* MT102 (pSB403) which is able to detect 3-oxo-C_{12}-HSL. Notice the remarkably diminished ability to secrete AHLs, including 3-oxo-C_{12}-HSL, in the *vqsR* mutant (vqsR-). In Fig. 1c, d lanes with AHL standards are indicated by s (B. Huber and L. Eberl).

3.1.4. VqsR affects secretion of extracellular virulence factors

Quorum sensing modulates the expression of a broad spectrum of virulence genes in *P. aeruginosa* (Passador *et al.*, 1993). To analyze the effect of the *vqsR* mutation on the production of bacterial extracellular virulence factors, I have investigated its impact on proteases, hemolysins and pyocyanin secretion.

Proteases:

The secretion of proteases was determined by growth of the investigated *P. aeruginosa* strains on M9 agar plates, which were supplemented with 0.75 % casein (see chapter 2.6.3.2). The easily visible halo on casein agar plates, indicating proteolytic activity was observed for the TB wild type, but not for its *vqsR* mutant. On the other side, in the complemented mutant, the secretion of proteases was restored according to our expectations (Figure 3.8).

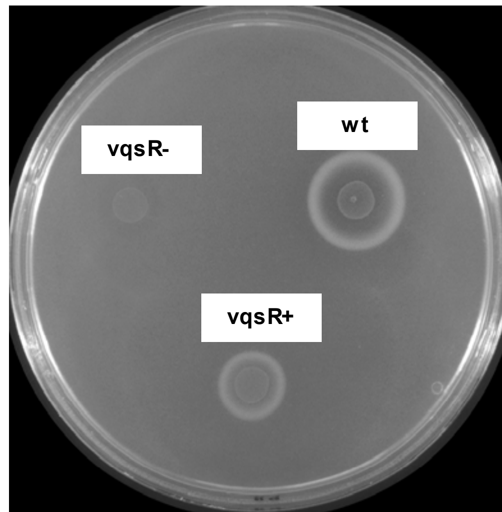


Fig. 3.8. Secretion of proteases by the *P. aeruginosa* TB wild type strain (wt), its Tn*5*::*vqsR* transposon mutant (vqsR-) and complemented mutant (vqsR+). No proteolytic activity of the mutant strain was detected on casein agar.

Casein is known to be degraded mainly by activity of LasB protease, alkaline protease and protease IV (Cowell *et al.*, 2003). All these three proteases play an important part in the infection process of *P. aeruginosa* (van Delden, 2004). Protease IV is implicated in the degradation of the components of the complement C1q and C3, fibrinogen, immunoglobulin G, plasmin and plasminogen (Engel *et al.,* 1998a) and in facilitation of the *P. aeruginosa* corneal infections (Engel *et al.,* 1998b). Alkaline protease was also proven to degrade complement components as well as fibrin and fibrinogen (Shibuya *et al.,* 1991) but its role in the tissue invasion is still unclear. LasB is a highly efficient protease with the proteolytic activity about ten times higher than *P. aeruginosa* alkaline protease and four times higher effectivity towards casein than trypsin (Galloway, 1991). LasB protease constitutes one of the major virulence determinants during acute infection due to its ability to destroy protein elastin (van Delden, 2004). Expression of both, LasB elastase and alkaline protease was shown to be controlled by the quorum sensing circuit in *P. aeruginosa* (Latifi *et al.,* 1995).

Intriguingly, the proteolytic activity of the *vqsR* mutant was not inducible even after exogenous addition of 0.3 µM 3-oxo-C_{12}–HSL and 0.4 µM C_4-HSL to the medium. Using the same concentrations of autoinducers, the extracellular proteolytic activity of a *lasI rhlI* PAO1 double mutant was restored to the level of the wild type. These data indicate that, in contrast to the inactivation of *lasI* and *rhlI*, the lack of AHL synthesis does not completely explain the phenotype of protease deficiency in the *vqsR* mutant.

To test specifically for elastase secretion, the modified elastin-Congo red assay was used (Rust *et al.,* 1994) (see chapter 2.6.3.3). This assay exploits elastin covalently linked to a Congo Red dye as substrate. Secreted elastase digests elastin-Congo red, thus releasing the red dye which causes an easily measurable colorimetric reaction. Using this assay, the *vqsR* mutant was found to be impaired in the ability to produce elastase which is known to be LasR-3-oxo-C_{12}-HSL dependent, whereas the ability of the complemented mutant to secrete elastase was restored almost to levels comparable to the wild type (Figure 3.9.a and 3.9.b).

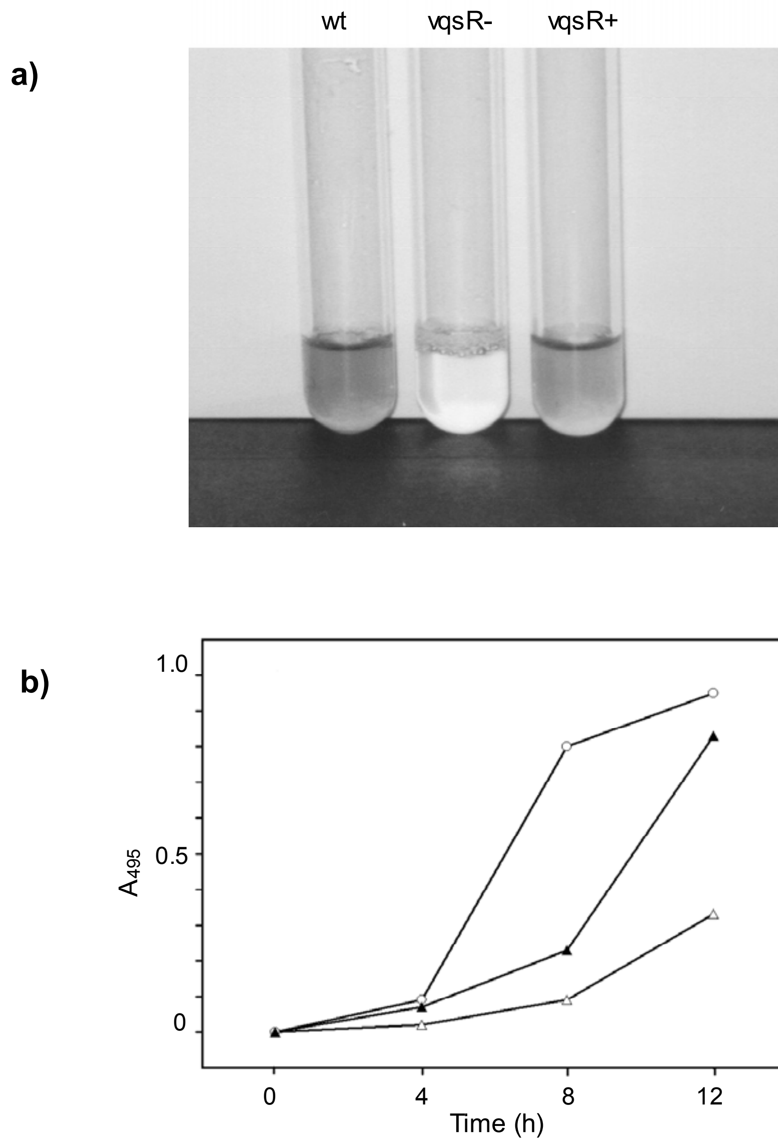


Fig. 3.9. Secretion of elastase by the *P. aeruginosa* TB wild type strain (wt), its Tn*5*::*vqsR* transposon mutant (vqsR-) and complemented mutant (vqsR+).

a) Elastase secreted by the wild type and the complemented mutant digests elastin-Congo red, thus releasing the red dye and causing an easily distinguishable coloured reaction. The ability of *vqsR* mutant to secrete elastase was reduced severely. The picture shows a result of a representative experiment after 8 hours of growth of tested strains in LB medium.

b) Kinetics of the secretion of elastase by examined *P. aeruginosa* strains. The amount of released red dye in the supernatant was quantified by measuring absorbance at 495 nm (A_{495}). ○, wt; △, vqsR-; ▲, vqsR.

Hemolysins:

The hemolytic activity was analysed by growth of the investigated *P. aeruginosa* strains on the Columbia blood agar (Becton Dickinson) (see chapter 2.6.3.4). The zone of clearance on Columbia agar plates was clearly visible in the wild type and in the complemented mutant. On the other hand, the zone of clearance around the analysed *vqsR* mutant was only small and hard to seen thus reflecting its significantly reduced hemolytic activity (Figure 3.10).

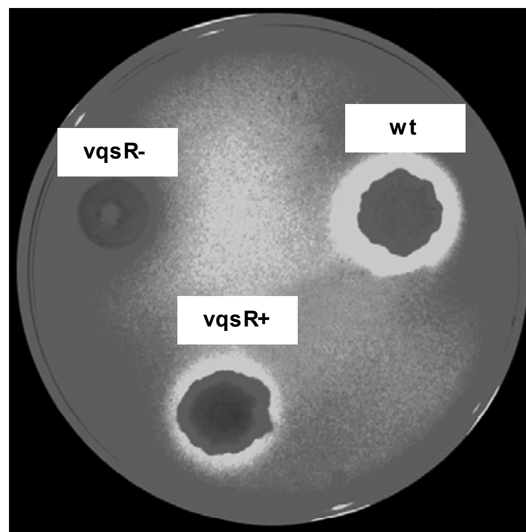


Fig. 3.10. Secretion of hemolysins by the *P. aeruginosa* TB wild type strain (wt), its Tn*5*::*vqsR* transposon mutant (vqsR-) and complemented mutant (vqsR+) on the Columbia agar plates. The mutant strains displayed significantly reduced hemolytic activity.

P. aeruginosa produces two different hemolysins: phospholipase C and rhamnolipids. Rhamolipids represent very potent biosurfactants, which act in concert with phospholipase C in order to dissolve lipids (for instance phospholipids of lung surfactant) and lecithin (van Delden, 2004). Furthermore phospholipase C was shown to induce release of the inflammatory mediators from human inflammatory effector cells (platelets, granulocytes, and monocytes), whereas rhamnolipids were shown to be crucial for the developments of biofilms (Konig *et al.,* 1996; Davey *et al.,* 2003). Both hemolysins: phospholipase C as well as rhamolipids were shown to be controlled by quorum sensing in *P. aeruginosa* (Passador *et al.*, 1993, Hentzer *et al.*, 2003).

Pyocyanin:

For determination of the pyocyanin secretion ability of the wild type, *vqsR* mutant and complemented mutant a special medium called King's medium A was used which favours the secretion of pyocyanin and simultaneously inhibits the secretion of siderophores (King *at al.,* 1954).

As shown below, measuring the amount of pyocyanin in the supernatants of the tested bacterial strains revealed a dramatic decrease of pyocyanin secretion in the *vqsR* mutant when compared to the wild type and complemented mutant (Figure 3.11.a and 3.11.b), thus revealing the important role of VqsR for the pyocyanin production by *P. aeruginosa*.

Pyocyanin is a blue phenazine pigment. It represents an important *P. aeruginosa* extracellular virulence factor due to its ability to generate reactive oxygen species (superoxide and hydrogen peroxidase), thus exposing host cells to oxidative stress (Muller, 2002). As a consequence, pyocyanin was shown to induce apoptosis of human neutrophils and inhibit lymphocyte proliferation (Usher *et al.,* 2002). Furthermore, the secretion of pyocyanin in *P. aeruginosa* was also found to be controlled by quorum sensing (Latifi *et al.,* 1995).

In all the experiments described above the mutation of analysed *vqsR* reduced significantly secretion of various exoproducts: proteases (including elastase), hemolysins and pyocyanin. On the other hand, in the complemented mutant, proteases, hemolysins as well as pyocyanin secretion ability were restored almost to levels comparable to the wild type. All exoproducts, whose secretion was investigated, are known to be implicated in the *P. aeruginosa* virulence and their secretion was shown to be controlled by quorum sensing circuitry. Thus, these experiments provided further evidence that VqsR constitutes an essential element of the *P. aeruginosa* cell-to-cell communication network.

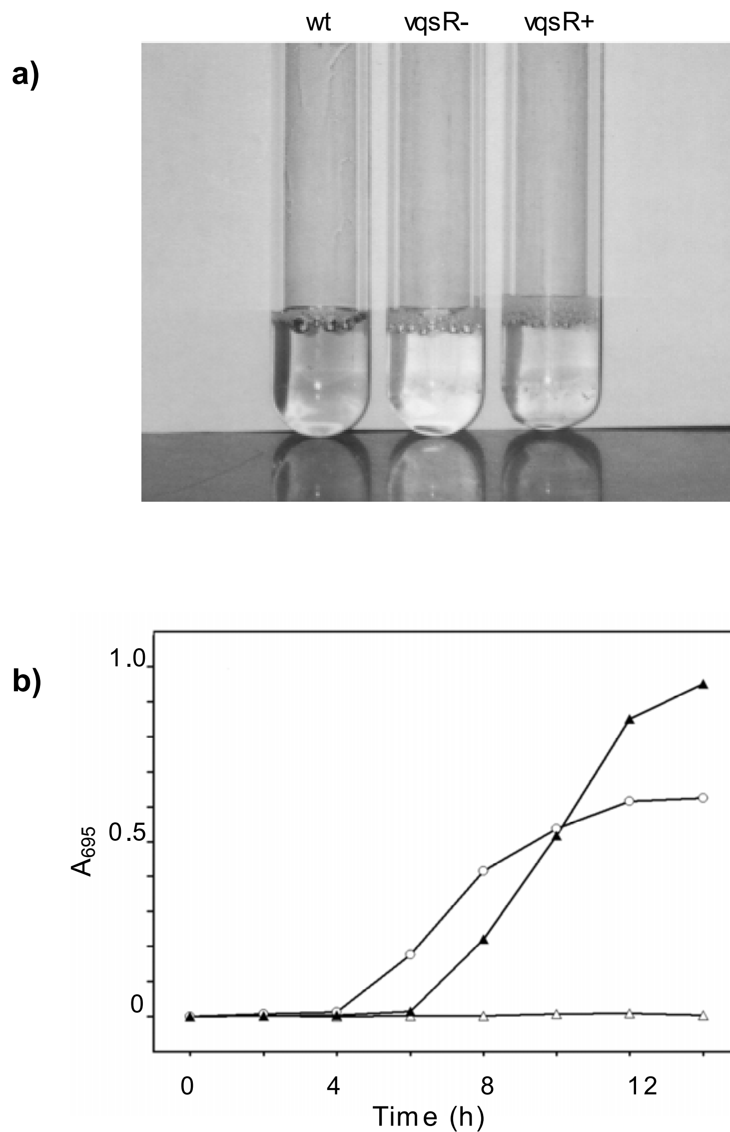


Fig. 3.11. Pyocyanin secretion by *P. aeruginosa* TB wild type (wt), its Tn*5*::*vqsR* transposon mutant (vqsR-) and complemented mutant (vqsR+). Strains were cultivated in the King's A medium.

a) Pyocyanin secreted by the complemented mutant and wild type caused an easily detectable coloured reaction, whereas the pyocyanin secretion ability of the *vqsR* mutant was reduced dramatically. The picture shows a result of a representative experiment after 8 hours of growth of tested strains in King's medium A.

b) Kinetics of the secretion of pyocyanin by examined *P. aeruginosa* strains. The amount of pyocyanin in the supernatant was quantified by measuring absorbance at 695 nm (A_{695}). ○, wt; △, vqsR-; ▲, vqsR.

3.1.5. VqsR has an impact on virulence in a *C. elegans* model

The nematode *C. elegans* has been used as a bacterial pathogenesis model for the identification of virulence-attenuated mutants in *P. aeruginosa* (Mahajan-Miklos *et al.,* 1999; Tan *et al.,* 1999). In fact, it has been shown that the analysis of the interaction with this invertebrate host has the potential to predict disease outcome in the mammalian host. Testing of the wild type and its Tn*5*::*vqsR* transposon mutant in the *C. elegans* killing model revealed a significant attenuation of the *vqsR* mutant compared to the wild type (Figure 3.12). The killing activity of the complemented mutant was restored to wild type levels (Figure 3.12), indicating an important role of the *vqsR* gene in virulence towards *C. elegans*.

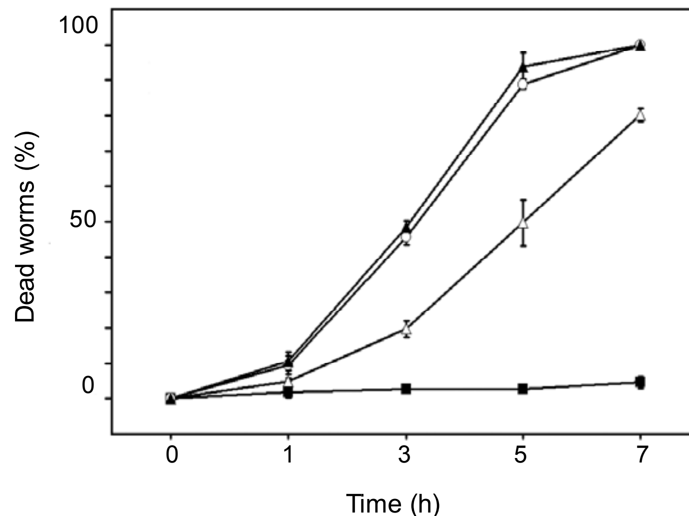


Fig. 3.12. Kinetics of the killing of *C. elegans* by TB wild type strain (wt), its Tn*5*::*vqsR* transposon mutant (vqsR-) and complemented mutant (vqsR+). 45 to 60 L4 larvae were placed in each well and scored for dead worms by microscopic examination. *E. coli* DH5α served as a negative control. Values are the mean ± SD of a representative experiment with triplicate values. ○, wt; Δ, vqsR-; ▲, vqsR+; ■, *E. coli* (D. Jordan).

3.1.6. Transcriptional analysis of *vqsR*

3.1.6.1. Expression of *vqsR* on Northern blot

The expression of VqsR mRNA was analyzed on Northern blots under various growth conditions. Standard conditions like growth in LB medium to early and late exponential phase were used as well as exposure to different stress conditions like growth in ABC minimal mineral medium, growth in the presence of oxidative stress generated by hydrogen peroxide, growth in the presence of serum and growth in the presence of PMNs.

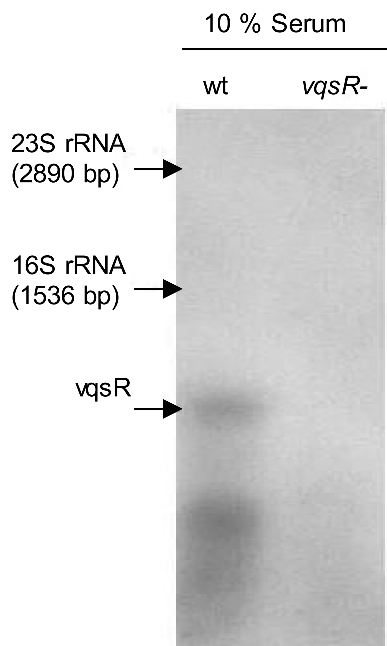


Fig. 3.13. Northern blot of *P. aeruginosa* TB wild type strain (wt) and its Tn*5*::*vqsR* transposon mutant (vqsR-) mRNA expression in the presence of 10% human blood serum. RNA isolated from both strains was hybridised with the 615 bp probe prepared from the *vqsR* sequence.

While exposing to the hydrogen peroxide generated stress, serum and PMNs, bacteria were grown in a dialysis bag with appropriate pore diameter to ensure continuous exchange of fluids. This was particularly convenient while growing bacteria in the presence of PMNs due to difficulties in the separation of eukaryotic and prokaryotic RNA. In addition to the degradation by bacteria, hydrogen peroxide concentration in the medium is also decreasing spontaneously. Dialysis bags in

combination with an excess of stressor (10 mM) were also used in order to measure the steady state response of bacteria to hydrogen peroxide. Above 5 mM of hydrogen peroxide was still present in the medium after 2 hours of incubation. After the incubation period, the cells were immediately recovered from the dialysis bag and subjected to RNA isolation (see chapter 2.4.3). The expression pattern of *vqsR* was examined by Northern blots.

Consistent with the hypothesis about its regulatory function, *vqsR* was found to be expressed only very lowly on Northern blots. Hybridization with a genomic *vqsR* probe gave a weak signal when *P. aeruginosa* TB had been cultured in the presence of H_2O_2 or human serum (Figure 3.13), but only barely detectable signals under the other tested conditions. The Tn*5*::*vqsR* mutant did not produce any detectable VqsR transcript under all chosen conditions.

3.1.6.2. GeneChip expression analysis of the VqsR regulon in the presence of serum and H_2O_2

P. aeruginosa GeneChips were used to investigate the effect of VqsR on global changes in the gene expression profile. Total RNA, extracted from bacterial cultures cultivated in the presence of 10 % human blood serum or 10 mM H_2O_2, was hybridized on the DNA microarrays in duplicate. The oligonucleotide-array comprises the whole genome of *P. aeruginosa* PAO1 (5549 ORFs, 18 tRNA genes and 1 rRNA operon, 199 probe sets for all intergenic regions exceeding 600 base pairs and 117 additional ORFs which are present in other *P. aeruginosa* strains).

The signals on the arrays representing the expression levels of individual genes were used to calculate the expression ratio between the wild type and mutant strain cultivated in the presence of H_2O_2 and serum. Only those genes were considered to be differentially expressed which exhibited a significant change according to Wilcoxon rank test, showed at least two fold change in expression in all four independent comparisons and passed through the Bonferroni correction for multiple comparisons. The genes having signal intensities below 100 were discarded due to large experimental variation. Numerous genes were differentially regulated in the *vqsR* mutant when compared to the wild type strain (Figure 3.14).

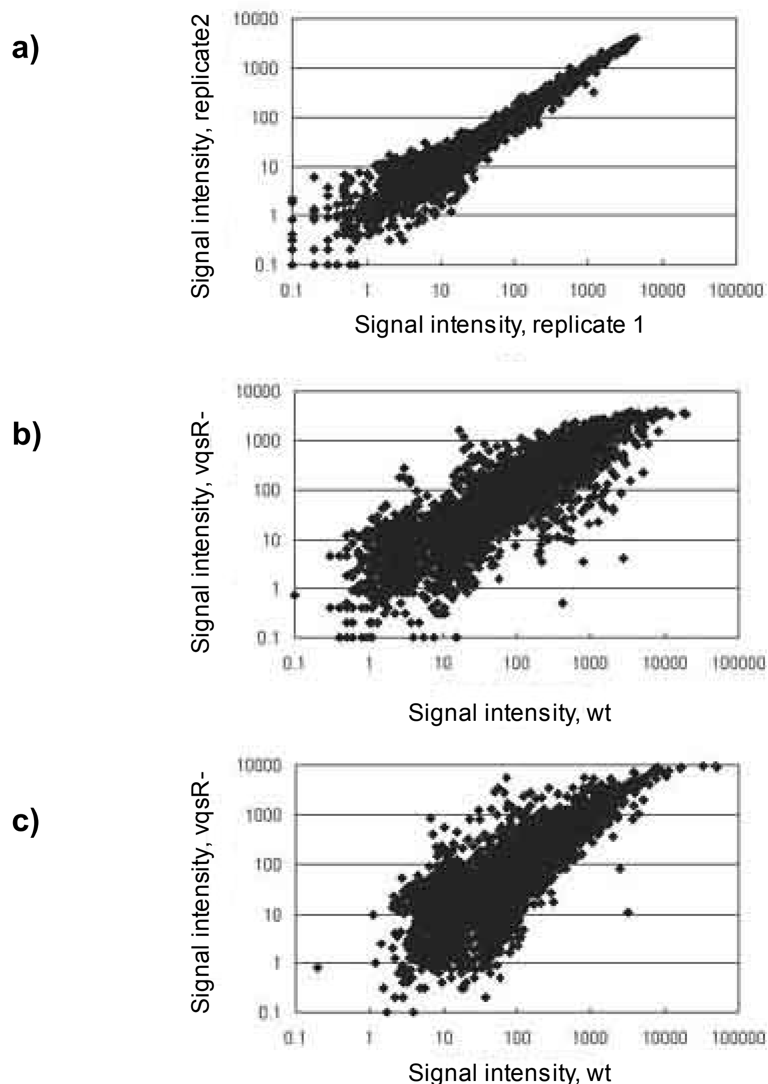


Fig. 3.14. Logarithmic scatter graph of absolute signal intensities of *P. aeruginosa* ORFs represented by 5900 individual array spots. The graph indicates that the expression of many genes was altered in the *vqsR* mutant under both tested conditions.

a) Global expression profiles of two TB wild type cultures cultivated under the same growth conditions. The absolute signal intensities of one replicate were plotted against the signal intensities of a second replicate.

b) Absolute signal intensities of TB wild type strain (wt) plotted against signal intensities of the Tn*5*::*vqsR* transposon mutant (vqsR-) when both cultures were treated with H_2O_2.

c) Signal intensities of the TB wild type (wt) plotted against Tn*5*::*vqsR* transposon mutant (vqsR-) obtained after cultivation of both cultures in the presence of human blood serum.

By applying the stringent criteria outlined above, the mutation of *vqsR* significantly influenced the expression of 151 genes in the presence of H_2O_2 and of 113 genes in the presence of human blood serum. Out of 151 genes differentially regulated in the presence H_2O_2, 55 genes were downregulated and 96 genes were upregulated in the *vqsR* mutant. Out of 113 genes differentially regulated in the presence of serum, the expression of 92 genes was repressed and the expression of remaining 21 genes was promoted in the mutant. Only 26 genes were significantly regulated under both conditions.

The differences in the gene expression of the wild type and the mutant ranged from 2-fold up to 130-fold (Figure 3.15). The expression of the majority of the genes (112 genes) was modified 4 to 8-fold, whereas the expression of only five genes was altered more than 64-fold.

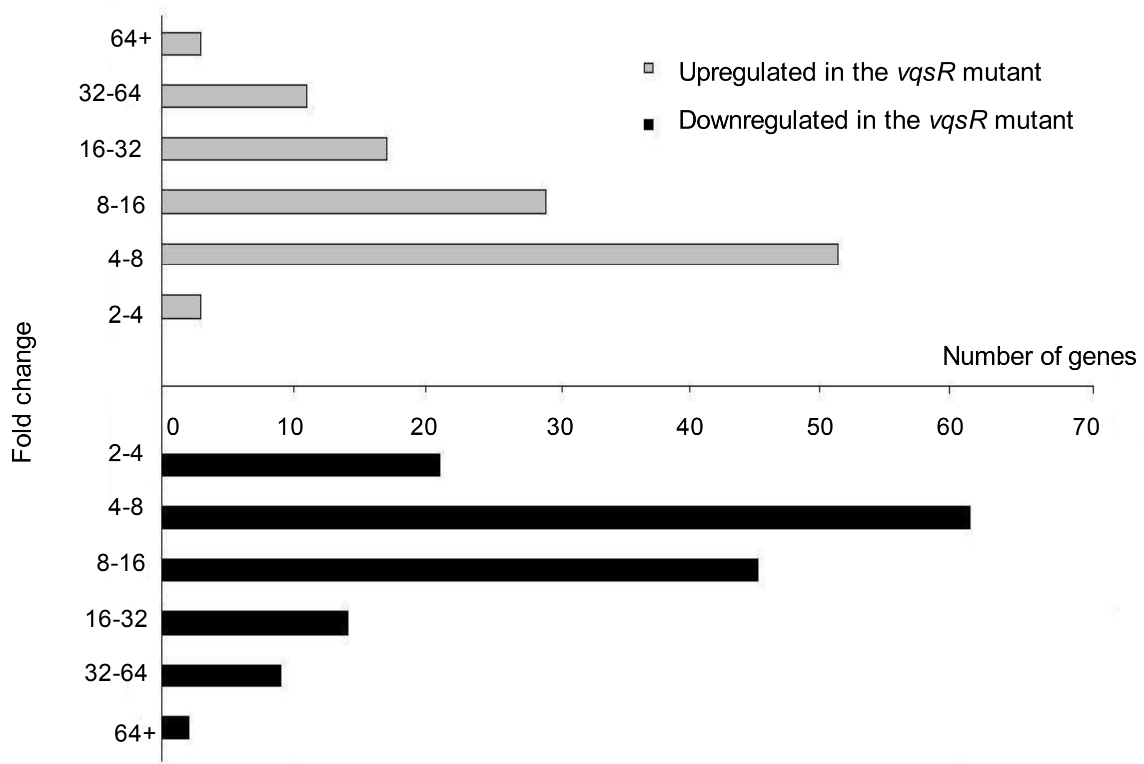


Fig. 3.15. Fold changes of the differentially regulated genes. Genes were separated according to the magnitude of differential expression.

Interestingly, almost 90 % from the genes whose expression was altered more than 32-fold were previously identified as quorum sensing regulated genes (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003).

Expression of a significant number of genes implicated in amino acid biosynthesis, antibiotic resistance, cell wall and lipopolysaccharide biosynthesis, carbon compound catabolism, central intermediary metabolism, chemotaxis, protein secretion, fatty acid and phospholipid metabolism, translation and post-translational modification, adaptation protection, biosynthesis of cofactors or those encoding putative enzymes and two-component regulatory systems were found to be differentially regulated in the examined *vqsR* mutant (Figure 3.16).

The largest proportion of all differentially regulated genes (43 %) belongs to the class of hypotheticals with unknown function. Expression of 49 genes out of these genes was upregulated in the mutant and expression of 64 genes was downregulated.

Expression of 36 genes (11 upregulated in the mutant and 25 downregulated in the mutant), which represent 24 % of the known ORFs and which are all involved in the transport of small molecules in *P. aeruginosa* was found to be controlled by VqsR. Other functional classes with the largest numbers of genes regulated by VqsR included the genes encoding membrane proteins (22 %), energy metabolism (22 %), secreted factors (22 %) and transcriptional regulators (19 %). Of particular interest are genes encoding secreted factors and proteins involved in the transport of small molecules due to their involvement in *P. aeruginosa* virulence (Stover *et al.,* 2000). The modulation of expression of a large number of other transcriptional regulators suggested an important regulatory function for VqsR.

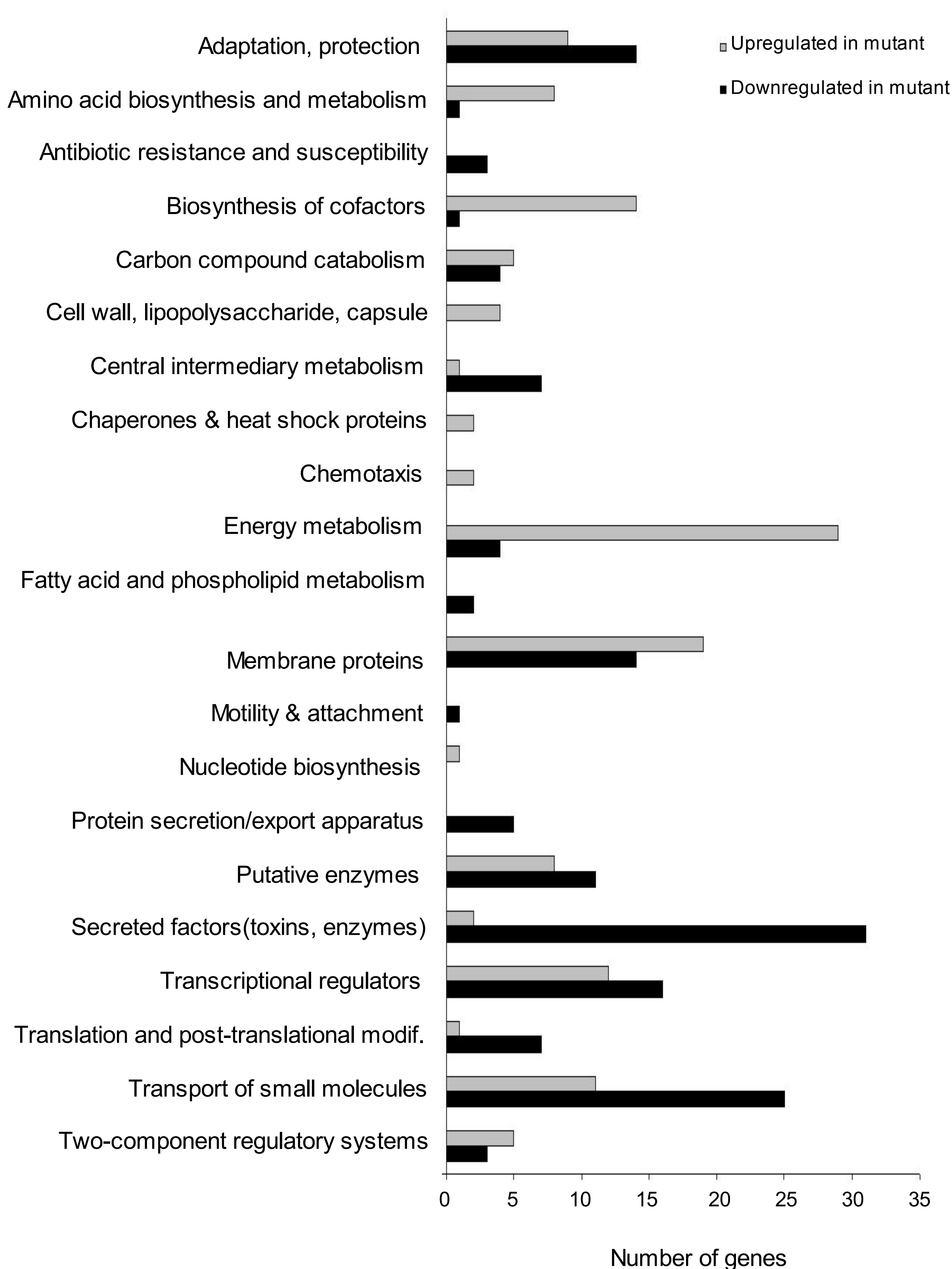


Fig. 3.16. Comparison of the transcriptome of *P. aeruginosa* TB and its *vqsR* Tn*5* transposon mutant during growth in the presence of serum and H_2O_2. The number of genes is classified by metabolic category as defined in the original publication on the PAO genome sequence (Stover *et al.,* 2000).

3.1.6.2.1. *vqsR* regulates a broad spectrum of quorum sensing genes

Recently three independent transcriptome analyses were published with the ambition to identify elements of the quorum sensing regulon in *P. aeruginosa* (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003). Comparison of the genes identified in these studies with those regulated by *vqsR* revealed that a huge proportion of the genes modulated by the investigated VqsR belong to the category of quorum sensing regulated. From the genes found to be under the control of VqsR in the presence of hydrogen peroxide and serum, nearly 40 % and 60 % respectively are previously identified quorum sensing genes (Figure 3.17).

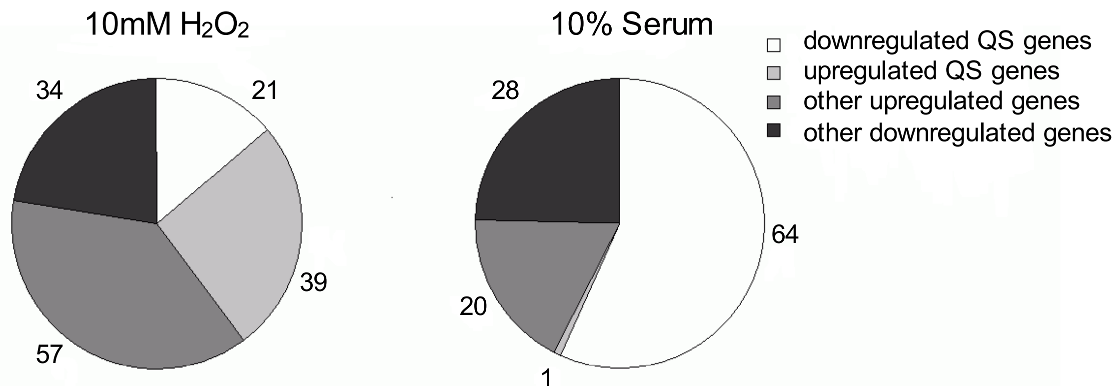


Fig. 3.17. Proportion of quorum sensing (QS) regulated genes among all differentially expressed genes in the *P. aeruginosa vqsR* mutant.

Thus this transcriptome analysis revealed that VqsR modulates the expression of a broad spectrum of quorum sensing and virulence genes and confirmed the hypothesis about the key role of VqsR in the population density dependent gene regulation in *P. aeruginosa.* Figures 3.18 and 3.19 represent an overview of all genes whose expression was found to be significantly up- and downregulated in the Tn*5*::*vqsR* mutant according to the results of the Genechip analyses, whereas Tables 3.1 and 3.2 list selectively only the previously identified quorum sensing genes giving the detailed information about their products.

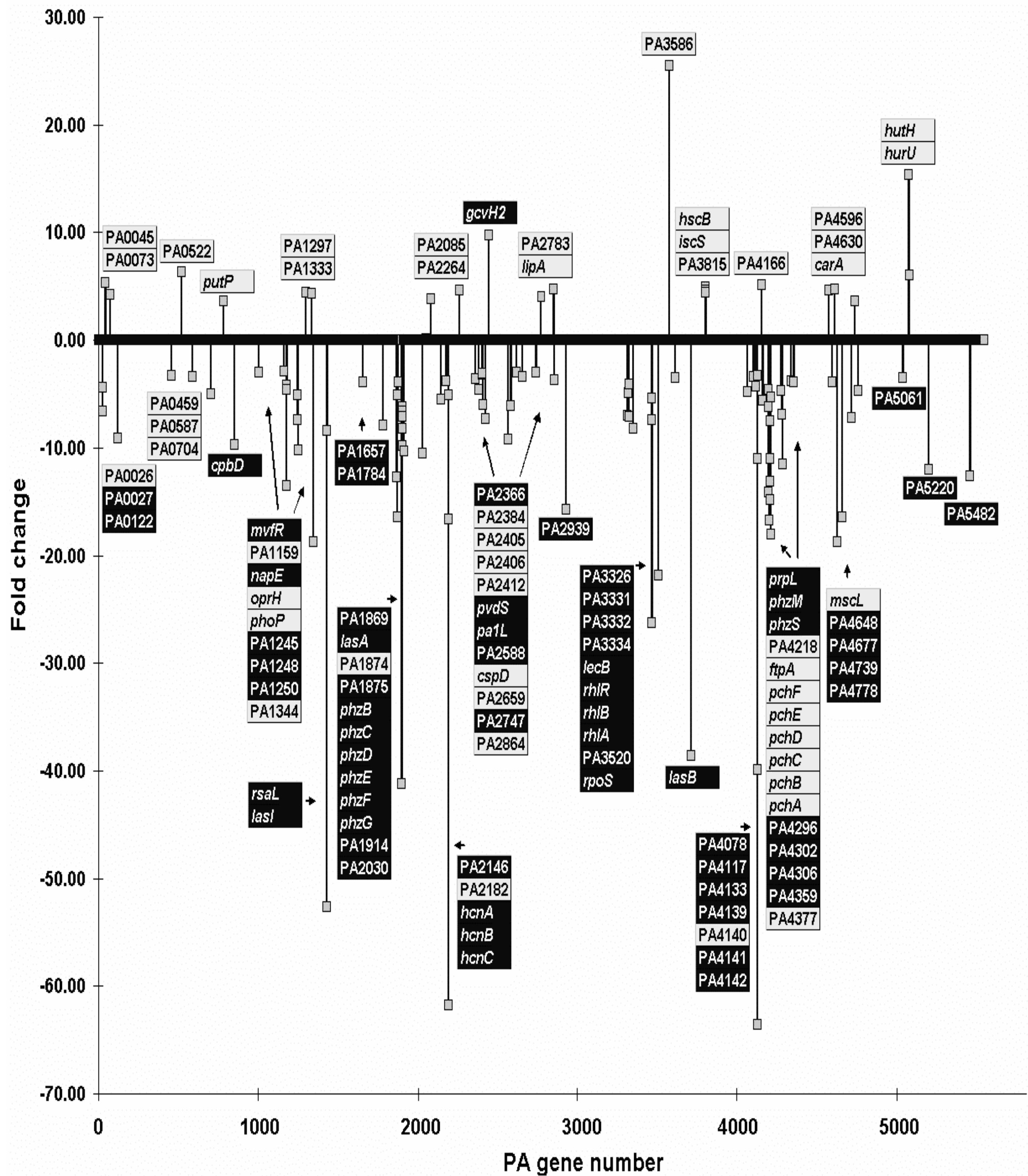


Fig. 3.18. Differential gene expression of the Tn*5*::*vqsR* transposon mutant compared to *P. aeruginosa* wild type strain TB in the presence of 10 % human blood serum. Positive values represent genes whose expression is upregulated in the mutant and negative numbers genes whose expression is downregulated in the mutant compared to the TB wild type strain. Quorum sensing regulated genes are indicated by black colour (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003).

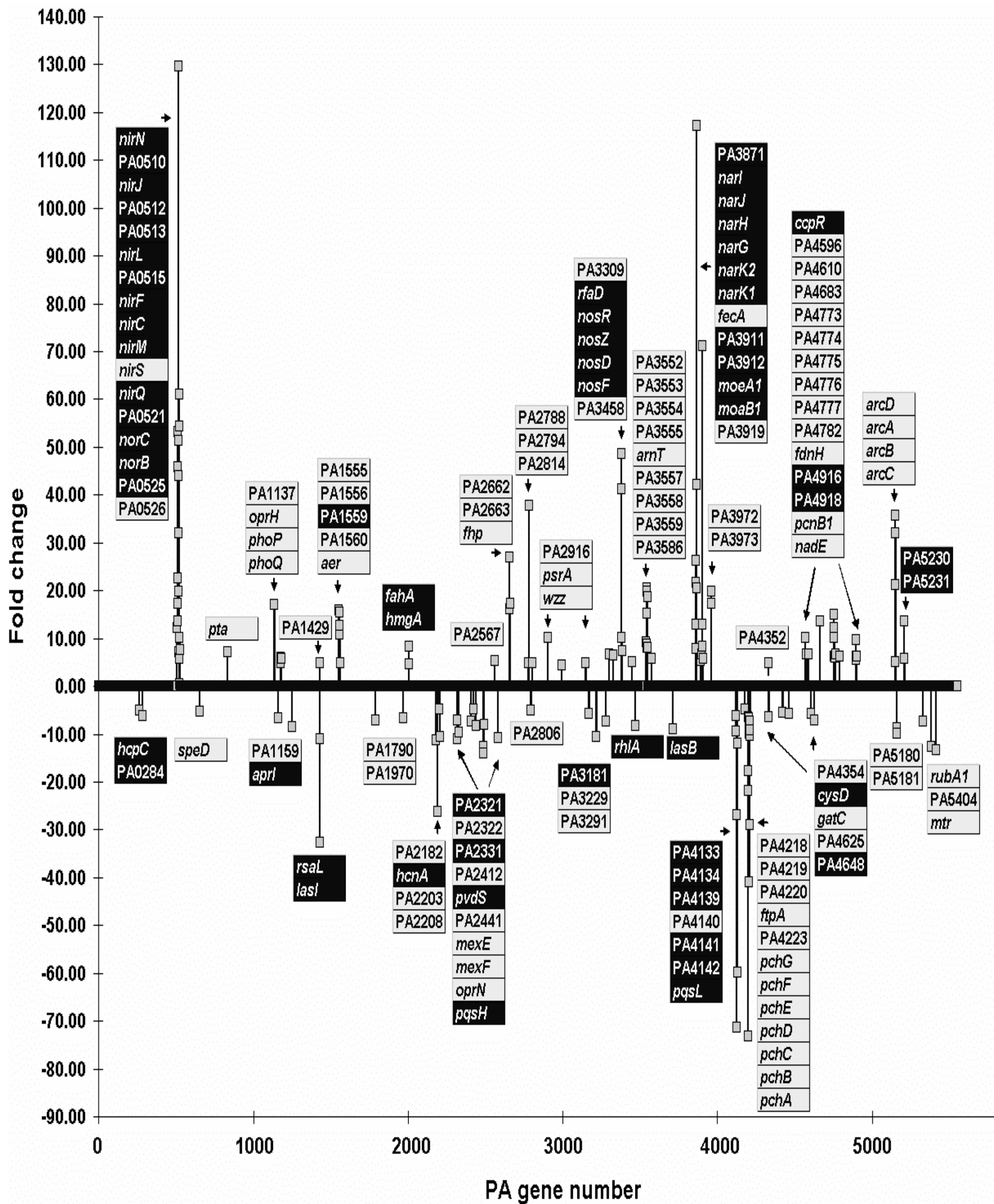


Fig. 3.19. Differential gene expression of the Tn*5*::*vqsR* transposon mutant compared to *P. aeruginosa* wild type strain TB in the presence of 10 mM H_2O_2. Positive values represent genes whose expression is upregulated in the mutant and negative numbers genes whose expression is downregulated in the mutant compared to the TB wild type strain. Quorum sensing regulated genes are indicated by black colour (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003).

Tab. 3.1. Quorum sensing genes with upregulated expression in the *vqsR* mutant in the presence of serum and H_2O_2 (Hentzer *et al*., 2003; Schuster *et al*., 2003; Wagner *et al*., 2003).

ORF[1]	Gene name	Fold change[2]		Protein description
		Serum	H_2O_2	
Nitrogen metabolism				
PA0509	*nirN*		12.1	probable c-type cytochrome
PA0510			53.1	probable uroporphyrin-III c-methyltransferase
PA0511	*nirJ*		13.5	heme d1 biosynthesis protein NirJ
PA0512			17.3	conserved hypothetical protein
PA0513			22.6	probable transcriptional regulator
PA0514	*nirL*		45.9	heme d1 biosynthesis protein NirL
PA0515			129.7	probable transcriptional regulator
PA0516	*nirF*		32.0	heme d1 biosynthesis protein NirF
PA0517	*nirC*		51.5	probable c-type cytochrome precursor
PA0518	*nirM*		43.9	cytochrome c-551 precursor
PA0520	*nirQ*		7.3	regulatory protein NirQ
PA0521			5.9	probable cytochrome c oxidase subunit
PA0523	*norC*		54.3	nitric-oxide reductase subunit C
PA0524	*norB*		61.0	nitric-oxide reductase subunit B
PA0525			10.3	probable dinitrification protein NorD
PA3391	*nosR*		41.2	regulatory protein NosR
PA3392	*nosZ*		48.6	nitrous-oxide reductase precursor
PA3393	*nosD*		10.2	NosD protein
PA3394	*nosF*		7.5	NosF protein
PA3872	*narI*		13.0	respiratory nitrate reductase gamma chain
PA3873	*narJ*		21.6	respiratory nitrate reductase delta chain
PA3874	*narH*		26.2	respiratory nitrate reductase beta chain
PA3875	*narG*		117.1	respiratory nitrate reductase alpha chain
PA3876	*narK2*		42.2	nitrite extrusion protein 2
PA3877	*narK1*		20.5	nitrite extrusion protein 1
PA3914	*moeA1*		71.1	molybdenum cofactor biosynthetic protein A1
PA3915	*moaB1*		13.0	molybdopterin biosynthetic protein B1
Other genes				
PA1559			15.5	hypothetical protein
PA2008	*fahA*		4.6	fumarylacetoacetase
PA2009	*hmgA*		8.3	homogentisate 1,2-dioxygenase
PA2446	*gcvH2*	9.8		glycine cleavage system protein H2
PA3337	*rfaD*		6.6	ADP-L-glycero-D-mannoheptose 6-epimerase
PA3871			7.8	prob. peptidyl-prolyl cis-trans isomerase, PpiC-type
PA3911			6.7	conserved hypothetical protein

ORF[1]	Gene name	Fold change[2]		Protein description
		Serum	H_2O_2	
Other genes				
PA3912			8.3	conserved hypothetical protein
PA4587	*ccpR*		10.2	cytochrome c551 peroxidase precursor
PA4916			5.5	hypothetical protein
PA4918			9.7	hypothetical protein
PA5230			13.5	probable permease of ABC transporter
PA5231			5.9	prob. ATP-binding/permease fusion ABC transporter

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons.

In the presence of serum, the *vqsR* mutant overexpressed only those genes that belong to the category of amino acid metabolism or the category of conserved hypotheticals, presumably just reflecting the serum as a protein-rich carbon source (Table 3.1).

Exposure to H_2O_2 activated genes by more than 10-fold in the mutant that are either conserved hypotheticals or encode components of anaerobic metabolism. Nitrite and nitrate respiration have been also previously reported to be repressed by quorum sensing (Table 3.1) (Wagner *et al.,* 2003).

On the other hand, the inactivation of *vqsR* decreased mRNA levels of some of the major regulators of the quorum sensing network in *P. aeruginosa*: LasI, RhlR, RsaL, RpoS and MvfR (Table 3.2). LasI and RhlR are the major components of two quorum sensing systems operating in this organism: the *las* system, which consists of the transcriptional activator LasR and the AHL synthase LasI, which directs the synthesis of *N*-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C_{12}-HSL) and the *rhl* system, which consists of the transcriptional regulator RhlR and the AHL synthase RhlI, which directs the synthesis of *N*-butanoyl-homoserine lactone (C_4-HSL) (Passador *et al.,* 1993; Latifi *et al.,* 1995; Pearson *et al.,* 1995; Pesci *et al.,* 1997). MvfR (PqsR) has been proven to be responsible for the production of the *P. aeruginosa* quinolone signal (PQS) molecule (Déziel *et al.,* 2004).

Tab. 3.2. Quorum sensing genes with downregulated expression in the *vqsR* mutant in the presence of serum and H_2O_2 (Hentzer *et al*., 2003; Schuster *et al*., 2003; Wagner *et al*., 2003).

ORF[1]	Gene name	Fold change[2]		Protein description
		Serum	H_2O_2	
***las* and *rhl* systems**				
PA1432	*lasI*	8.4	11.0	autoinducer synthesis protein LasI
PA3477	*rhlR*	5.4		transcriptional regulator RhlR
Global regulators of QS				
PA1003	*mvfR*	3.0		transcriptional regulator
PA1431	*rsaL*	52.6	32.7	regulatory protein RsaL
PA3622	*rpoS*	3.5		sigma factor RpoS
PQS synthesis				
PA2587	*pqsH*		10.8	probable FAD-dependent monooxygenase
PA4190	*pqsL*		4.9	probable FAD-dependent monooxygenase
Proteases biosynthesis and secretion				
PA1248	*aprF*	7.4		alkaline protease secretion protein AprF
PA1250	*aprI*	10.2	8.4	alkaline proteinase inhibitor AprI
PA1871	*lasA*	16.4		LasA protease precursor
PA1875		3.9		probable outer membrane protein precursor
PA3724	*lasB*	38.6	9.0	elastase LasB
PA4142		3.3	12.1	probable secretion protein
PA4175	*prpL*	5.6		probable endoproteinase Arg-C precursor
Phenazine biosynthesis				
PA1900	*phzB2*	41.3		probable phenazine biosynthesis protein
PA1901	*phzC2*	6.2		phenazine biosynthesis protein PhzC
PA1902	*phzD2*	8.2		phenazine biosynthesis protein PhzD
PA1903	*phzE2*	9.8		phenazine biosynthesis protein PhzE
PA1904	*phzF2*	6.6		probable phenazine biosynthesis protein
PA1905	*phzG2*	7.1		probable pyridoxamine 5'-phosphate oxidase
PA4209	*phzM*	6.2		probable O-methyltransferase
PA4217	*phzS*	14.1		probable FAD-dependent monooxygenase
Rhamnolipid biosynthesis				
PA3478	*rhlB*	7.4		rhamnosyltransferase chain B
PA3479	*rhlA*	26.3	8.2	rhamnosyltransferase chain A
Hydrogen cyanide biosynthesis				
PA2193	*hcnA*	61.8	26.2	hydrogen cyanide synthase HcnA
PA2194	*hcnB*	16.6		hydrogen cyanide synthase HcnB
PA2195	*hcnC*	5.1		hydrogen cyanide synthase HcnC
Lectins				
PA2570	*pa1L*	9.3		PA-I galactophilic lectin

ORF[1]	Gene name	Fold change[2]		Protein description
		Serum	H_2O_2	
Lectins				
PA3361	*lecB*	8.2		fucose-binding lectin PA-IIL
Other genes				
PA0027		6.6		PPI ase, chaperone
PA0122		9.1		conserved hypothetical protein
PA0263	*hcpC*		5.1	secreted protein Hcp
PA0284			6.1	hypothetical protein
PA0852	*cpbD*	9.7		chitin-binding protein CbpD precursor
PA1177	*napE*	4.2		periplasmic nitrate reductase protein NapE
PA1245		5.1		hypothetical protein
PA1657		3.9		conserved hypothetical protein
PA1784		7.9		hypothetical protein
PA1869		12.7		probable acyl carrier protein
PA1914		10.3		conserved hypothetical protein
PA2030		10.5		hypothetical protein
PA2146		5.5		conserved hypothetical protein
PA2321			7.2	gluconokinase
PA2331			9.7	hypothetical protein
PA2366		3.6		conserved hypothetical protein
PA2588		6.1		probable transcriptional regulator
PA2747		3.0		hypothetical protein
PA2939		15.7		probable aminopeptidase
PA3181			5.7	2-keto-3-deoxy-6-phosphogluconate aldolase
PA3326		7.0		probable Clp-family ATP-dependent protease
PA3331		4.9		cytochrome P450
PA3332		4.1		conserved hypothetical protein
PA3334		7.1		probable acyl carrier protein
PA3520		21.9		hypothetical protein
PA4078		4.8		probable nonribosomal peptide synthetase
PA4117		3.4		probable bacteriophytochrome
PA4133		4.3	9.5	cytochrome c oxidase subunit (cbb3-type)
PA4134			6.3	hypothetical protein
PA4139		63.6	71.4	hypothetical protein
PA4141		40.0	59.9	hypothetical protein
PA4296		4.7		probable two-component response regulator
PA4302		6.9		probable type II secretion system protein
PA4306		11.6		hypothetical protein
PA4359		3.8		conserved hypothetical protein
PA4443	*cysD*		5.5	ATP sulfurylase small subunit

ORF[1]	Gene name	Fold change[2]		Protein description
		Serum	H_2O_2	
Other genes				
PA4648		18.8	7.2	hypothetical protein
PA4677		16.4		hypothetical protein
PA4739		7.3		conserved hypothetical protein
PA4778		4.7		probable transcriptional regulator
PA5061		3.5		conserved hypothetical protein
PA5220		12.0		hypothetical protein
PA5482		12.6		hypothetical protein

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons.

The stationary sigma factor RpoS was demonstrated to be involved in the modulation of expression of a large number of quorum sensing genes as well as the type III secretion system and anti-host effector proteins (Hogardt *et al.,* 2004; Schuster *et al.,* 2004).

Downregulation of the expression of LasI encoding 3-oxo-C_{12}-HSL autoinducer molecules in the transcriptome assay confirmed the previously shown experimental results with AHL biosensor strains (see chapter 3.1.3).

Repressed in the *vqsR* mutant were also *pqsH* and *pqsL*, which are both involved in the *P. aeruginosa* PQS signalling. Of particular interest is more than 10-fold downregulation of *pqsH* which converts PQS precursor 4-hydroxy-2-heptylquinoline into active PQS and whose expression is controlled by the *las* system (Gallagher *et al.,* 2004; Déziel *et al.,* 2004).

Also consistent with the results of the phenotypic assays shown above in chapter 3.1.4, mutation of *vqsR* led to the downregulation of the genes responsible for the expression of different extracellular virulence factors (Table 3.2).

These include various proteases (*aprF, aprI, lasA, lasB, prpL*), rhamnolipids (*rhlA, rhlB*) which are one of the two known *P. aeruginosa* hemolysins (van Delden, 2004) as well as the huge operon implicated in the biosynthesis of phenazine (*phzB2, phzC2, phzD2, phzE2, phzF2, phzG2, phzM, phzS*). Phenazine is the precursor

molecule of the blue *P. aeruginosa* pigment pyocyanin (Mavrodi *et al.,* 2001) whose expression was also proven to be repressed in the *vqsR* mutant by phenotypic analyses.

In addition to the number of genes hypotheticals of unknown function, mutation of *vqsR* also negatively affected the expression of other known virulence genes: lectins (*pa1L, lecB*) and hydrogen cyanide (*hcnA, hcnB, hcnC*).

The galactophilic lectin PA-IL, encoded by *pa1L* as well as the fucose-binding lectin PA-II L encoded by *lecB* are involved in the adherence to epithelial cells and mucin and for colonization of the host tissues and surfaces. Adherence of *P. aeruginosa* to epithelial cells is mainly mediated by type IV pili, covered with the PA-1L or PA-IIL lectins, which act as a ligands reacting with complementary sequences on host cells (Hahn, 1997). The expression of both lectins was shown to be controlled by quorum sensing in *P. aeruginosa* (Winzer *et al.,* 2000).

Hydrogen cyanide is a potent poison that blocks cytochrome oxidase, which in turn leads to inhibition of mitochondrial respiration. This secondary metabolite was shown to be responsible for rapid paralytic killing of the nematode *C. elegans* (Gallagher and Manoil, 2001). Thus, rather massive downregulation of hydrogen cyanide synthases (5 to more than 60-fold) would explain the significant attenuation of the *vqsR* mutant virulence towards *C. elegans* compared to the wild type in this study (see chapter 3.1.5). The role of hydrogen cyanide for human infections is still unclear, but it is hypothesized that it could participate in tissue destruction and may contribute to toxicity in *P. aeruginosa* infected burn wounds (Goldfarb and Margraf, 1967; van Delden, 2004)

Thus, in summary the *vqsR* mutant produced significantly less mRNA species of genes that are necessary for the synthesis of quorum sensing quinolones and AHLs and of virulence factors under control thereof.

As shown previously (Wagner *et al.,* 2003), variable responses in the expression of quorum sensing-regulated genes to different environmental stimuli may be caused by alterations in the expression of the regulators themselves. When comparing mRNA chip expression data with those of the known major regulators of AHL-mediated quorum sensing (*lasR, lasI, rhlR, rhlI*), *vqsR* was the only gene with a comparatively higher expression (2.5-fold) in the presence of serum than in LB medium (Table 3.3).

The GeneChip data confirm the results of the Northern blot experiments showing that the expression of *vqsR* is more strongly activated by an environment that contains human serum, as it happens in *Pseudomonas* septicaemia or burn wounds.

Tab. 3.3. GeneChip normalized signal intensities of mRNA transcripts involved in quorum sensing regulation in different environments. Numbers in brackets indicate relative expression values in 10 mM H_2O_2 and 10 % serum compared to growth in LB medium.

Gene	ORF[1]	Expression of mRNA transcripts				
		LB	10 mM H_2O_2		10 % serum	
lasR	PA1430	722.3	360.4	(0.5)	456.4	(0.6)
lasI	PA1432	377.1	239.7	(0.6)	335.7	(0.9)
rhlR	PA3477	1754.2	221.9	(0.1)	769.8	(0.4)
rhlI	PA3476	222.6	84.7	(0.4)	182.8	(0.8)
vqsR	PA2591	120.1	65.2	(0.5)	293.5	(2.4)

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

3.1.6.2.2. *vqsR* links quorum sensing and iron uptake

The GeneChip analysis revealed an important role of VqsR for the modulation of expression of many iron-regulated genes.

In addition to the regulation of the production of the siderophores pyochelin and pyoverdine, iron availability in *P. aeruginosa* modulates also the expression of some virulence factors, including the extracellular protease PrpL and the exotoxin A. In fluorescent pseudomonads, the expression of pyoverdine biosynthesis genes is altered by the alternative sigma factor PvdS, the gene which is known to be controlled by the general iron-co-factored repressor Fur (Lamont *et al.,* 2002; Ravel & Cornelis, 2003).

Recently, two independent microarray analyses (Ochsner *et al.,* 2002; Palma *et al.,* 2003) identified genes induced by iron limitation in *P. aeruginosa*. Comparison of these genes with those found to be VqsR dependent in this study, revealed that in total, 25 genes found to be iron-regulated are also repressed in a *vqsR* mutant. Interestingly, out of these iron-regulated genes, some are also regulated by quorum sensing (Cornelis and Aendekerk, 2004) (Table 3.4).

In the *vqsR* mutant, the *pvdS* gene is downregulated together with other genes belonging to the pyoverdine biosynthetic locus. In addition, the genes which are responsible for the biosynthesis and uptake of the second siderophore, pyochelin, are also downregulated in the *vqsR* mutant.

An overlap of quorum sensing regulated functions and the iron regulon in *P. aeruginosa* was also reported by Arevalo-Ferro *et al.,* 2003 using proteomics, but was not apparent in any of the three previous DNA microarray analyses (Hentzer *et al*., 2003; Schuster *et al*., 2003; Wagner *et al*., 2003). The most plausible explanation for these discrepancies is the fact that media used to grow the bacteria contained iron above a concentration of 10 μM resulting in repression of most of the iron-limitation-induced genes (Vasil, 2003; Cornelis and Aendekerk, 2004). Another reason for these discrepancies could be the usage of planctonic *P. aeruginosa* PAO1 in the previous three transcriptome analyses. In a recent study (Hentzer *et al.,* 2004), the quorum sensing regulon of sessile *P. aeruginosa* cells was mapped by transcriptomics and in this study many genes were identified, which were previously shown to be iron- regulated. According to authors of that study, quorum sensing influences the response to iron-limitation when cells are in the biofilm mode of growth. Because *P. aeruginosa* strain TB used in our work is known to form microcolonies and biofilm-like structures even when growing in the planctonic culture, this could be another explanation why the iron regulon was affected by the mutation of *vqsR*, but was not apparent in the previous DNA microarray analyses.

This genome-wide transcriptome analysis provided clear evidence that certain sets of genes are only affected by quorum sensing when particular environmental conditions prevail. The complexity of the quorum sensing circuitry may be therefore the result of the demand for very precise regulation of certain sets of genes in response to both environmental stimuli and population density. This genetic flexibility may be one of the key elements responsible for the tremendous environmental versatility of *P. aeruginosa*.

Tab. 3.4. QS and iron limitation. Genes known to be induced by iron limitation as detected by transcriptome analyses (Ochsner *et al.,* 2002; Palma *et al.,* 2003) with downregulated expression in a *vqsR* mutant in the presence of serum and H_2O_2 (Cornelis and Aendekerk, 2004).

ORF[1]	Gene name	Fold change[2]		Protein description
		Serum	H_2O_2	
PA0026[3]		4.4		hypothetical protein
PA0027[3]		6.6		PPIase, chaperone
PA1003[3]	*mvfR*	3.0		transcriptional regulator
PA1245		5.1		hypothetical protein
PA1248[3]	*aprF*	7.4		alkaline protease secretion protein AprF
PA1431[3]	*rsaL*	52.6	32.7	regulatory protein RsaL
PA2384		4.6		Fur-like regulator
PA2405		3.2		pyoverdine biosynthesis
PA2406		3.1		pyoverdine biosynthesis
PA2412	*mbtH*	6.0	7.3	pyoverdine biosynthesis
PA2426	*pvdS*	7.3	4.8	sigma factor PvdS
PA4175[3]	*prpL*	5.6		probable endoproteinase Arg-C precursor
PA4218		4.6	6.5	probable transporter
PA4219			21.9	hypothetical protein
PA4221	*ftpA*	16.8	17.8	Fe(III)-pyochelin receptor precursor
PA4223			6.7	probable ATP-binding component of ABC transporter
PA4224	*pchG*		8.9	pyochelin biosynthetic protein PchG
PA4225	*pchF*	7.5	9.9	pyochelin synthetase
PA4226	*pchE*	14.9	41.0	dihydroaeruginoic acid synthetase
PA4228	*pchD*	13.1	10.3	pyochelin biosynthesis protein PchD
PA4229	*pchC*	11.1	7.3	pyochelin biosynthetic protein PchC
PA4230	*pchB*	18.0	29.0	salicylate biosynthesis protein PchB
PA4231	*pchA*	5.3	9.3	salicylate biosynthesis isochorismate synthase
PA4359	*FeoA*	3.8		iron(II) uptake

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons.

[3] Genes also known to be regulated by quorum sensing.

3.1.6.2.3. Additional genes regulated by *vqsR*

Tables 3.5 and 3.6 list genes regulated by VqsR which were not previously identified as being quorum sensing or iron regulated by transcriptome analyses (Hentzer *et al*., 2003; Schuster *et al*., 2003; Wagner *et al*., 2003; Ochsner *et al.,* 2002; Palma *et al.,* 2003). Out of these additional VqsR dependent genes, the major upregulated genes in the *vqsR* mutant are implicated in the antimicrobial peptide resistance, chemotaxis, proton pumping, arginine metabolism and transport of small molecules. On the other hand, disruption of *vqsR* downregulated one multidrug efflux system, genes responsible for the antimicrobial peptide resistance and transport.

However, some of these, as for instance the multidrug efflux systems were already proven to be involved in the quorum sensing in *P. aeruginosa* (Aendekerk *et al.,* 2002; Maseda *et al.,* 2004). Consistent with that, MexEF-OprN, the multidrug efflux system which affects cell-to-cell signalling (Kohler *et al.,* 2001) was also downregulated in the *vqsR* mutant (Table 3.6).
Moreover, the *nirS* gene (PA0509), which is implicated in the denitrification was upregulated in the presence of H_2O_2 in this study (Table 3.5). Even if nitrite and nitrate respiration have been previously reported to be repressed by quorum sensing (Wagner *et al.,* 2003), the *nirS* gene was so far not identified in the transcriptome analyses (Hentzer *et al*., 2003; Schuster *et al*., 2003; Wagner *et al*., 2003). Therefore many of these ORFs may represent an additional, novel quorum sensing genes whose expression was not detected by the previous transcriptome analyses due to their strict VqsR and/or environmental dependency.

A good example of the importance of the environment for regulation of the quorum sensing genes is the different modulation of the *oprH-phoP-phoQ* (PA1178-PA1179) operon, which is known to be involved in the antimicrobial peptide resistance. In our study, this operon was downregulated by VqsR in the presence of serum and upregulated in the presence of oxidative stress. This is consistent with the observation about the slightly different pools of quorum sensing regulated genes in the independent transcriptome analyses (Vasil, 2003) and with the knowledge about the fine tuning of the *P. aeruginosa las* and *rhl* quorum sensing systems by global regulators.

Tab. 3.5. Genes with upregulated expression in the *vqsR* mutant.

ORF[1]	Gene name	Fold change[2]		Protein description
		Serum	H_2O_2	
Antimicrobial peptide resistance				
PA1178	*oprH*		4.9	outer membrane protein H1 precursor
PA1179	*phoP*		6.1	two-component response regulator PhoP
PA1180	*phoQ*		5.7	two-component sensor PhoQ
PA3553			20.4	probable glycosyl transferase
PA3554			19.8	conserved hypothetical protein
PA3555			8.0	conserved hypothetical protein
PA3556	*arnT*		8.9	Prot.name: inner membr. L-Ara4N transferase ArnT
PA3557			15.3	conserved hypothetical protein
PA3558			8.1	hypothetical protein
Chemotactic transducers				
PA1561	*aer*		4.8	aerotaxis receptor Aer
PA2788			4.8	probable chemotaxis transducer
Proton pumps, energy metabolism				
PA1555			15.9	probable cytochrome c
PA1556			10.8	probable cytochrome c oxidase subunit
PA1560			12.7	hypothetical protein
PA2664	*fhp*		17.2	flavohemoprotein
PA4811	*fdnH*		6.3	nitrate-inducible formate dehydrogenase, beta subunit
PA4919	*pcnB1*		6.3	nicotinate phosphoribosyltransferase
PA4920	*nadE*		6.3	NH3-dependent NAD synthetase
Transport of small molecules				
PA0073		4.2		probable ATP-binding component of ABC transporter
PA0783	*putP*	3.6		sodium/proline symporter PutP
PA1297		4.4		probable metal transporter
PA1429			4.8	probable cation-transporting P-type ATPase
PA3901	*fecA*		5.3	Fe(III) dicitrate transport protein FecA
Arginine metabolism				
PA5170	*arcD*		5.1	arginine/ornithine antiporter
PA5171	*arcA*		21.3	arginine deiminase
PA5172	*arcB*		35.7	ornithine carbamoyltransferase, catabolic
PA5173	*arcC*		32.0	carbamate kinase
Denitrification				
PA0519	*nirS*		19.8	nitrite reductase precursor
Other genes				
PA0045		5.4		hypothetical protein
PA0522		6.3		hypothetical protein

ORF[1]	Gene name	Fold change[2]		Protein description
		Serum	H_2O_2	
Other genes				
PA0526			7.8	hypothetical protein
PA0835	*pta*		7.3	phosphate acetyltransferase
PA1137			17.2	probable oxidoreductase
PA1333		4.3		hypothetical protein
PA2085		3.8		probable ring-hydroxylating dioxygenase small subunit
PA2264		4.7		conserved hypothetical protein
PA2567			5.3	hypothetical protein
PA2662			27.1	conserved hypothetical protein
PA2663			16.0	hypothetical protein
PA2783		4.0		hypothetical protein
PA2794			37.9	hypothetical protein
PA2814			4.9	hypothetical protein
PA2862	*lipA*	4.7		lactonizing lipase precursor
PA2916			10.2	hypothetical protein
PA3006	*psrA*		4.3	transcriptional regulator PsrA
PA3160	*wzz*		4.8	O-antigen chain length regulator
PA3309			6.8	conserved hypothetical protein
PA3458			5.1	probable transcriptional regulator
PA3552			9.2	conserved hypothetical protein
PA3559			18.6	probable nucleotide sugar dehydrogenase
PA3586		25.5	5.7	probable hydrolase
PA3811	*hscB*	4.9		heat shock protein HscB
PA3814	*iscS*	4.6		L-cysteine desulfurase (pyridoxal phosph.-dependent)
PA3815		4.4		conserved hypothetical protein
PA3919			5.9	conserved hypothetical protein
PA3972			17.3	probable acyl-CoA dehydrogenase
PA3973			19.8	probable transcriptional regulator
PA4166		5.1		probable acetyltransferase
PA4352			4.9	conserved hypothetical protein
PA4596		4.6	6.7	probable transcriptional regulator
PA4610			6.6	hypothetical protein
PA4630		4.8		hypothetical protein
PA4683			13.5	hypothetical protein
PA4758	*carA*	3.6		carbamoyl-phosphate synthase small chain
PA4773			6.2	hypothetical protein
PA4774			15.1	hypothetical protein
PA4775			12.2	hypothetical protein
PA4776			10.3	probable two-component response regulator

ORF[1]	Gene name	Fold change[2] Serum	H_2O_2	Protein description
Other genes				
PA4777			6.1	probable two-component sensor
PA4782			6.7	hypothetical protein
PA5098	*hutH*	15.4		histidine ammonia-lyase
PA5100	*hutU*	6.0		urocanase

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons.

Tab. 3.6. Genes with downregulated expression in the *vqsR* mutant.

ORF[1]	Gene name	Fold change[2] Serum	H_2O_2	Protein description
Multidrug efflux system				
PA2493	*mexE*		12.6	RND multidrug efflux membr. fusion prot. MexE prec.
PA2494	*mexF*		14.0	RND multidrug efflux transporter MexF
PA2495	*oprN*		8.1	outer membrane protein OprN precursor
Antimicrobial peptide resistance				
PA1178	*oprH*	13.5		outer membrane protein H1 precursor
PA1179	*phoP*	4.6		two-component response regulator PhoP
Transport of small molecules				
PA2322			10.9	gluconate permease
PA4614	*mscL*	3.9		conductance mechanosensitive channel
PA5434	*mtr*		13.3	tryptophan permease
Other genes				
PA0459		3.3		probable ClpA/B protease ATP binding subunit
PA0587		3.4		conserved hypothetical protein
PA0654	*speD*		5.3	S-adenosylmethionine decarboxylase proenzyme
PA0704		5.0		probable amidase
PA1159		2.9	6.8	probable cold-shock protein
PA1344		18.8		probable short-chain dehydrogenase
PA1790			7.2	hypothetical protein
PA1874		5.1		hypothetical protein
PA1970			6.7	hypothetical protein
PA2182		3.8	11.3	hypothetical protein
PA2203			4.9	probable amino acid permease

ORF[1]	Gene name	Fold change[2]		Protein description
		Serum	H_2O_2	
Other genes				
PA2208			10.6	hypothetical protein
PA2441			8.3	hypothetical protein
PA2622	*cspD*	3.0		cold-shock protein CspD
PA2659		3.4		hypothetical protein
PA2806			5.1	conserved hypothetical protein
PA2864		3.8		conserved hypothetical protein
PA3229			10.6	hypothetical protein
PA3291			7.5	hypothetical protein
PA4140		11.0	27.0	hypothetical protein
PA4220			73.1	hypothetical protein
PA4354			6.5	conserved hypothetical protein
PA4377		3.9		hypothetical protein
PA4482	*gatC*		5.7	Glu-tRNA(Gln) amidotransferase subunit C
PA4625			5.7	hypothetical protein
PA5180			8.7	conserved hypothetical protein
PA5181			9.9	probable oxidoreductase
PA5351	*rubA1*		7.4	rubredoxin
PA5404			12.7	hypothetical protein

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons.

3.1.6.3. GeneChip expression analysis of the VqsR regulon in the ABC minimal medium

AB minimal medium was previously shown to induce the expression of the *vir* operon of *Agrobacterium tumefaciens*, an operon which is known to be implicated in the pathogenicity of that bacterium in different infection models (Gray *et al.,* 1992; Wirawan *et al.,* 1993). Furthermore, the same medium supplemented with 10 mM citrate (ABC minimal medium) was extensively used in previous studies when investigating the quorum sensing dependent secretion of virulence factors and biofilm formation of *P. aeruginosa* (Huber *et al.,* 2001; Huber *et al.,* 2003). The enhanced virulence properties of *P. aeruginosa* in minimal medium were also reflected by the large number of differentially expressed genes in the investigated VqsR mutant. The differences in the gene expression of wild type and VqsR mutant ranged from 2-fold

to up to more than 400-fold. In our experimental setting, the mutation of *vqsR* altered the expression of 731 genes more than 5-fold, 457 of which were upregulated and 274 downregulated.

3.1.6.3.1. VqsR and modulation of general metabolism

Figure 3.20 depicts the number of differentially regulated genes classified by metabolic categories (Stover *et al.,* 2000). The largest proportion of all regulated genes (43 %) belongs to the class of hypotheticals with unknown function. Expression of 456 out of these hypothetical genes was upregulated in the mutant, whereas the expression of 131 genes was downregulated. In the VqsR mutant the expression of genes was downregulated that are involved in amino acid, energy and fatty acid metabolism, nucleotide biosynthesis, transcription, translation and post-translational processing and secretion of proteins. On the other hand the disruption of *vqsR* led to the upregulation of genes implicated in carbon compound catabolism, transport and membrane constituents. Moreover, numerous transcriptional regulators, σ^{70}-factors and two-component systems were upregulated demonstrating that VqsR is a key regulatory protein of the *P. aeruginosa* cell. In summary, the central anabolic pathways were downregulated, whereas transport and catabolic pathways were upregulated in the investigated VqsR mutant (Figure 3.20, Appendix I). This observation suggests that besides the impact on virulence, quorum sensing and iron homeostasis (Juhas *et al.,* 2004), VqsR also plays an important role in the regulation of the general metabolism of *P. aeruginosa.*

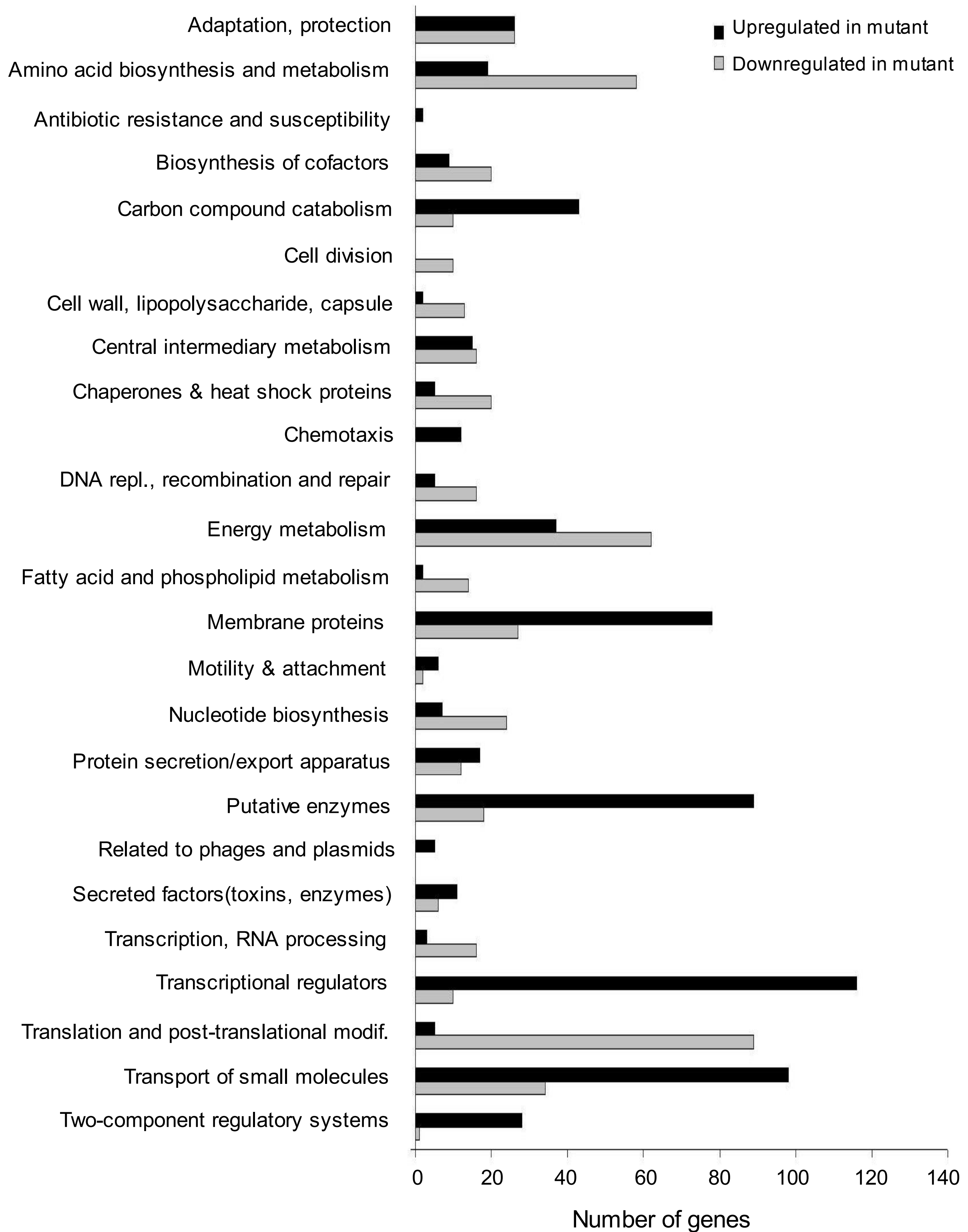


Fig. 3.20. Comparison of the transcriptome of *P. aeruginosa* TB and its *vqsR* Tn*5* transposon mutant during growth in ABC medium. The number of genes with significantly different cDNA hybridization signals on the Affymetrix *P. aeruginosa* GeneChip is classified by metabolic category as defined in the original publication on the PAO genome sequence (Stover *et al.,* 2000).

3.1.6.3.2. VqsR regulates the expression of ECF sigma factors and additional group of quorum sensing genes

Seven genes encoding different sigma factors (PA0149, PA0472, PA1350, PA1912, PA2468, PA3899, PA4896) which belong to the extracytoplasmic function (ECF) subfamily of the σ^{70}– factors were found to be upregulated more than 5-fold in the VqsR mutant. Interestingly, six of them are adjacent to genes involved in the iron metabolism, thus suggesting that VqsR partially exerts its impact on iron uptake and metabolism through the direct antagonism on these ECF sigma factors.

Disruption of *vqsR* differentially affected the expression of numerous genes of the *las* and *rhl* quorum sensing network in the ABC minimal medium. Of the genes identified as being quorum sensing regulated by previous transcriptome analyses (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003), 125 genes were upregulated and 58 genes were downregulated in the VqsR mutant (Figure 3.21). The expression of a few quorum sensing genes which were previously shown to belong to the VqsR regulon was not altered in the ABC minimal medium. This group comprises genes implicated in phenazine and pyocyanin biosynthesis (*phzB, phzC, phzD, phzE, phzF, phzG, phzM, phzS*), in the synthesis of lectins (*pa1L, lecB*) and of the stationary sigma factor RpoS as well as few genes involved in the biosynthesis of other bacterial exoproducts. The most plausible explanation for these discrepancies is the fact that for this study, the bacteria were grown into exponential phase (OD_{600} = 1.0), whereas in previous transcriptome analyses the cultures were investigated in the stationary phase (OD_{600} = 5.0). The stationary sigma factor RpoS as well as many virulence exoproducts of *P. aeruginosa*, including pyocyanin and lectins are known to be only produced at the high cell concentrations of the stationary phase.

On the other side, a large number of previously undescribed VqsR-regulated quorum sensing genes was identified. Tables 3.7 and 3.8 list those quorum sensing genes which were not previously categorized in the VqsR-regulon but whose expression was altered significantly in the VqsR mutant in the examined ABC minimal medium. In addition to numerous hypotheticals of unknown function, the mutation of *vqsR* affected positively the expression of components of a multidrug efflux pump *mexG, opmD* as well as of *lasR*.

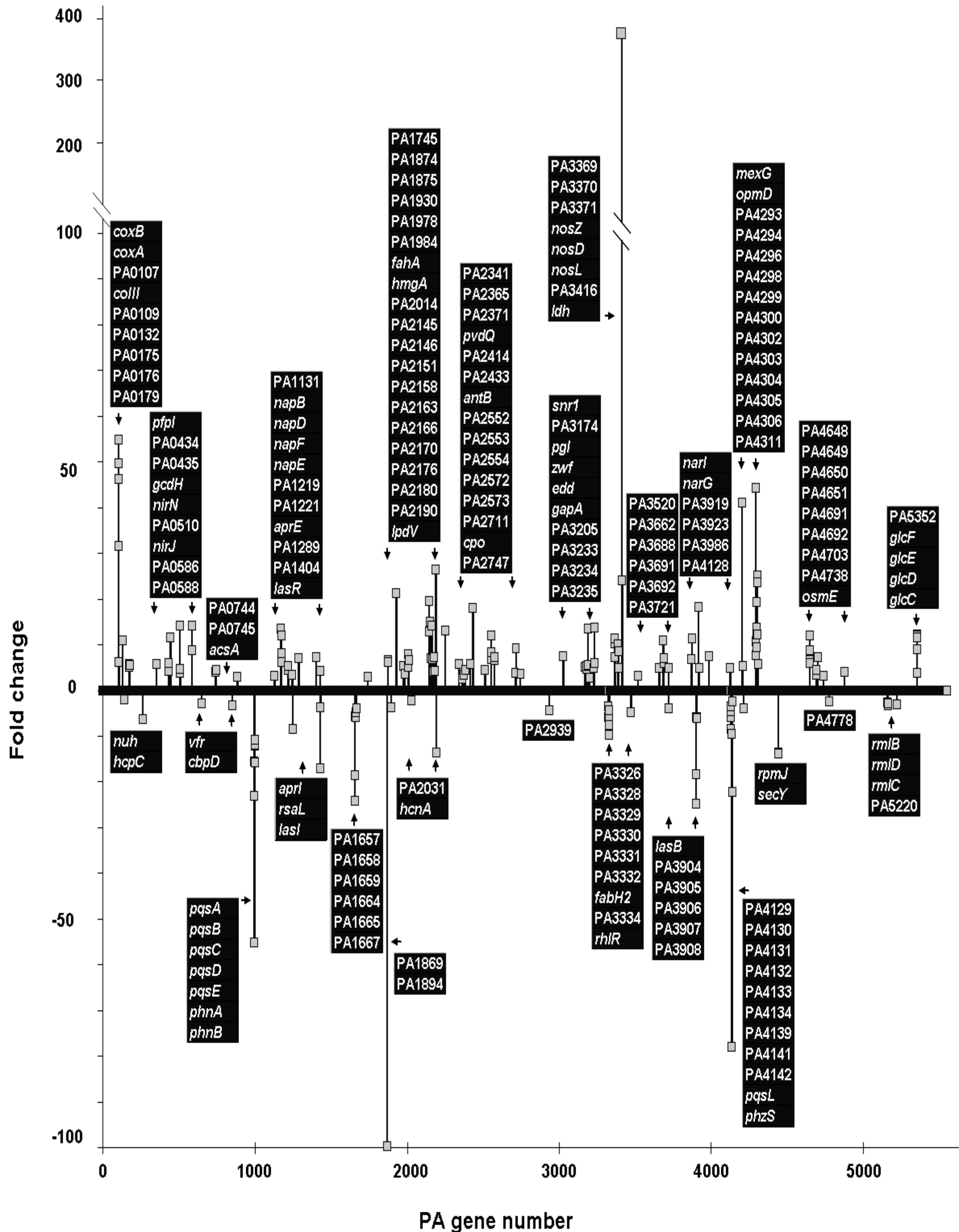


Fig. 3.21. VqsR-regulated quorum sensing genes in ABC minimal medium. The figure depicts the subgroup of VqsR-regulated genes that in previous GeneChip analyses on *lasRI rhlIR* mutants had been identified to be regulated by the *las* and *rhl* regulon (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003). Positive values represent quorum sensing genes whose expression is upregulated in the mutant, whereas negative values represent quorum sensing genes whose expression is downregulated in the mutant compared to the TB wild type strain.

The influence of VqsR on the expression of LasR, together with the presence of a *las* box in the promoter region of VqsR, underlines the existence of an autoregulatory feedback loop between VqsR and *las* quorum sensing circuit. Besides numerous hypothetical genes, the disruption of *vqsR* negatively affected the expression of the PQS biosynthesis operon (*pqsA, pqsB, pqsC, pqsD, pqsE, phnA, phnB*) that is implicated in the modulation of quorum sensing and virulence and of the transcriptional regulator Vfr that controls type III mediated secretion of virulence factors (Wolfgang *et al.,* 2003).

In conclusion, this analysis identified novel genes belonging to the VqsR-regulon of *P. aeruginosa*. Results presented show that VqsR plays an even more important role in the virulence and quorum sensing network of *P. aeruginosa* than concluded from GeneChip expression analysis in the presence of serum and H_2O_2. Furthermore, VqsR was shown to be involved in the regulation of ECF sigma factors genes and major metabolic pathways, such as nucleotide biosynthesis, transcription, protein biosynthesis and processing.

Tab. 3.7. Upregulated quorum sensing genes. QS genes (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003) upregulated in the *vqsR* mutant cultured in ABC minimal medium, which were not identified as being VqsR regulated in the presence serum and H_2O_2.

ORF[1]	Gene name	Fold change[2]	Protein description
PA0105	*coxB*	31.5	cytochrome c oxidase, subunit II
PA0106	*coxA*	54.7	cytochrome c oxidase, subunit I
PA0107		49.6	conserved hypothetical protein
PA0108	*coIII*	46.2	cytochrome c oxidase, subunit III
PA0109		6.2	hypothetical protein
PA0132		10.8	beta-alanine-pyruvate transaminase
PA0175		5.0	probable chemotaxis protein methyltransferase
PA0176		5.8	probable chemotaxis transducer
PA0179		5.5	probable two-component response regulator
PA0355	*pfpI*	5.8	protease PfpI
PA0434		4.2	hypothetical protein
PA0435		5.9	hypothetical protein
PA0447	*gcdH*	11.5	glutaryl-CoA dehydrogenase
PA0586		14.2	conserved hypothetical protein

ORF[1]	Gene name	Fold change[2]	Protein description
PA0588		8.7	conserved hypothetical protein
PA0744		4.0	probable enoyl-CoA hydratase/ isomerase
PA0745		4.3	probable enoyl-CoA hydratase/ isomerase
PA0887	*acsA*	2.9	acetyl-coenzyme A synthetase
PA1131		3.0	probable MFS transporter
PA1173	*napB*	13.5	cytochrome c-type protein NapB precursor
PA1175	*napD*	8.1	NapD protein of periplasmic nitrate reductase
PA1176	*napF*	8.2	ferredoxin protein NapF
PA1219		4.5	hypothetical protein
PA1221		5.2	hypothetical protein
PA1247	*aprE*	3.4	alkaline protease secretion protein AprE
PA1289		7.0	hypothetical protein
PA1404		7.3	hypothetical protein
PA1430	*lasR*	4.2	transcriptional regulator LasR
PA1745		2.9	hypothetical protein
PA1930		21.2	probable chemotaxis transducer
PA1978		5.3	probable transcriptional regulator
PA1984		3.6	probable aldehyde dehydrogenase
PA2011		3.3	hydroxymethylglutaryl-CoA lyase
PA2014		6.7	probable acyl-CoA carboxyltransferase beta chain
PA2145		19.6	hypothetical protein
PA2151		14.9	conserved hypothetical protein
PA2158		7.0	probable alcohol dehydrogenase (Zn-dependent)
PA2163		14.2	hypothetical protein
PA2166		6.8	hypothetical protein
PA2170		4.1	hypothetical protein
PA2176		7.3	hypothetical protein
PA2180		4.3	hypothetical protein
PA2190		26.5	conserved hypothetical protein
PA2250	*lpdV*	13.1	lipoamide dehydrogenase-Val
PA2341		5.6	probable component of ABC maltose/mannitol transporter
PA2385	*pvdQ*	4.4	probable acylase
PA2414		5.9	L-sorbosone dehydrogenase
PA2433		18.0	hypothetical protein
PA2513	*antB*	4.3	anthranilate dioxygenase small subunit
PA2552		6.9	probable acyl-CoA dehydrogenase
PA2553		12.0	probable acyl-CoA thiolase
PA2554		8.4	probable short-chain dehydrogenase
PA2572		6.6	probable two-component response regulator

ORF[1]	Gene name	Fold change[2]	Protein description
PA2573		7.3	probable chemotaxis transducer
PA2711		3.8	probable periplasmic spermidine/putrescine-binding protein
PA2717		9.1	chloroperoxidase precursor
PA3032	*snr1*	7.5	cytochrome c Snr1
PA3174		5.1	probable transcriptional regulator
PA3182	*pgl*	4.6	6-phosphogluconolactonase
PA3183	*zwf*	5.3	glucose-6-phosphate 1-dehydrogenase
PA3194	*edd*	5.6	phosphogluconate dehydratase
PA3195	*gapA*	13.5	glyceraldehyde 3-phosphate dehydrogenase
PA3233		4.8	hypothetical protein
PA3234		5.9	probable sodium:solute symporter
PA3235		13.8	conserved hypothetical protein
PA3369		10.1	hypothetical protein
PA3370		11.3	hypothetical protein
PA3371		7.1	hypothetical protein
PA3396	*nosL*	8.6	NosL protein
PA3416		397.8	probable pyruvate dehydrogenase E1 component, beta chain
PA3418	*ldh*	24.1	leucine dehydrogenase
PA3662		4.8	hypothetical protein
PA3688		11.0	hypothetical protein
PA3691		5.8	hypothetical protein
PA3692		7.0	probable outer membrane protein precursor
PA3721		4.9	probable transcriptional regulator
PA3923		4.8	hypothetical protein
PA3986		7.5	hypothetical protein
PA4128		4.8	hypothetical protein
PA4205	*mexG*	41.0	membrane protein MexG
PA4208	*opmD*	5.3	probable outer membrane protein precursor
PA4293		44.2	probable two-component sensor
PA4294		10.8	hypothetical protein
PA4298		12.2	hypothetical protein
PA4299		12.6	hypothetical protein
PA4300		13.7	hypothetical protein
PA4303		9.4	hypothetical protein
PA4304		25.1	probable type II secretion system protein
PA4305		12.1	hypothetical protein
PA4311		5.8	conserved hypothetical protein
PA4649		8.8	hypothetical protein
PA4650		6.6	hypothetical protein

ORF[1]	Gene name	Fold change[2]	Protein description
PA4651		6.0	probable pili assembly chaperone
PA4691		3.4	hypothetical protein
PA4692		4.4	conserved hypothetical protein
PA4703		7.3	hypothetical protein
PA4738		3.0	conserved hypothetical protein
PA4876	*osmE*	4.1	osmotically inducible lipoprotein OsmE
PA5352		12.2	conserved hypothetical protein
PA5353	*glcF*	12.0	glycolate oxidase subunit GlcF
PA5354	*glcE*	11.5	glycolate oxidase subunit GlcE
PA5355	*glcD*	9.0	glycolate oxidase subunit GlcD

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons.

Tab. 3.8. Downregulated quorum sensing genes. QS genes (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003) downregulated in the *vqsR* mutant cultured in ABC minimal medium, which were not identified as being VqsR regulated in the presence serum and H_2O_2.

ORF[1]	Gene name	Fold change[2]	Protein description
PA0652	*vfr*	2.9	transcriptional regulator
PA0996	*pqsA*	15.7	probable coenzyme A ligase
PA0997	*pqsB*	23.2	hypothetical protein
PA0998	*pqsC*	55.2	hypothetical protein
PA0999	*pqsD*	12.2	3-oxoacyl-[acyl-carrier-protein] synthase III
PA1000	*pqsE*	12.0	hypothetical protein
PA1001	*phnA*	15.8	anthranilate synthase component I
PA1002	*phnB*	11.0	anthranilate synthase component II
PA1658		18.6	conserved hypothetical protein
PA1659		5.6	hypothetical protein
PA1664		5.9	hypothetical protein
PA1665		4.9	hypothetical protein
PA1667		4.0	hypothetical protein
PA1894		3.3	hypothetical protein
PA3328		3.5	probable FAD-dependent monooxygenase
PA3329		8.1	hypothetical protein
PA3330		10.0	probable short chain dehydrogenase

ORF[1]	Gene name	Fold change[2]	Protein description
PA3333	*fabH2*	4.5	3-oxoacyl-acyl-carrier-protein-synthase II
PA3904		24.9	hypothetical protein
PA3905		18.4	hypothetical protein
PA3906		6.0	hypothetical protein
PA3907		5.9	hypothetical protein
PA3908		6.1	hypothetical protein
PA4129		5.9	hypothetical protein
PA4130		4.7	probable sulfite or nitrite reductase
PA4131		8.5	probable iron-sulfur protein
PA4132		3.6	conserved hypothetical protein
PA4242	*rpmJ*	21.9	50S ribosomal protein L36
PA4243	*secY*	15.5	secretion protein SecY

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons.

3.2. Characterization of *gltR* and *47D7*

At this time, only one source of information is available about the function of *gltR*, which presents this gene as a regulator implicated in the transport of glucose (Sage *et al.,* 1996). On the other hand there is nothing known about the product of the *47D7* gene.

The disruption of *gltR* by Tn*5* transposon mutagenesis by previous Ph.D. student (Wiehlmann, 2001) led to decreased resistance of the mutant to human blood serum and to the protease secretion deficiency. The disruption of *47D7* led to complete loss of protease secretion ability of the mutant. These preliminary experiments suggested an important role of the genes *gltR* and *47D7* in *P. aeruginosa* virulence.

To confirm or refute this hypothesis, genes *gltR* and *47D7* were comprehensively analysed.

3.2.1. Features of the *gltR* (PA3192) gene

The sequence of *gltR* (729 bp = 242 amino acids) corresponds to the ORF PA3192 of the *P. aeruginosa* PAO1 genome (http://www.pseudomonas.com) (Figure 3.22).

According to the information stored in the PAO1 database, *gltR* (PA3192) encodes a two-component response regulator GltR which is involved in the carbon compound catabolism and gluconeogenesis. *gltR* (PA3192) is placed in a cluster of genes which encode other glucose phosphorylative pathway enzymes or genes which are responsible for the regulation of this pathway. The end of *gltR* (PA3192) ORF could possibly form a hairpin-like structure but due to its rather low AT-content it would constitute a very weak terminator. Therefore it is more likely that the following ORFs (PA3191*, glk, edd*) are transcribed together with *gltR* (PA3192) on one RNA transcript.

gltR (PA3192) harbours a DNA binding domain in its carboxyl-terminus that is typical for transcriptional regulators and one additional domain that corresponds to the receiver domain of response regulators (Figure 3.23).

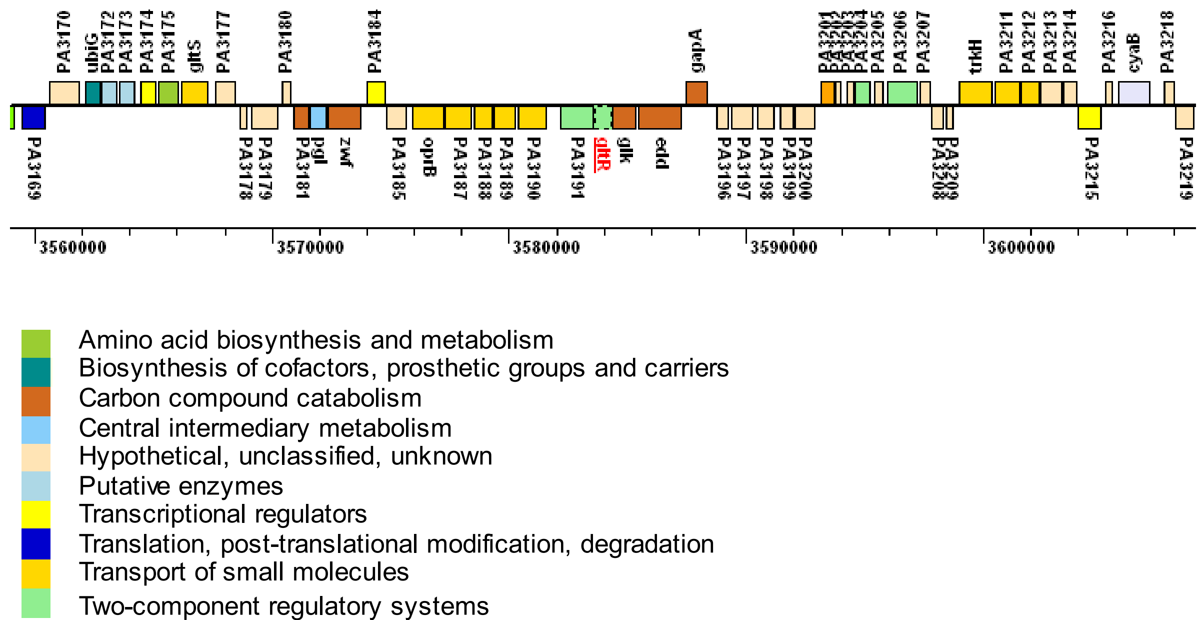


Fig. 3.22. The map showing the part of the *P. aeruginosa* chromosome area around the investigated *gltR* (PA3192) gene (http://www.pseudomonas.com).

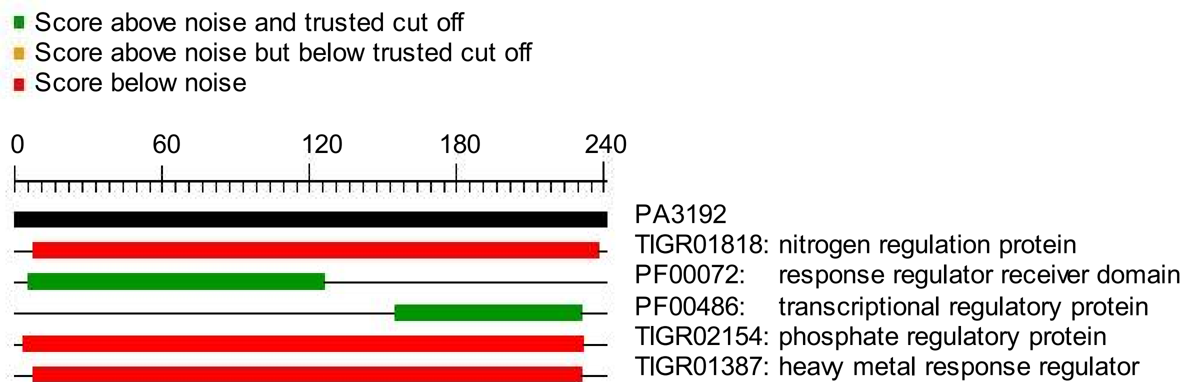


Fig. 3.23. The scheme showing the pfam domains of the *gltR* (PA3192) gene. *gltR* maintains a DNA binding domain in its carboxyl-terminus that is typical for a transcriptional regulator and a domain which corresponds to the receiver domain of response regulators (http://www.tigr.org).

3.2.2. Features of the *47D7* gene

The *47D7* gene has no homologous ORF in the sequenced *P. aeruginosa* PAO1 genome. It shares only a very weak homology ($2\ e^{-19}$) on the protein level with the hypothetical ORF from *Salmonella typhimurium.*

Southern hybridisation revealed that this sequence is not specific only for strain TB, but that it can be also found in two other *P. aeruginosa* isolates (CSGB8 and SG17M) (Wiehlmann, 2001). Sequencing of the *47D7* gene revealed that it comprises a 1308 bp long ORF (Figure 3.24).

```
CTATTCGGTATCGGCTACAGATTCCGGTACGTCTTCGCCGGCGGTTTCTTCATTTTCTCTACTGCGGT
AATCGATCGTTCGTTCTGGGAAGCCTGGGATCCCCTCCCCTTTGGTATCGATGAAGGCATCCCAGTGC
CACCAGCCAGCGGAACCGAACTGACCGTTGTACCAACGAATCTTTCTCTGTCGGCGCGCCTGGTACGT
CGGCGTATTCATCCCGAATACCTCCAGGGGATATTGCGTGTCACGGCCCATGTCTAGCAGCCAGAAGT
CAGGAAAAATTTCATCCTCAGCTTCAAAGCGTGGGGGCTTGAAGAAGGCACGTCCCTCGGCGTGGAGC
TTTTCCTCAATTGTGGCTTCGTAGCCGGAGTCGAGCGGAATCATCCGGTCGCTGACGCGCATCAGCGC
CAAATCCAGGACATCCGCGTAGCGCCCCTTCTTCAGATTCAGCTGCGCGATCGCAATAACCTCGTGCC
CAGACTCCCACGCGACTAGCTCAGAAGCAAAGCTACGTTCAACTCGGCGCCGAACCTCCGCTCGAATG
TCCATCGTCGGCATCCCGAAGGGTCCCGAGACAGAAAGCGTCAAGAGATTGCGTCGTTCCGAGTTGAA
GGACGCCAGCGGCGACACCGCAATGAGCCTGCGCGAATGCTCCTGCGCCAGCTCCACGACCTCAGCAT
TGCGCTTCGCCATGCGGGAGTTCTTCTTGGCCGACATCAGCAAGACGTCGAACACCGTCATTCGGCTG
GCCCGGATACGCTTTGCGGCTTCAGATACCCAATAGGCAACATTCGCGGGCGTTCGCTTGCCTTCCAT
CAGTGGGTACCAGTTGGCCAGTCCAGCCTCTAACCACAGCAGCTGGAGCAGCCCGAGCAGACGTATCG
AAGGCTTCTTAACCCCCGGCGTACGCGGAACGTCGGCCACGTCATCGGCGGCGGGAGACGCTTCTTTG
ACTCGAAGGCCATGGGCTAGCCGGATGCGCAGGGTGTTGTCATCGGCCTCCTCTACAACTCCCTCAGC
GTAGCCCTGCAGGCCTGATCGCTCTGGGGCGTGAGCATGGAATCGGCAGTCGTTTGCATGCTCCGGAC
CGGTGTTGGCGTACCGGGCAAGGTGATAGTTGTCGCTCCCCTCCCGATGTTTCACCGAGAGATAGCGA
TTACCCTTGCCGGGGCACAGGCAGACCACAGGGGTACCGTGTGCTCGTTGCAGGACCGATTTCCAGCC
ATCCTCGAATTCGTTCTTGACCTGGAATTCCCTGGAATATTTCTTCTGGGTACTGACCACCCGTACTT
CGTAAGTTTTCGACAT
```

Fig. 3.24. Complete sequence of the investigated non-PAO1 gene *47D7*.

3.2.3. Complementation of the *gltR* and *47D7* mutants

The *P. aeruginosa gltR* and *47D7* mutants were generated by Tn*5* transposon mutagenesis (Wiehlmann 2001). Subsequently these mutants were complemented *in trans* to ensure that the observed phenotypes (decreased resistance to human blood serum and protease secretion deficiency) were caused by the transposon inactivation of the respective genes and not by any other secondary genetic event.

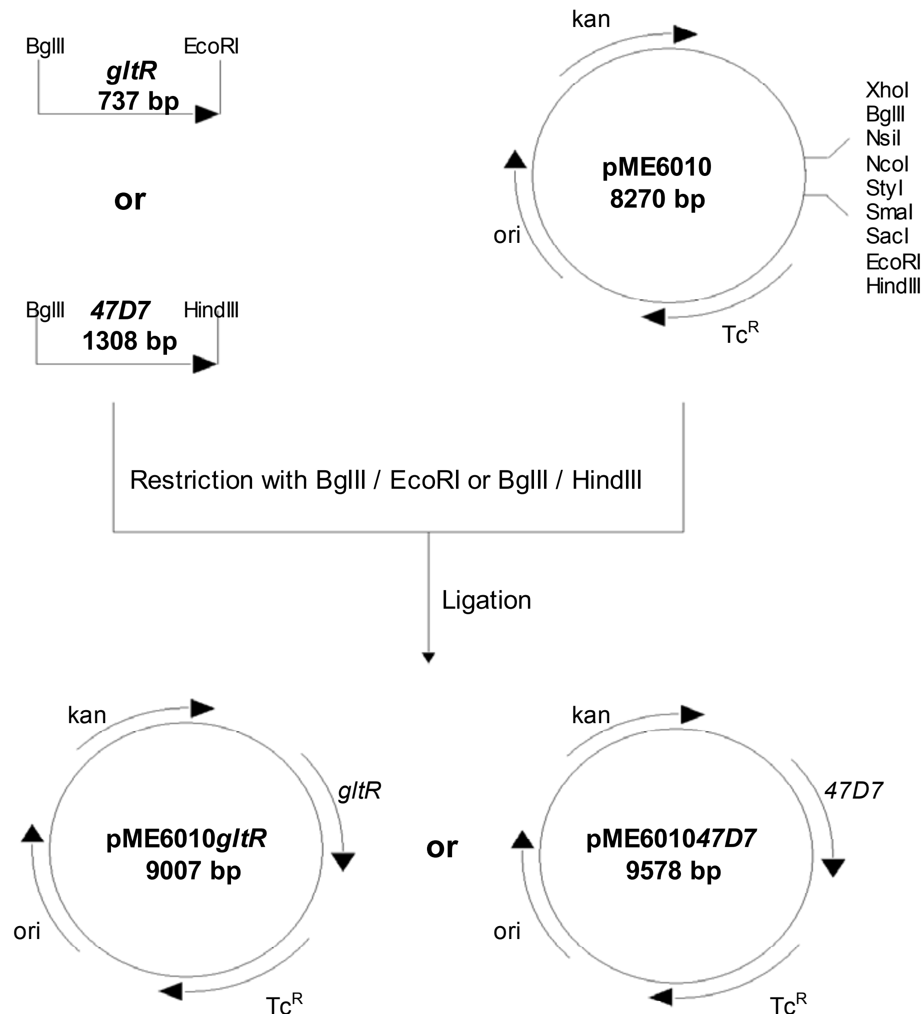


Fig. 3.25. Scheme of the cloning of the genes *gltR* and *47D7* into pME6010 vector.
The PCR fragments with the lengths of 737 bp and 1308 bp comprising the analysed genes *gltR* and *47D7*, respectively were cloned in the broad host range vector pME6010 maintaining the tetracycline resistance. The recombinant plasmids pME6010*gltR* and pME6010*47D7* were subsequently used for complementation of the phenotypes of the investigated *P. aeruginosa* mutants.

The pME6010*gltR* plasmid used for complementation *in trans* was constructed by cloning the 737 bp of the *gltR* gene generated by PCR with primers: 5'-GAT TAT AGA TCT GTG AGC GCG AAC GGA CG-3', 5'-GTC ATG AAT TCG CTC ATG GCT GCA GGT-3' into the BglII/EcoRI-restricted pME6010 shuttle vector. The pME6010*47D7* plasmid was constructed by cloning the 1308 bp of the *47D7* gene generated by PCR with primers: 5'-GAT TAT AGA TCT ATG TCG AAA ACT TAC-3', 5'-GTA TAT AAG CTT CTA TTC GGT ATC GGC-3' into the BglII/HindIII - restricted pME6010 shuttle vector (Figure 3.25). The constructed recombinant plasmids pME6010*gltR* and pME6010*47D7* were introduced into *E. coli* OneShot®TOP10 chemically competent cells (Invitrogen) by transformation and subsequently into the *P. aeruginosa* mutant *gltR* and *47D7* via electroporation (see chapter 2.3.). The restriction digest of the recombinant plasmids pME6010*gltR* and pME6010*47D7* isolated from the complemented *P. aeruginosa* strains confirmed genetic complementation of the *gltR* and *47D7* mutations (Figures 3.26 and 3.27).

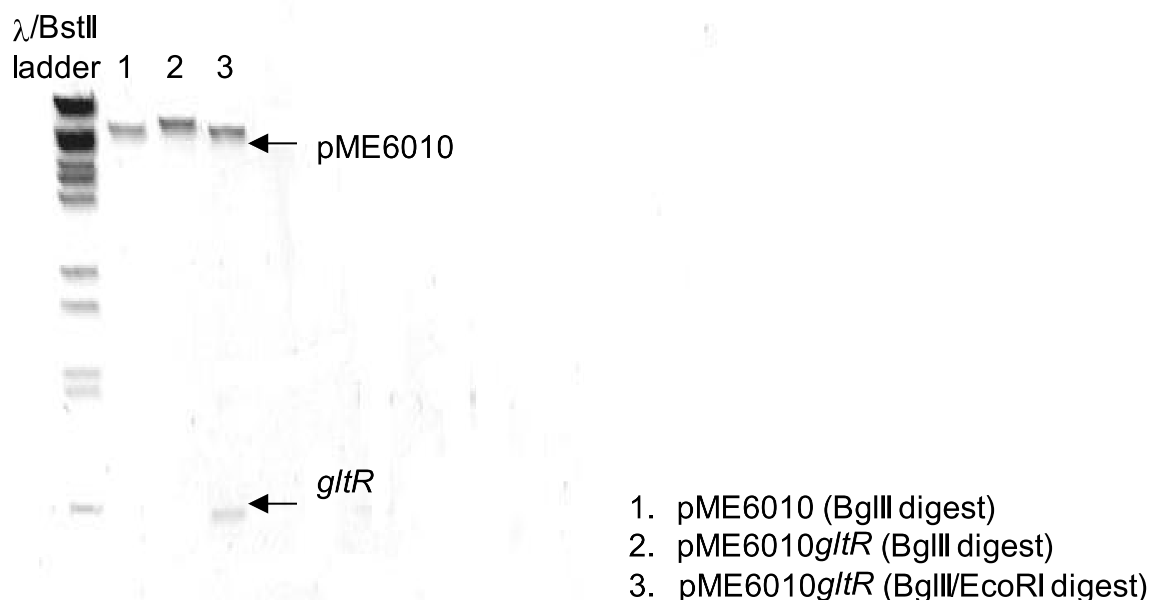
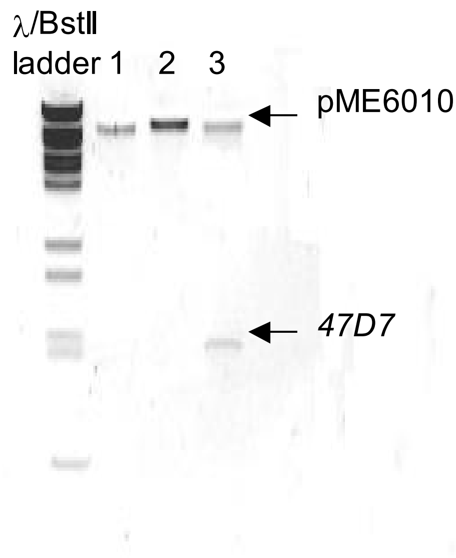


Fig. 3.26. The restriction digest of the recombinant plasmid pME6010*gltR*. pME6010*gltR* was isolated from the complemented *P. aeruginosa* TB*gltR*(pME6010*gltR*) mutant strain.



1. pME6010 (BglII digest)
2. pME6010*47D7* (BglII digest)
3. pME6010*47D7* (BglII/HindIII digest)

Fig. 3.27. The restriction digest of the recombinant plasmid pME6010*47D7*. pME6010*47D7* was isolated from the complemented *P. aeruginosa* TB*47D7*(pME6010*47D7*) mutant strain.

3.2.4. Phenotypic complementation of the *gltR* and *47D7* mutants

Disruption of *gltR* and *47D7* by Tn*5* transposon mutagenesis (Wiehlmann, 2001) caused decreased resistance to human blood serum as well as the significant protease secretion deficiency of the *gltR* mutant and the complete loss of the protease secretion ability of the *47D7* mutant. To test whether these striking phenotypes were caused by transposon inactivation of the *gltR* and *47D7* genes or by a secondary genetic event, the same experiments were performed with all five strains: TB wild type, *gltR* and *47D7* mutants and both constructed complemented mutants.

Susceptibility to serum:

The tested *P. aeruginosa* strains were grown for 2 hours in the presence of human AB serum and the number of survived bacteria was evaluated as described in the chapter 2.6.2.

In this assay, *gltR* mutant showed decreased resistance to serum when comparing to the wild type strain, but the phenotype of the complemented mutant was not restored to the levels comparable to the wild type strain (Figure 3.28). In fact, the resistance of the complemented mutant to serum was even lower than the resistance of the *gltR* mutant, thus confirming that GltR is not important for the survival of *P. aeruginosa* in serum.

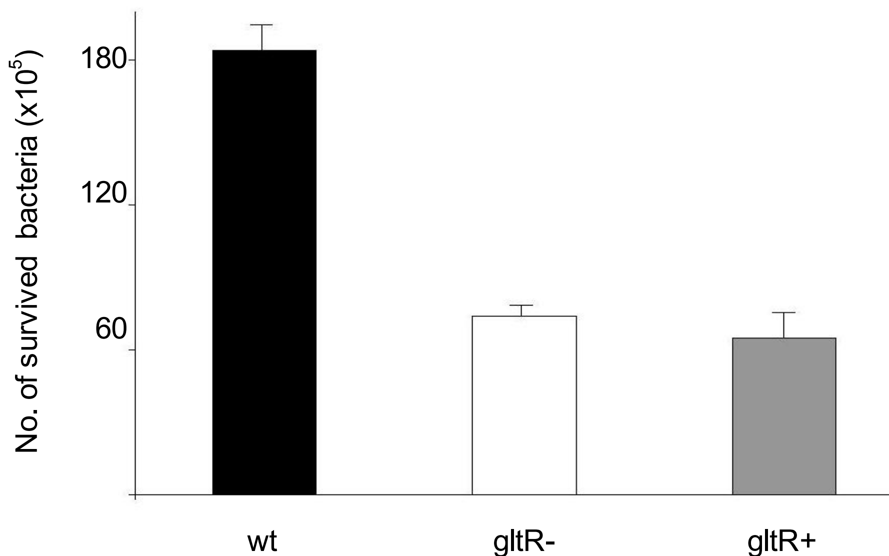


Fig. 3.28. Susceptibility to serum of the *P. aeruginosa* TB wild type strain (wt), its Tn*5*::*gltR* transposon mutant (gltR-) and complemented mutant (gltR+). The survival ability of the complemented mutant was not restored to the levels comparable to the wild type.

Proteases:

The secretion of proteases was determined by growth of the investigated *P. aeruginosa* strains on M9 agar plates supplemented with 0.75 % casein (see chapter 2.6.3.2).

The easily visible halo on casein agar plates, indicating proteolytic activity was observed for the TB wild type strain and in the *gltR* mutant the secretion of proteases was slightly reduced as expected (Figure 3.29). However, in the complemented mutant strain the proteolytic activity was not restored as it would be expected in case that the protease secretion deficiency was caused by mutation of *gltR* (Figure 3.29).

Thus this result revealed that reduced proteolytic activity of the *gltR* mutant was caused by a secondary genetic event and not by the mutation of *gltR* as it was hypothesized by Wiehlmann, 2001.

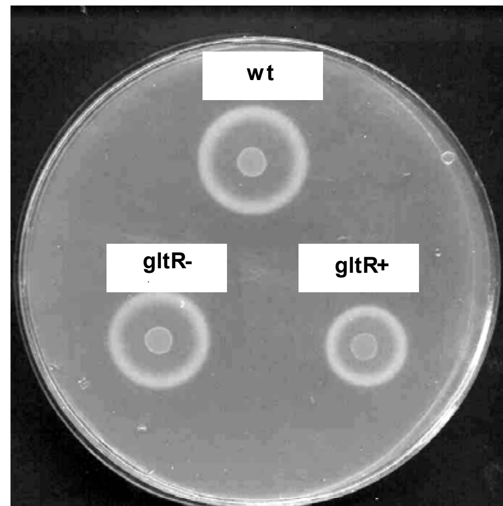


Fig. 3.29. Secretion of proteases by the *P. aeruginosa* TB wild type strain (wt), its Tn*5*::*gltR* transposon mutant (gltR-) and complemented mutant (gltR+). Restoration of the proteolytic activity was not detected for the complemented mutant strain on casein agar.

The easily visible halo on casein agar plates was observed for the TB wild type and in the *47D7* mutant the secretion of proteases was completely switched-off (Figure 3.30). However, in the complemented mutant the proteolytic activity was not restored as it would be expected in case that the protease secretion deficiency was caused by the mutation of *47D7* (Figure 3.30). Thus this experiment confirmed that reduced proteolytic activity of the *47D7* mutant was not caused by mutation of *47D7*, but by a secondary genetic event elsewhere in the genome.

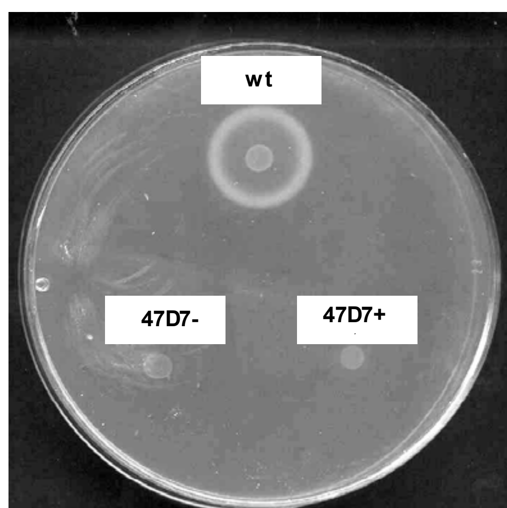


Fig. 3.30. Secretion of proteases by the *P. aeruginosa* TB wild type strain (wt), its Tn*5*::*47D7* transposon mutant (47D7-) and complemented mutant (47D7+). Restoration of the proteolytic activity was not detected for the complemented mutant on casein agar.

3.3. Global regulation of oxidative stress response by IcsF

3.3.1. Features of the *icsF* (PA1572) gene

The sequence of *icsF* (1146 bp = 382 amino acids) corresponds to the ORF PA1572 of the *P. aeruginosa* PAO1 genome (http://www.pseudomonas.com) (Figure 3.31). According to the information stored in the PAO1 database, *icsF* (PA1572) encodes a protein with 56 % homology to a hypothetical protein of *Pyrococcus horikoshii* with the length of 377 amino acids. Like its homologue in *P. horikoshii*, the IcsF protein also belongs to the class of hypotheticals with unknown function, therefore opening a wide field for future investigations.

icsF (PA1572) is placed in a cluster of genes which are implicated in the energy metabolism of *P. aeruginosa* (Figure 3.31). The last 20 bp of the *icsF* (PA1572) build together with the first bases of the following ORF PA1573 a typical terminator structure. This suggests, that the following genes are not transcribed together with *icsF* (PA1572) on a single RNA transcript.

The G+C content of this gene (69,38 mol%) is in the range of average G+C content of the *P. aeruginosa* genome (61,80 mol% - 70,14 mol%) (Stover *et al.*, 2000), thus suggesting that like the *vqsR* gene, *icsF* was not acquired by phylogenetically recent horizontal transfer, but is rather a part of the core *P. aeruginosa* genome.

A blast search revealed that *icsF* (PA1572) harbours a domain that is typical for ATP-NAD kinases (Figure 3.32) and further investigation confirmed that IcsF shares strong homology with the NAD kinase from *E. coli.*

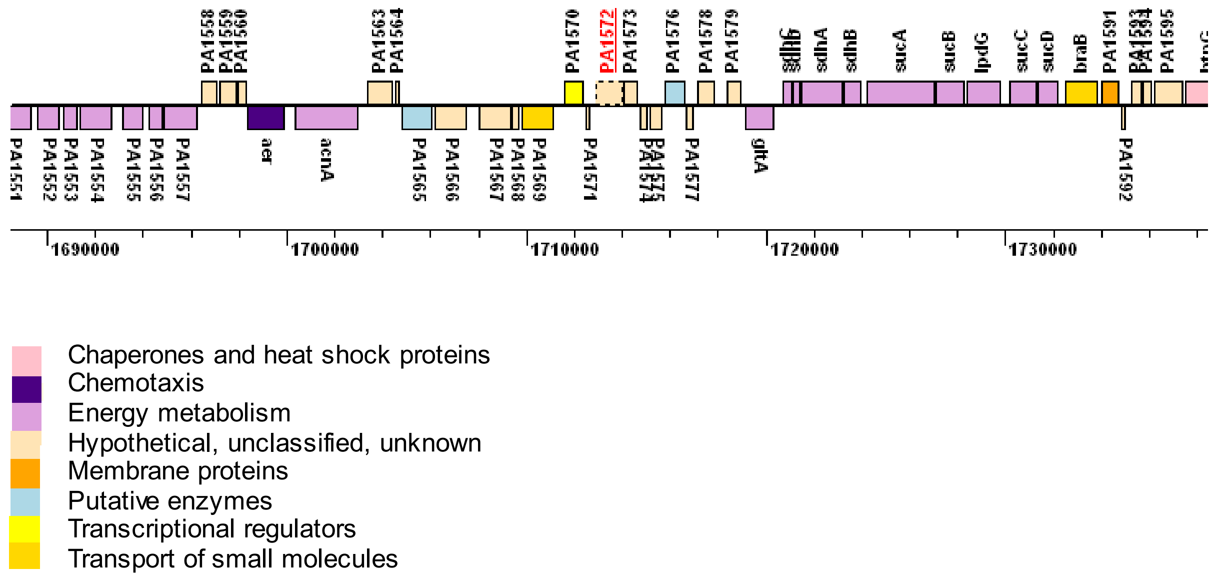


Fig. 3.31. The map showing the part of the *P. aeruginosa* chromosome area around the investigated *icsF* (PA1572) gene (http://www.pseudomonas.com).

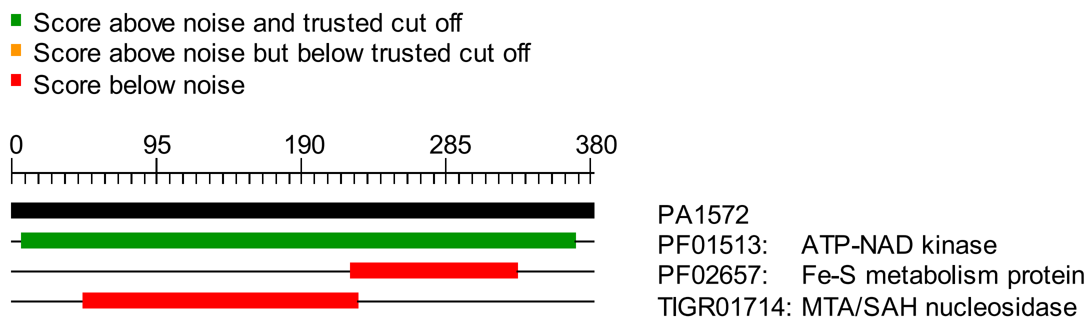


Fig. 3.32. The scheme showing the pfam domains of the *icsF* (PA1572) gene (http://www.tigr.org). *icsF* (PA1572) maintains a domain that is typical for ATP-NAD kinases.

3.3.2. Complementation of the *icsF* gene

The *P. aeruginosa icsF* mutant was generated by Tn*5* signature transposon mutagenesis as described previously (Wiehlmann *et al.,* 2002). This mutant was complemented *in trans* to ensure that all the subsequently observed striking phenotypes (see chapters 3.3.3 – 3.3.6) were caused by the transposon inactivation of the *icsF* gene and not by any other secondary genetic event.

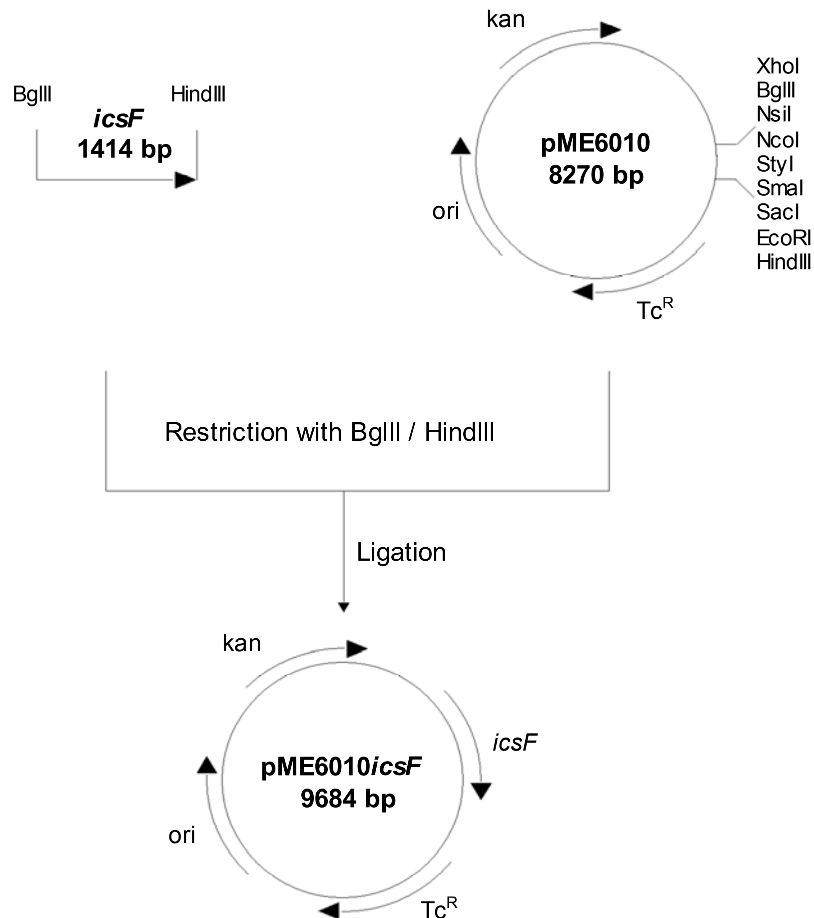


Fig. 3.33. Scheme of the cloning of the *icsF* gene into pME6010 vector.
The PCR fragment with the length of 1414 bp comprising the analysed *icsF* gene was cloned into the broad host range vector pME6010 maintaining the tetracycline resistance. The recombinant plasmid pME6010*icsF* was used for complementation of the phenotype of the *P. aeruginosa icsF* mutant.

The pME6010*icsF* plasmid used for complementation *in trans* was constructed by cloning the 1414 bp of the *icsF* gene generated by PCR with primers: 5'-TAT TAG ATC TAT GGA CAT GTT TCG CCT GG-3', 5'-CTA TAT TAA GCT TCT GTA GAT CCA GCC CC-3' into the *Bgl*II/HindIII-restricted pME6010 shuttle vector (Figure

3.33). The recombinant pME6010*icsF* plasmid was introduced into *E. coli* OneShot®TOP10 chemically competent cells (Invitrogen) by transformation and subsequently into the *P. aeruginosa icsF* mutant via electroporation (see chapter 2.3). The recombinant plasmid pME6010*icsF* was afterwards isolated from electroporated *P. aeruginosa* cells and subjected to restriction digest in order to confirm genetic complementation of the *icsF* mutation (Figure 3.34).

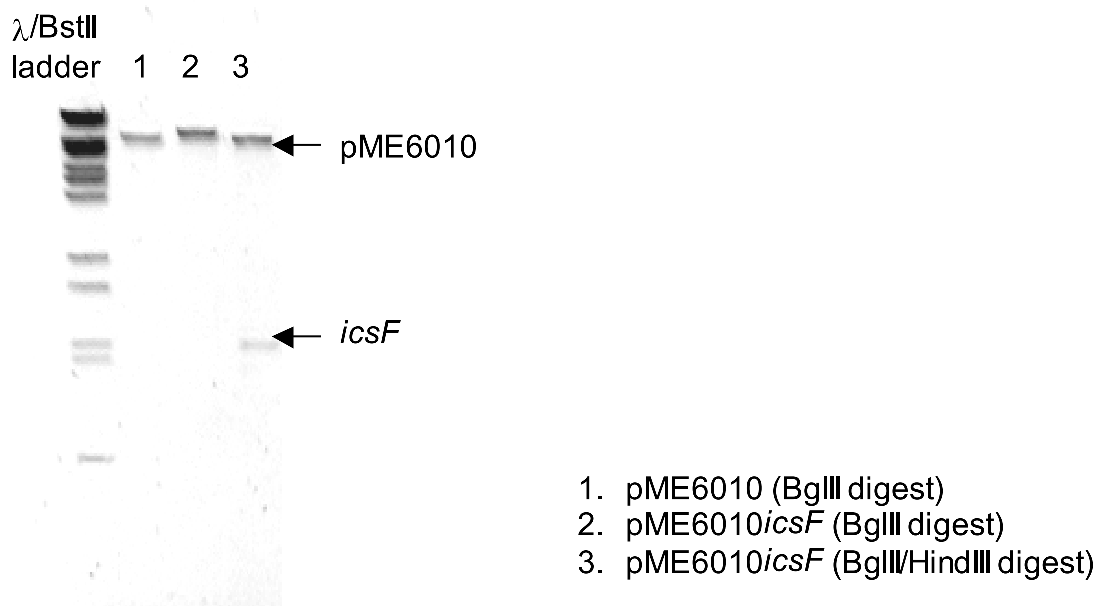


Fig. 3.34. The restriction digest of the recombinant plasmid pME6010*icsF*. pME6010*icsF* was isolated from the complemented *P. aeruginosa* TB*icsF*(pME6010*icsF*) mutant strain.

3.3.3. IcsF is crucial for the intracellular survival in PMNs

PMN-mediated phagocytosis is known as the host's most proficient antipseudomonal weapon (Döring *et al.*, 1995). To investigate the impact of IcsF on the ability of *P. aeruginosa* TB to survive intacellularly in PMNs, wild type strain, Tn*5*::*icsF* transposon mutant and complemented mutant were cultivated for 2 hours in the presence of PMNs. Subsequently PMNs were lysed and the number of intracellular viable bacteria was determined by counting the cfu (see chapter 2.6.1).

As shown in Figure 3.35, this experiment revealed that the ability of the investigated *icsF* mutant to survive intracellularly in PMNs was significantly decreased when compared to the wild type. In fact, the intracellular survival ability of the *icsF* mutant was less than 10 % of the wild type. On the other hand, the intracellular survival ability of the complemented mutant was completely restored and was even slightly higher than that of the wild type (Figure 3.35).

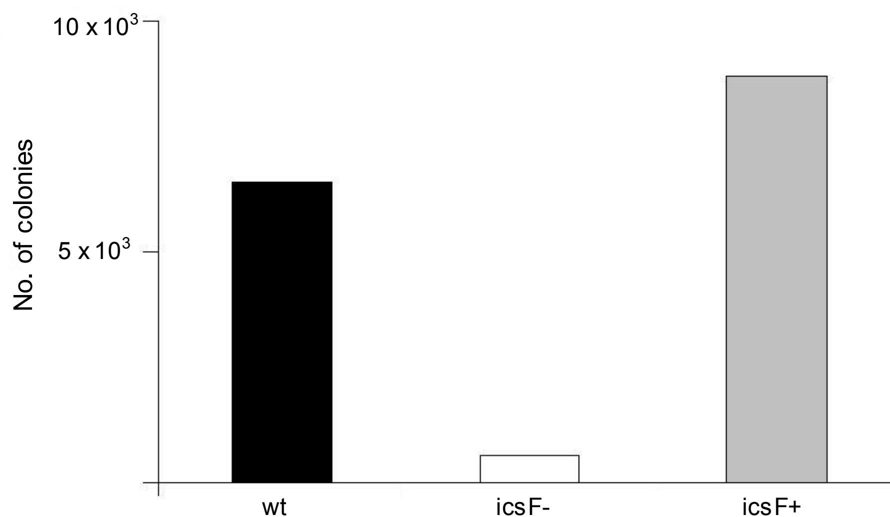


Fig. 3.35. Intracellular survival of the *P. aeruginosa* TB wild type strain (wt), its Tn*5*::*icsF* transposon mutant (icsF-) and complemented mutant (icsF+) in PMNs. The investigated strains were cultured for 2 hours in the presence of PMNs. Subsequently PMNs were lysed and the number of intracellular viable bacteria was determined by counting of cfu.

TB (TBCF10839 isolate) is a highly virulent strain of *P. aeruginosa*, which was isolated in 1983 from the sputum of a CF patient who had been suffering a severe chronic infection with *P. aeruginosa.* The reason for the high virulence of this strain is its ability to survive and replicate in PMNs (Miethke, 1985; Wiehlmann, 2001). All known evidence indicated that some novel mechanisms operate in the *P. aeruginosa* TB which allows this bacterium to thrive in PMNs. So far the genetic basis for the intracellular survival of *P. aeruginosa* strain TB in PMNs remained an enigma; however, the experimental results presented above suggests that the investigated *icsF* gene constitutes one of the crucial elements responsible for this striking phenotype.

3.3.4. IcsF affects production of autoinducer molecules

As mentioned before, quorum sensing in *P. aeruginosa* employs AHLs as autoinducer signalling molecules (Pearson *et al.,* 1994). In order to investigate the impact of the analysed *icsF* gene on AHL production, we streaked the *P. aeruginosa* TB wild type strain and its Tn*5*::*icsF* transposon mutant close to the GFP-based broad range AHL sensor *E. coli* JM105(pJBA89) (Andersen *et al.,* 2001) (Figure 3.36). The AHL secretion of the examined *icsF* mutant was reduced when comparing to the wild type (Figure 3.36).

Two different quorum sensing acylhomoserine lactones *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butyryl homoserine lactone (C_4-HSL) constitute the major components of the *P. aeruginosa las* and *rhl* systems, respectively (Wagner *et al*., 2003). Therefore, the cross-streak experiments were also performed with the biosensor *P. putida* F117 (pKR-C12), which only detects a narrow range of long chain AHLs, being most sensitive for 3-oxo-C_{12}-HSL (Steidle *et al.,* 2001). As with *E. coli* (pJBA89), the investigated *icsF* mutant secreted remarkably lower amounts of AHLs (Figure 3.37).

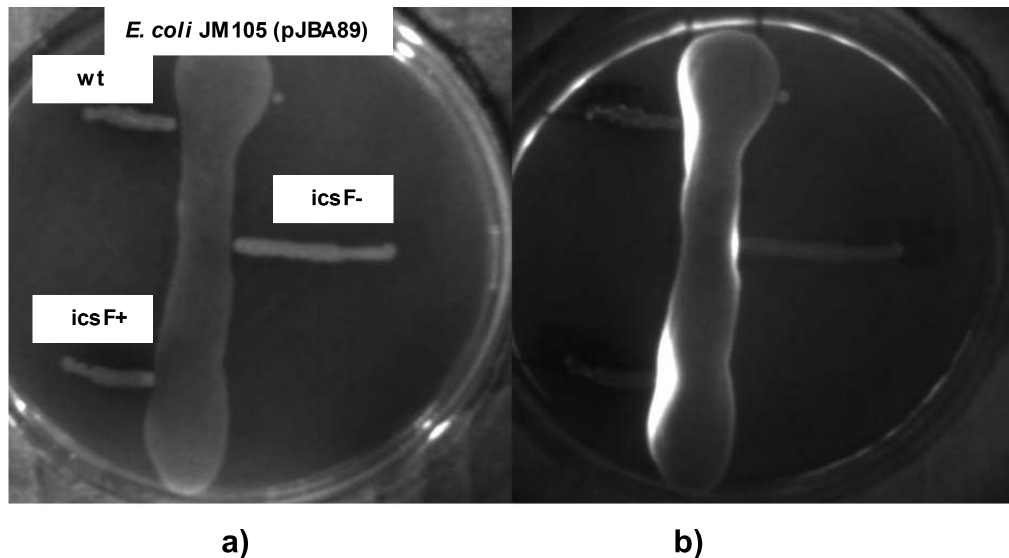


Fig. 3.36. AHLs secretion by *P. aeruginosa* TB wild type (wt), its Tn*5*::*icsF* transposon mutant (icsF-) and complemented mutant (icsF+). The tested strains were cultivated with the GFP-based broad range AHL sensor *E. coli* JM105 (pJBA89).

a) tested *P. aeruginosa* strains were illuminated with normal light.
b) tested strains illuminated with blue light exciting GFP. *icsF* mutant showed diminished AHL secretion when comparing to the wild type.

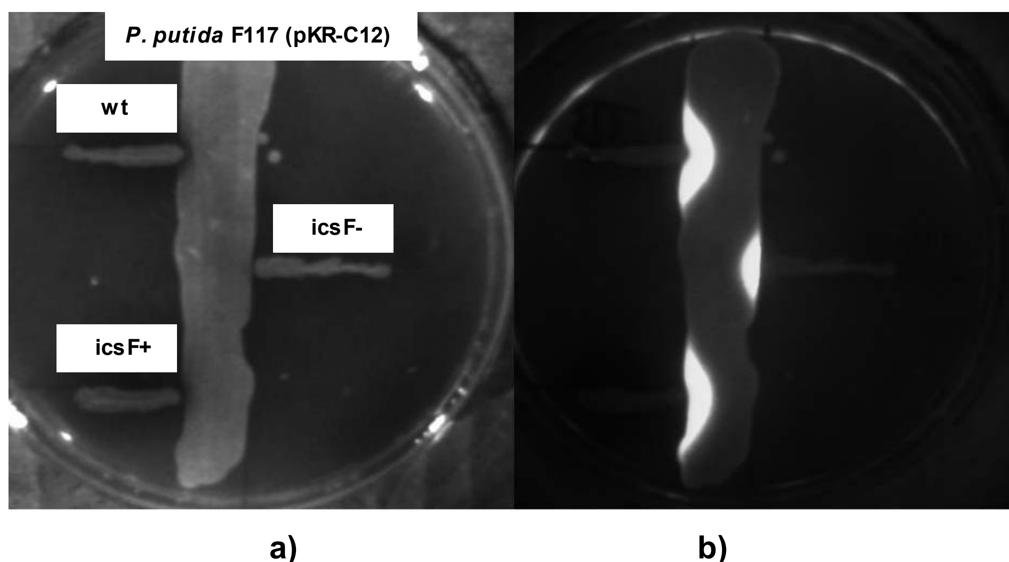


Fig. 3.37. AHLs secretion by *P. aeruginosa* TB wild type (wt), its Tn*5*::*icsF* transposon mutant (icsF-) and complemented mutant (icsF+). All strains were cultivated with *P. putida* F117 (pKR-C12), which contains a translational fusion of the *lasB* promoter to *gfp* and the *lasR* under control of a lac-type promoter.

a) tested *P. aeruginosa* strains were illuminated with normal light.
b) tested strains illuminated with blue light exciting GFP. *icsF* mutant showed reduced AHL secretion when comparing to the wild type.

For more detailed analysis, thin-layer chromatography (TLC) was performed with AHL biosensors *E. coli* MT102 (pSB403) and *Chromobacterium violaceum* CV026 (McClean *et al.,* 1997; Shaw *et al.,* 1997; Winson *et al.,* 1998; Geisenberger *et al.,* 2000). Using this technique, the *icsF* mutant was confirmed to be impaired in the production of AHLs, including both C_4-HSL and 3-oxo-C_{12}-HSL (Figure 3.38). Complementation *in trans* (see chapter 3.3.2) restored the AHL secretion ability of the *icsF* mutant to levels comparable to the wild type thus providing further evidence that in this case the phenotype of the mutant was caused by the transposon inactivation of the *icsF* gene and not by any other secondary genetic event (Figures 3.36, 3.37 and 3.38).

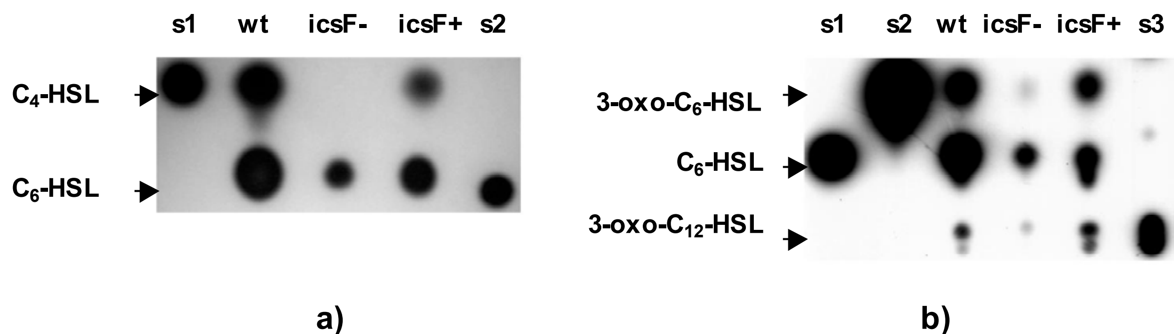


Fig. 3.38. TLC analysis of AHLs secreted by *P. aeruginosa* TB wild type (wt), its Tn*5*::*icsF* transposon mutant (icsF-) and complemented mutant (icsF+). Lanes with standards are indicated by s.

a) using AHL biosensor *Ch. violaceum* CV026 which is able to detect C_4-HSL. Note the significantly diminished ability to secrete AHLs, including C_4-HSL (upper spot) in the *icsF* mutant (icsF-).
b) using AHL biosensor *E. coli* MT102 (pSB403) which is able to detect 3-oxo-C_{12}-HSL. Notice the reduced ability to secrete AHLs, including 3-oxo-C_{12}-HSL, in the *icsF* mutant (icsF-) (B.Huber and L. Eberl).

Hence these experiments revealed the impact of IcsF on the production and secretion of quorum sensing autoinducers. The disruption of *icsF* did not shut-down the AHL secretion completely, as in the case of *vqsR* mutation (see chapter 3.1.3), but the ability of bacterium to secrete AHLs was reduced significantly when compared to the wild type.

3.3.5. IcsF affects secretion of extracellular virulence factors

Evidence has accumulated over the last few years that quorum sensing modulates the expression of a broad spectrum of virulence genes in *P. aeruginosa* (Passador *et al.*, 1993). As IcsF was shown to have an influence on the secretion of quorum sensing AHL signalling molecules I hypothesized that the secretion of extracelular virulence factors will be also diminished in the IcsF mutant. To investigate the effect of the *icsF* mutation on the production of bacterial extracellular virulence factors, I investigated its impact on protease and pyocyanin secretion.

Proteases:

The secretion of proteases was determined by growth of the investigated *P. aeruginosa* strains on M9 agar plates which were supplemented with 0.75 % casein (see chapter 2.6.3.2). The easily visible halo on casein agar plates, indicating proteolytic activity was observed for the TB wild type and in the *icsF* mutant the secretion of proteases was slightly reduced as expected (Figure 3.39).

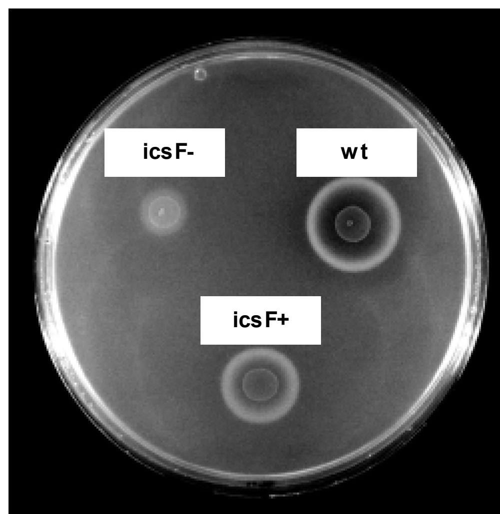


Fig. 3.39. Secretion of proteases by the *P. aeruginosa* TB wild type strain (wt), its Tn*5*::*icsF* transposon mutant (icsF-) and complemented mutant (icsF+). In the mutant the secretion of proteases was reduced when comparing to the wild type.

On the other side, in the complemented mutant, the secretion of proteases was restored according to our expectations (Figure 3.39).

All three casein degrading proteases, LasB elastase, alkaline protease and protease IV, have an important part in the infection process of *P. aeruginosa* (van Delden, 2004). Moreover, the expression of LasB elastase and alkaline protease was shown to be controlled by the quorum sensing in *P. aeruginosa* (Latifi *et al.,* 1995). To test specifically for elastase secretion, the modified elastin-Congo red assay was used (Rust *et al.,* 1994) (see chapter 2.6.3.3). Using this assay, the *icsF* mutant was found to be slightly impaired in the ability to produce elastase, whereas the ability of the complemented mutant to secrete elastase was restored almost to levels comparable to the wild type (Figure 3.40).

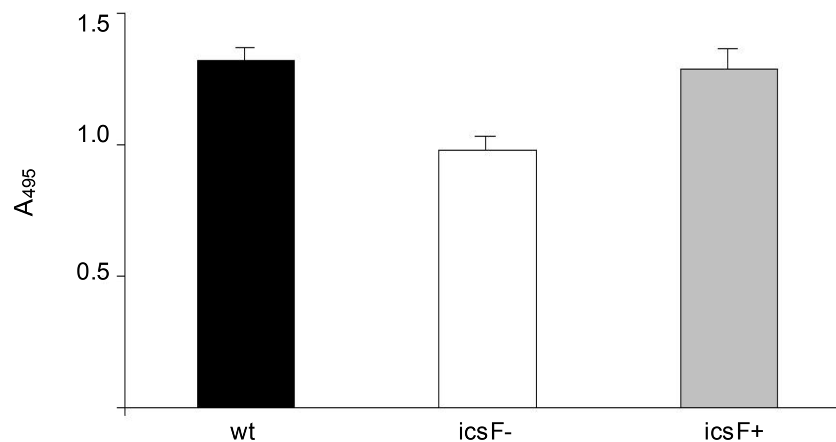


Fig. 3.40. Secretion of elastase by the *P. aeruginosa* TB wild type strain (wt), its Tn*5*::*icsF* transposon mutant (icsF-) and complemented mutant (icsF+). The amount of released red dye in the supernatant after 8 hours of growth of tested strains in LB medium was quantified by measuring absorbance at 495 nm (A_{495}).

Pyocyanin:

The special medium, King's medium A, was used for investigation of the pyocyanin secretion ability of the wild type, *icsF* mutant and the complemented mutant (see chapter 2.6.3.1), which favours the secretion of pyocyanin and simultaneously inhibits the secretion of siderophores (King *at al.,* 1954). As mentioned before, the secretion of pyocyanin in *P. aeruginosa* was also found to be controlled by quorum sensing (Latifi *et al.,* 1995).

As shown below, measuring the amount of pyocyanin in the supernatants of the tested bacterial strains revealed a significant decrease of pyocyanin secretion in the *icsF* mutant when compared to the wild type and complemented mutant (Figure 3.41) thus revealing the role of IcsF for the pyocyanin production.

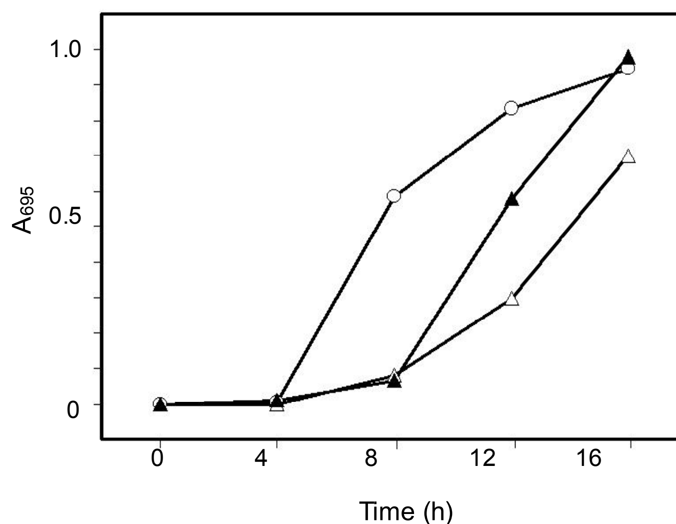


Fig. 3.41. Pyocyanin secretion by *P. aeruginosa* TB wild type (wt), its Tn*5*::*icsF* transposon mutant (icsF-) and complemented mutant (icsF+). Strains were cultivated in the King's A medium and the amount of pyocyanin in the supernatant was quantified by measuring absorbance at 695 nm (A_{695}). ○, wt; △, icsF-; ▲, icsF.

The mutation of *icsF* significantly reduced the secretion of proteases (including elastase) and pyocyanin, whereas in the complemented mutant protease as well as pyocyanin secretion ability were restored almost to levels comparable to the wild type. These exoproducts are known to be implicated in the *P. aeruginosa* virulence

and their secretion was shown to be controlled by quorum sensing circuitry. Thus, these experiments provide further evidence that IcsF affects the *P. aeruginosa* cell-to-cell communication network.

3.3.6. IcsF influences virulence towards *C. elegans*

As shown in the previous chapter, disruption of *icsF* decreased secretion of extracellular virulence factors protease and pyocyanin, thus suggesting the significant impact of IcsF on the overall virulence of *P. aeruginosa*. To prove this hypothesis, wild type strain, Tn*5*::*icsF* transposon mutant and complemented mutant were tested for their virulence towards the nematode *C. elegans*, which has been frequently used before as a bacterial pathogenesis model for the identification of virulence-attenuated mutants in *P. aeruginosa* (Mahajan-Miklos *et al.,* 1999; Tan *et al.,* 1999). This experiment revealed a significant attenuation of virulence of the *icsF* mutant when compared to the wild type (Figure 3.42). On the other hand, the killing activity of the complemented mutant was restored to wild type levels (Figure 3.42), confirming the important role of the *icsF* gene in *P. aeruginosa* virulence towards *C. elegans*.

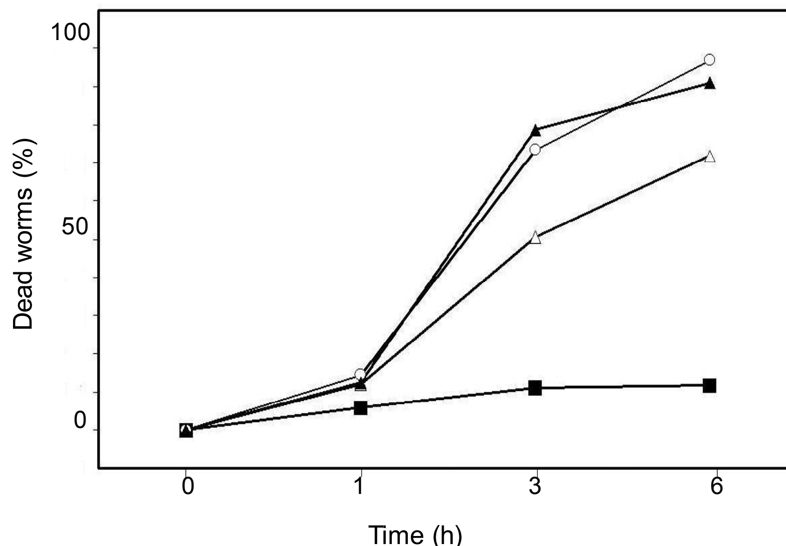


Fig. 3.42. Kinetics of the killing of *C. elegans* by TB wild type strain (wt), its Tn*5*::*icsF* transposon mutant (icsF-) and complemented mutant (icsF+). 45 to 60 L4 larvae were placed in each well and scored for dead worms by microscopic examination. *E. coli* DH5α served as a negative control. Values are the mean ± SD of a representative experiment with triplicate values. ○, wt; △, icsF-; ▲, icsF+; ■, *E. coli* (D. Jordan).

3.3.7. Transcriptional analysis of *icsF*

3.3.7.1. Northern blot expression analysis of *icsF*

The expression of IcsF mRNA was analyzed by Northern blots under various growth conditions. Standard conditions like growth in LB medium to early and late exponential phase were used as well as exposure to different stress conditions like growth in the presence of oxidative stress generated by hydrogen peroxide, growth in the presence of serum, growth in the presence of PMNs, and growth in ABC minimal mineral medium.

While exposing to the oxidative stress generated by hydrogen peroxide, blood serum and PMNs, bacteria were cultivated in a dialysis bag with appropriate pore diameter to ensure continuous exchange of fluids. After the incubation period, the cells were immediately recovered from the dialysis bag and subjected to RNA isolation (see chapter 2.4.3.). The expression pattern of *icsF* was examined by Northern blots. Interestingly, *icsF* was found to be expressed only very lowly on Northern blots. Hybridization with a genomic *icsF* probe gave a weak signal when *P. aeruginosa* TB had been cultured in the presence of H_2O_2 or PMNs. Under all other examined growth condition, *icsF* expression signal was completely undetectable on Northern blots. The Tn*5*::*icsF* mutant did not produce any detectable IcsF transcript under all chosen conditions.

3.3.7.2. GeneChip expression analysis of *icsF*

P. aeruginosa GeneChips from Affymetrix were used to investigate the impact of IcsF on global changes in the gene expression profile. Total RNA, extracted from bacterial cultures cultivated in the presence of 10 % human blood serum or PMNs, was hybridized on the DNA microarrays in duplicate (see chapter 2.5). The signals on the arrays representing the expression levels of individual genes were used to calculate the expression ratio between the wild type and mutant strain cultivated in the presence of H_2O_2 and serum. Numerous genes were differentially regulated in the *icsF* mutant when compared to the wild type strain, particularly in the presence of H_2O_2 (Figure 3.43).

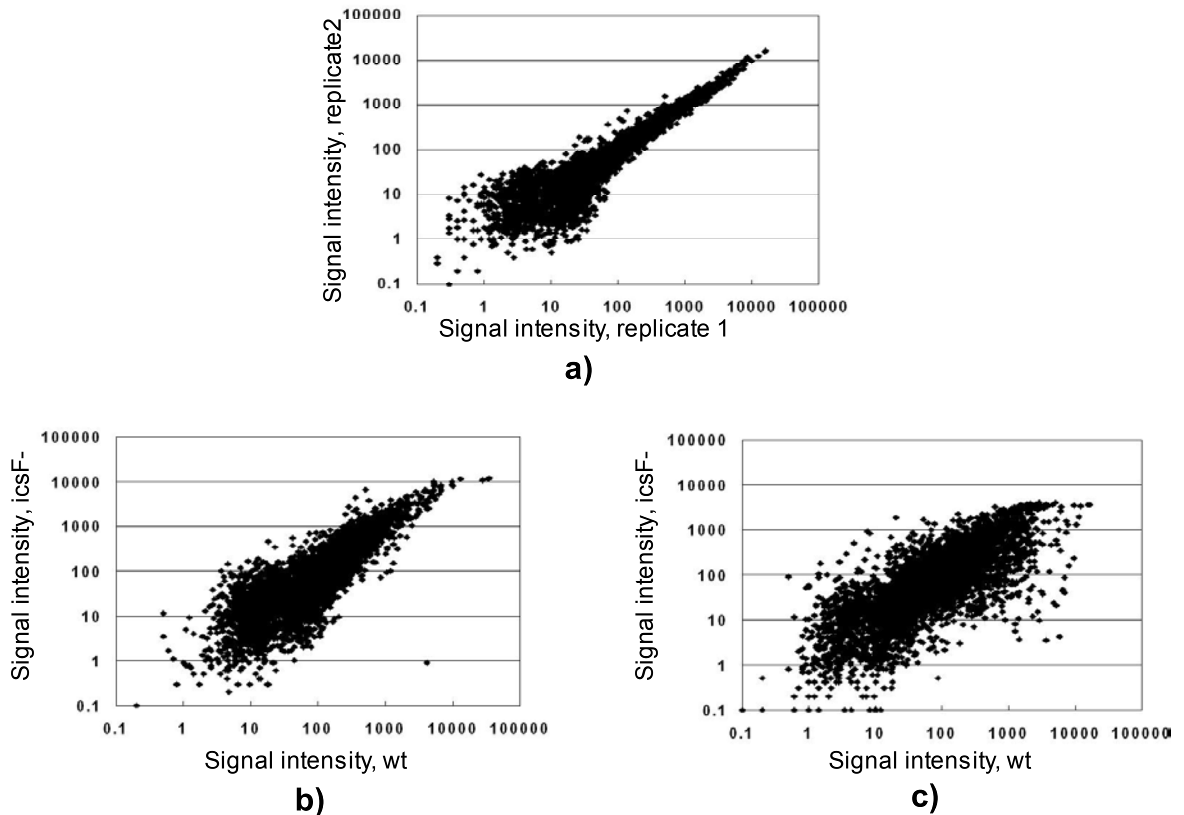


Fig. 3.43. Logarithmic scatter graph of absolute signal intensities of *P. aeruginosa* ORFs represented by 5900 individual array spots. The graph indicates that the expression of a number of genes was altered in the *icsF* mutant, particularly in the presence of H_2O_2.

a) Global expression profiles of two TB wild type cultures cultivated under the same growth conditions. The absolute signal intensities of one replicate were plotted against the signal intensities of a second replicate.

b) Absolute signal intensities of TB wild type strain (wt) plotted against signal intensities of the Tn*5*::*icsF* transposon mutant (icsF-) when both cultures were treated with H_2O_2.

c) Signal intensities of the TB wild type (wt) plotted against Tn*5*::*icsF* transposon mutant (icsF-) obtained after cultivation of both cultures in the presence of PMNs.

Confirming the results from Northern blots, *icsF* was found to be only very lowly expressed on DNA microarrays under all examined growth conditions with signal intensities ranging from 90 to 180. Such low expression combined with the large number of differentially expressed genes in the investigated *icsF* mutant underlines the important global regulatory function of IcsF.

GeneChip expression analysis revealed that only few genes were differentially expressed in the *icsF* mutant in the presence of PMNs, whereas a large number of genes was differentially expressed in the presence of H_2O_2. By applying the stringent criteria outlined above, the mutation of *icsF* significantly influenced the expression of 904 genes in the presence of H_2O_2 and of 110 genes in the presence of PMNs (see Appendix III and IV). Out of 904 genes differentially regulated in the presence H_2O_2, 401 genes were downregulated and 503 genes were upregulated in the *icsF* mutant. Out of 111 genes differentially regulated in the presence of PMNs, the expression of 53 genes was repressed and the expression of remaining 57 genes was promoted in the mutant. The differences in the gene expression of the wild type and the mutant ranged from 2-fold up to more than 1000-fold.

Expression of a significant number of genes involved in amino acid biosynthesis, biosynthesis of cofactors, carbon compound catabolism, central intermediary metabolism, chemotaxis, and translation and post-translational modification was found to be differentially regulated in the investigated *icsF* mutant (Figure 3.44). The largest proportion of all differentially regulated genes (36 %) belongs to the class of hypotheticals with unknown function. Expression of 259 genes out of these genes was upregulated in the mutant and expression of 197 genes was downregulated. Expression of 96 genes (69 upregulated in the mutant and 28 downregulated in the mutant), which represent 8 % of the known ORFs and which are all involved in the energy metabolism of *P. aeruginosa* was found to be controlled by IcsF. Other functional classes with the largest numbers of genes regulated by IcsF included the genes encoding membrane proteins (7 %), genes involved in transport of small molecules (7 %), putative enzymes (5 %), genes involved in adaptation and protection (5 %) and transcriptional regulators (4 %).

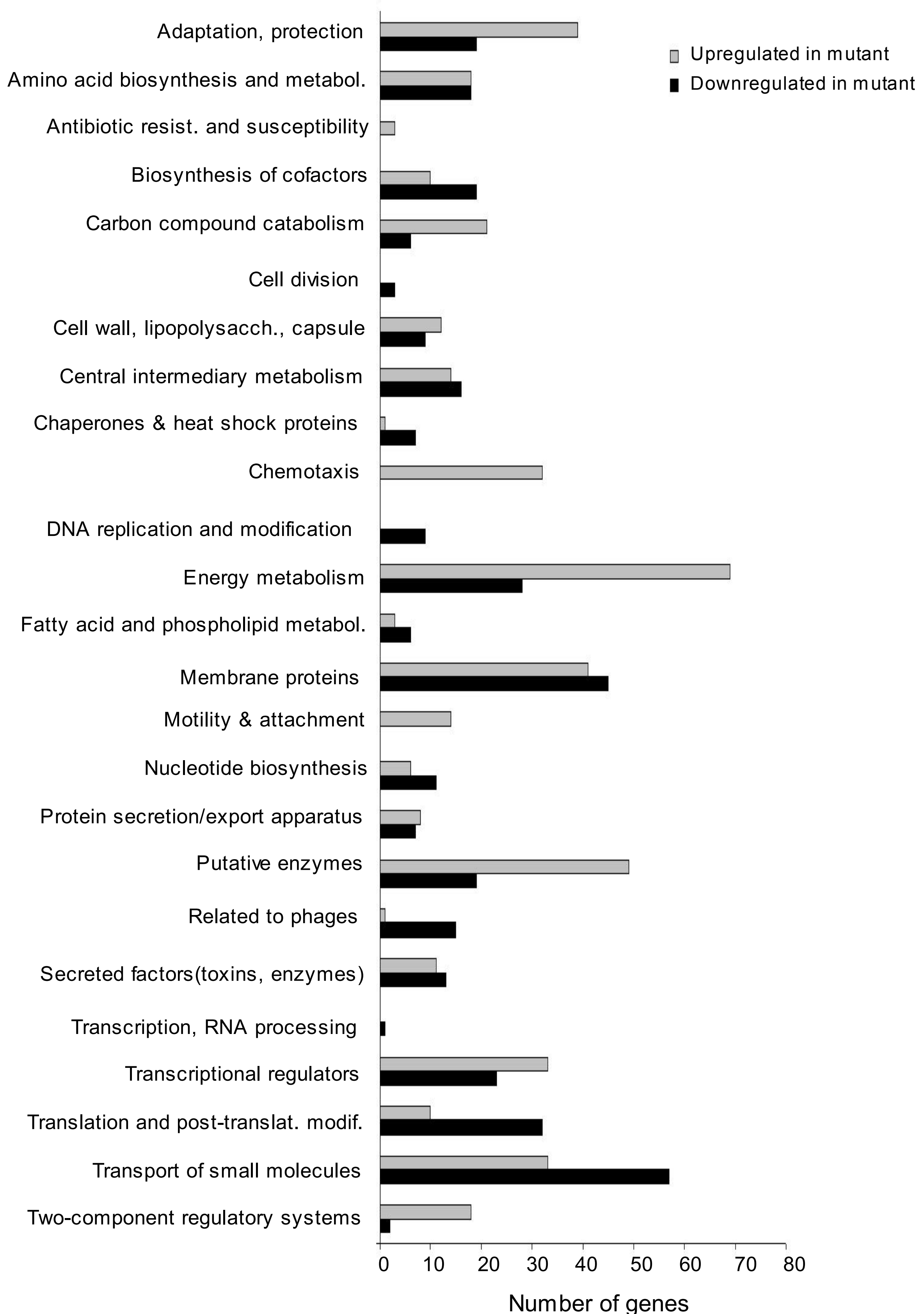


Fig. 3.44. Comparison of the transcriptome of *P. aeruginosa* TB and its *icsF* transposon mutant grown in the presence of PMNs and H_2O_2. The number of genes is classified by metabolic category as defined in the original publication on the PAO genome sequence (Stover *et al.,* 2000).

3.3.7.2.1. *icsF* regulates a large number of oxidative stress response genes

As mentioned above, only few genes were differentially expressed in the *icsF* mutant in the presence of PMNs, whereas a huge number of genes was differentially expressed in the presence of H_2O_2. This result suggested that the expression of *icsF* is not activated by cytokines released by PMNs, but rather that it needs a direct contact with PMNs and that its expression is triggered with maximal intensity when bacterium deals with the oxidative stress in the intracellular compartments of PMNs.

Knowing this, the impact of IcsF on the expression of oxidative stress genes was investigated. In the first step, the GeneChip expression profile of the wild type strain *P. aeruginosa* TB cultured in the presence of 10 mM H_2O_2 was compared with the GeneChip expression profile of the same strain grown in LB medium without addition of H_2O_2. This experiment led to the identification of whole group of genes differentially expressed by the wild type strain in the presence of H_2O_2, which are responsible for dealing with the oxidative stress in *P. aeruginosa*. In the second step, this gene array dataset was compared with the group of genes that was found to be regulated by IcsF in the presence of H_2O_2 and PMNs. Intriguingly, almost all genes controlled by IcsF are identified oxidative stress genes. Out of 110 genes regulated by IcsF in the presence of PMNs, 55 % (61 genes) belong to oxidative stress genes (see Appendix III) and in the presence of H_2O_2 this number is even higher. Out of 401 genes downregulated in the analysed *icsF* mutant in the presence of H_2O_2, about 80 % (317 genes) are oxidative stress genes. Similarly, out of 503 genes upregulated in the *icsF* mutant in the presence of H_2O_2, about 80 % (405 genes) are oxidative stress genes (see Appendix IV). Only 182 genes controlled by IcsF in the presence of H_2O_2 (98 upregulated and 84 downregulated) do not belong to the category of oxidative stress genes. On the other hand, this comparison revealed that out of the whole group of genes, which are implicated in the oxidative stress response of the *P. aeruginosa* strain TB (1318 genes), 55 % are controlled by IcsF.

Figure 3.45 underlines IcsF as the major regulator of oxidative stress response in *P. aeruginosa* showing that only 14 out of 164 more than 10-fold differentially regulated genes in the investigated *icsF* mutant do not belong to oxidative stress genes.

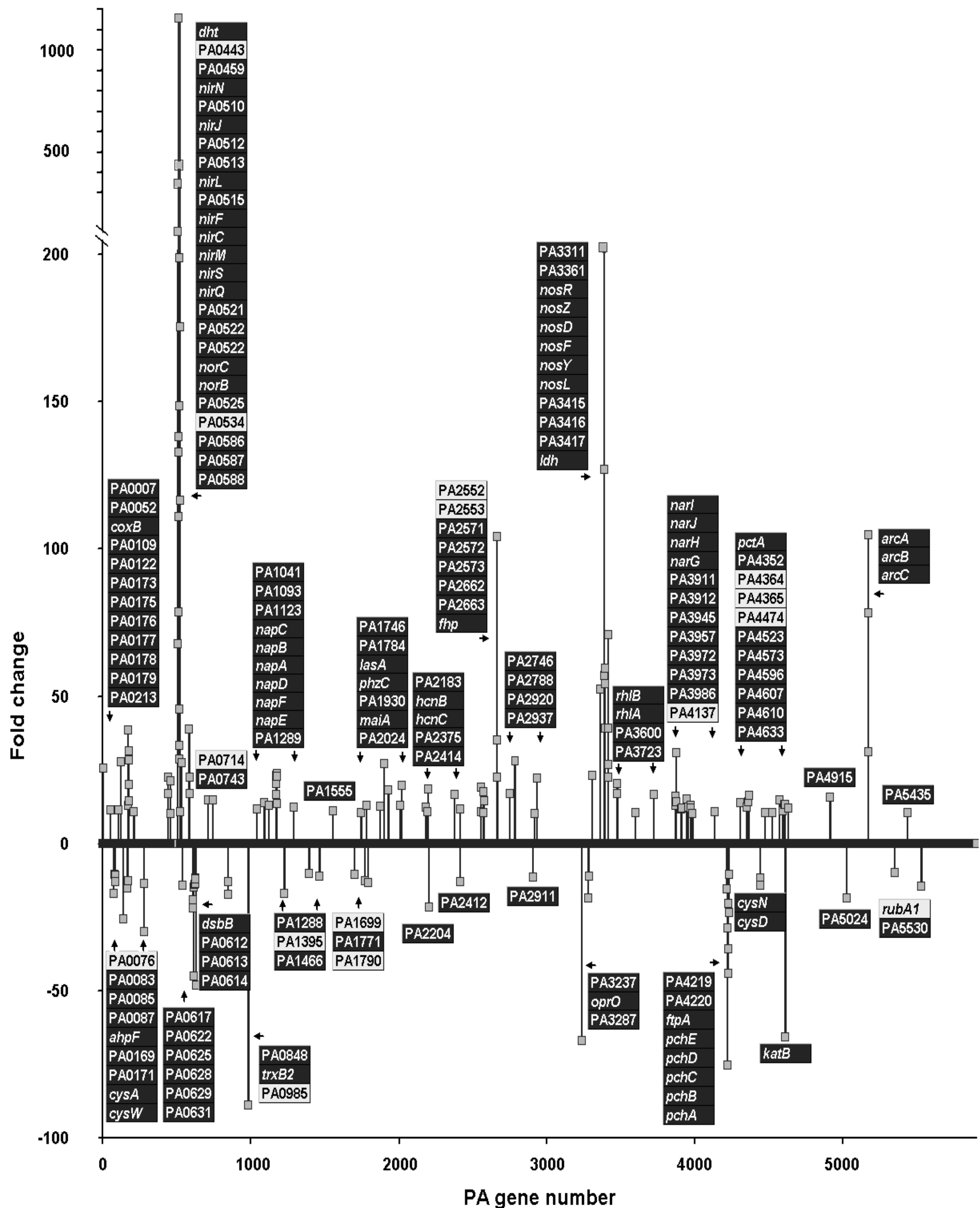


Fig. 3.45. Differential gene expression of the Tn*5*::*icsF* transposon mutant compared to *P. aeruginosa* wild type strain TB in the presence of 10 mM H_2O_2. Positive values represent genes whose expression is upregulated in the mutant and negative numbers genes whose expression is downregulated in the mutant compared to the TB wild type strain. Only genes whose expression is regulated more than 10-fold are shown. Oxidative stress genes are indicated by black colour.

The most differentially expressed in the investigated *icsF* mutant grown in the presence of H_2O_2 are large operons implicated in the nitrogen metabolism (Figure 3.45 and Table 3.9). Interestingly, all these genes were previously reported to be controlled by quorum sensing in *P. aeruginosa* thus highlighting the importance of quorum sensing as a switch for preferential usage of cellular pathways and for anaerobic growth (Wagner *et al.,* 2003). Results of the GeneChip expression analysis presented in this thesis shows that besides quorum sensing, the nitrogen metabolism is also implicated in the oxidative stress response of *P. aeruginosa*. Moreover, this analysis provided evidence that the investigated *icsF* gene, which influences both, intracellular survival and quorum sensing of *P. aeruginosa*, may be the factor that has a major impact on the expression of these operons.

Tab. 3.9. Oxidative stress response genes involved in nitrogen metabolism whose expression was found to be upregulated in the *icsF* mutant in the presence of H_2O_2.

ORF[1]	Gene name	Fold change[2]	Protein description
PA0509[3]	*nirN*	67.8	probable c-type cytochrome
PA0510[3]		414.9	probable uroporphyrin-III c-methyltransferase
PA0511[3]	*nirJ*	78.4	heme d1 biosynthesis protein NirJ
PA0512[3]		111.0	conserved hypothetical protein
PA0513[3]		138.1	probable transcriptional regulator
PA0514[3]	*nirL*	224.0	heme d1 biosynthesis protein NirL
PA0515[3]		1153.9	probable transcriptional regulator
PA0516[3]	*nirF*	132.8	heme d1 biosynthesis protein NirF
PA0517[3]	*nirC*	198.9	probable c-type cytochrome precursor
PA0518[3]	*nirM*	148.6	cytochrome c-551 precursor
PA0519	*nirS*	45.5	nitrite reductase precursor
PA0520[3]	*nirQ*	33.1	regulatory protein NirQ
PA0521[3]		28.7	probable cytochrome c oxidase subunit
PA0522[3]		10.6	hypothetical protein
PA0523[3]	*norC*	175.4	nitric-oxide reductase subunit C
PA0524[3]	*norB*	342.3	nitric-oxide reductase subunit B
PA0525[3]		116.3	probable dinitrification protein NorD
PA1172[3]	*napC*	20.1	cytochrome c-type protein NapC
PA1173[3]	*napB*	16.5	cytochrome c-type protein NapB precursor
PA1174	*napA*	23.1	periplasmic nitrate reductase protein NapA

ORF[1]	Gene name	Fold change[2]	Protein description
PA1175	*napD*	13.4	NapD protein of periplasmic nitrate reductase
PA1176	*napF*	23.6	ferredoxin protein NapF
PA1177	*napE*	22.8	periplasmic nitrate reductase protein NapE
PA3032[3]	*snr1*	2.7	cytochrome c Snr1
PA3391[3]	*nosR*	126.8	regulatory protein NosR
PA3392[3]	*nosZ*	219.9	nitrous-oxide reductase precursor
PA3393[3]	*nosD*	56.8	NosD protein
PA3394[3]	*nosF*	54.2	NosF protein
PA3395[3]	*nosY*	39.0	NosY protein
PA3396[3]	*nosL*	59.4	NosL protein
PA3872[3]	*narI*	12.9	respiratory nitrate reductase gamma chain
PA3873[3]	*narJ*	15.8	respiratory nitrate reductase delta chain
PA3874[3]	*narH*	14.1	respiratory nitrate reductase beta chain
PA3875[3]	*narG*	30.8	respiratory nitrate reductase alpha chain
PA3876[3]	*narK2*	5.5	nitrite extrusion protein 2
PA3879	*narL*	2.9	two-component response regulator NarL

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons.

[3] Quorum sensing regulated genes (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003).

3.3.7.2.2. *icsF* regulates the key oxidative stress response genes

Bacteria have developed a variety of strategies to cope with the oxidative stress in the intracellular compartments of PMNs. In particular, superoxid dismutase (SOD) and catalase are implicated in these oxidative stress mediated reactions (Hassett and Cohen, 1989). SOD generates hydrogen peroxide and molecular oxygen out of superoxide anions and in subsequent reaction catalase converts toxic hydrogen peroxide into water and molecular oxygen (Fridovich, 1978). Besides genes encoding superoxid dismutases (*sodB, sodM*) and catalases (*katA, katB, katE, katN*) (Brown *et al.,* 1995; Hassett *et al.,* 1995), the *P. aeruginosa* genome comprises also a few genes for alkyl hydroperoxide reductases (*ahpA, ahpB, ahpCF, ohr,* PA0848) as well as *oxyR* and *soxR* which encode the key modulators of the oxidative stress response in bacteria (Ochsner *et al.,* 2000; Ochsner *et al.,* 2001).

Table 3.10 shows the GeneChip signal intensities of the major oxidative stress response genes of *P. aeruginosa* in the wild type strain TB in the absence and presence of H_2O_2 compared with the signal intensities of the investigated *icsF* mutant grown in the presence of H_2O_2.

Under the experimental conditions used in this study the alkylhydroperoxide reductase AhpB, catalases KatE and KatN, regulator SoxR and the superoxid dismutase SodM were constitutively expressed at low levels in wild type in the absence and presence of H_2O_2, suggesting that under used experimental settings these proteins do not play a role in the response of *P. aeruginosa* to oxidative stress caused by H_2O_2. On the other hand, *ahpC*, *bfrB*, *bfrA*, or *katA* are already expressed at such high levels in the wild type strain, even without addition of the H_2O_2 to growth medium, that any further upregulation upon exposure to H_2O_2 would not be biologically meaningful for the bacterium. Most interesting is therefore the last category, which comprises the key oxidative stress response genes *ahpF,* PA0848, *trxB2, ohr, sodB, katB* and *oxyR*, whose mRNA transcripts expression was increased remarkably in the wild type strain after addition of 10 mM H_2O_2 to growth medium, but their expression in the examined *icsF* mutant cultured under the same condition remained almost completely unaltered. This is the group of genes whose expression is strongly dependent on the presence of IcsF protein. The differences in the expression of mRNA transcripts between wild type strain and *icsF* mutant cultivated in the presence of H_2O_2 were best visible in the case of alkyl hydroperoxide reductases *ahpF,* PA0848 and *ohr* (above 25-fold, 18-fold and 4-fold difference, respectively), thioredoxin reductase *trxB2* (15-fold difference) and catalase *katB* (around 100-fold difference). Noteworthy is also the slight modulation of the OxyR expression, which represents one of the well-conserved bacterial oxidative stress response regulators. Moreover, the effect of the *icsF* mutation on the expression of superoxide dismutase SodB suggests an important role of IcsF in the first step of oxidative stress response of *P. aeruginosa*, in the conversion of superoxide anions into H_2O_2. However, most striking is particularly the huge impact of IcsF on the expression of catalase KatB, which pinpoints IcsF as a principal protein responsible for regulation of H_2O_2 detoxification in *P. aeruginosa.*

Table 3.10. Expression of mRNA transcripts known to be involved in the oxidative stress response. GeneChip normalized signal intensities in the *P. aeruginosa* strain TB wild type in the absence (H_2O_2-) and presence (H_2O_2+) of 10 mM hydrogen peroxide and its Tn*5*::*icsF* transposon mutant (IcsF-) in the presence of 10 mM hydrogen peroxide.

ORF[1]	**Gene name**	**Signal intensity**		
		H_2O_2-	**H_2O_2+**	**IcsF-**
PA0139	*ahpC*	2320	3894	2510
PA0140	*ahpF*	63	1650	60
PA0847	*ahpB*	13	4	19
PA0848		66	1212	57
PA0849	*trxB2*	51	1227	82
PA2025	*gor*	392	330	334
PA2147	*katE*	4	4	7
PA2185	*katN*	11	12	21
PA2273	*soxR*	10	6	5
PA2850	*ohr*	56	201	55
PA3531	*bfrB*	2224	1710	1433
PA4235	*bfrA*	1964	710	1397
PA4236	*katA*	1770	2374	1406
PA4366	*sodB*	1490	2053	1250
PA4468	*sodM*	30	75	68
PA4613	*katB*	23	1736	17
PA5344	*oxyR*	229	378	207

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

4. Conclusions and perspectives

The objective of this work was to integrate four potential regulatory virulence genes (*vqsR, gltR, 47D7, icsF*) from the STM library of *P. aeruginosa* TB (Wiehlmann, 2001) into regulatory networks and pathways. To achieve this goal the mutants were complemented *in trans* and comprehensively analysed using combined approach of up-to-date technologies of functional genomics, genetics *in silico* and various bioassays.

4.1. *vqsR*

In summary, the disruption of *vqsR* repressed the expression of genes that are known to be promoted by quorum sensing and activated the expression of genes that are known to be repressed by quorum sensing. Moreover, the *vqsR* mutant harboured less mRNA transcript for the production of iron regulated genes and membrane-bound elements of antibiotic resistance (Figure 4.1). Besides the cytotoxicity caused by elements of the type III secretion system (Frank, 1997), antibiotic resistance and extracellular virulence factors are the major determinants for morbidity and prognosis of infections with *P. aeruginosa* in humans. The protein encoded by *vqsR* (PA2591) regulates several traits of pathogenicity, hence the name *vqsR*, (virulence and quorum sensing regulator), was assigned to PA2591.

Inactivation of *vqsR* abrogated the production of AHLs and decreased LasI mRNA by about 10-fold. In addition, *vqsR* contains a *las* box in its upstream region. Considering these data, I would like to conclude that *vqsR* is an essential element of the quorum sensing hierarchy whose inactivation disrupts the production of AHLs, including 3-oxo-C_{12}-HSL and C_4-HSL. On the other hand, the presence of the *las* box located upstream of *vqsR* places this gene under the direct control of the regulatory LasR-3-oxo-C_{12}-HSL complex, thus creating an autoinducer feedback loop. The influence of VqsR on the expression of LasR in ABC minimal medium further underlines the existence of the autoregulatory feedback loop between *vqsR* and *las* quorum sensing circuit.

This hypothesis is also supported by the GeneChip experiment performed by Wagner *et al.* (2003) where transcription of the *vqsR* gene (PA2591) was found to be reduced 5.7 fold in the *P. aeruginosa* mutant strain deficient in the 3-oxo-C_{12}-HSL and C_4-HSL synthesis.

As 3-oxo-C_{12}-HSL and C_4-HSL are involved in the initiation of quorum sensing, the disruption of 3-oxo-C_{12}-HSL and C_4-HSL production and secretion in the *vqsR* mutant inevitably has a negative effect on the whole quorum sensing cascade. Indeed, the GeneChip experiments revealed significant downregulation of the whole battery of quorum sensing genes in the *vqsR* transposon mutant.

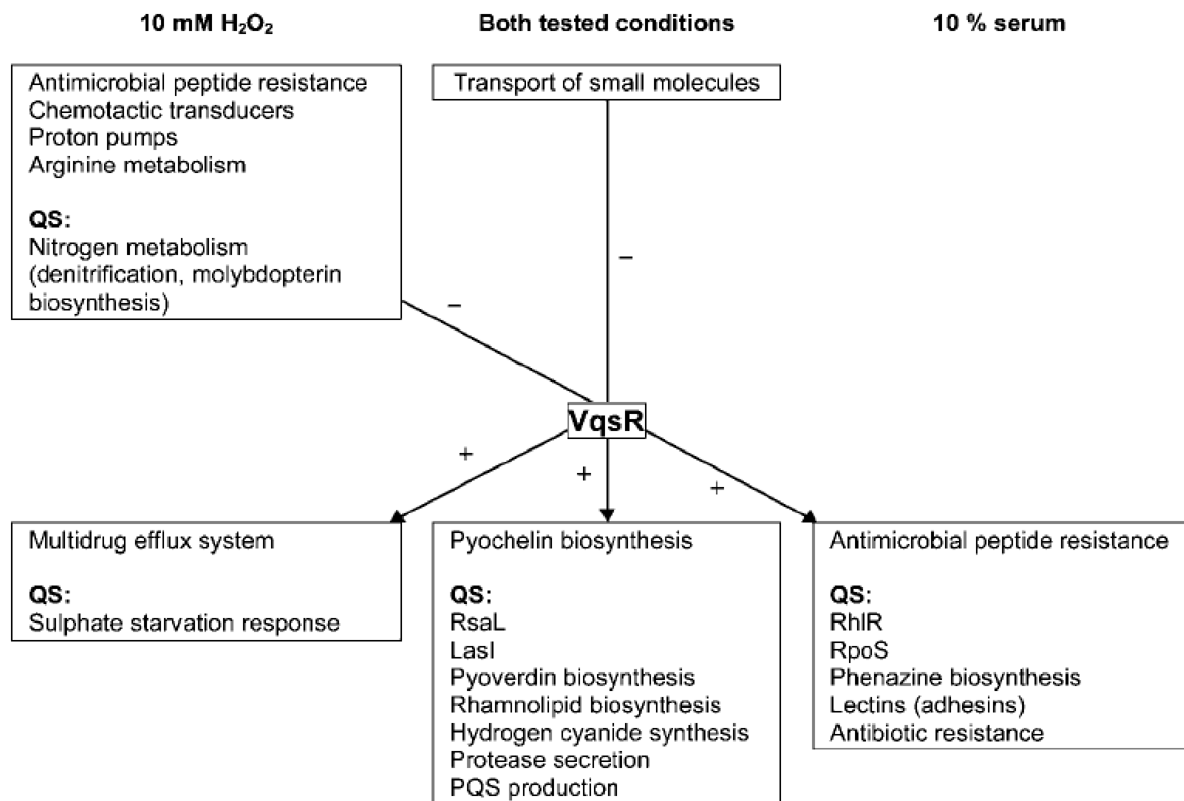


Fig. 4.1. Major gene categories and metabolic pathways regulated by VqsR in the presence of 10 mM H_2O_2 and 10 % serum.

Correspondingly the mutant was compromised in phenotypic traits that are under quorum sensing control such as protease (elastase), hemolysin and pyocyanin secretion and virulence in the *C. elegans* infection model.

In summary, *vqsR* is a new member of the LuxR family that is involved in cell-to-cell communication and virulence of *P. aeruginosa*. It constitutes an essential element of the quorum sensing cascade, which, in concert with additional regulators, represents a tool for the fine tuning of the quorum sensing in *P. aeruginosa* (Figure 4.2).

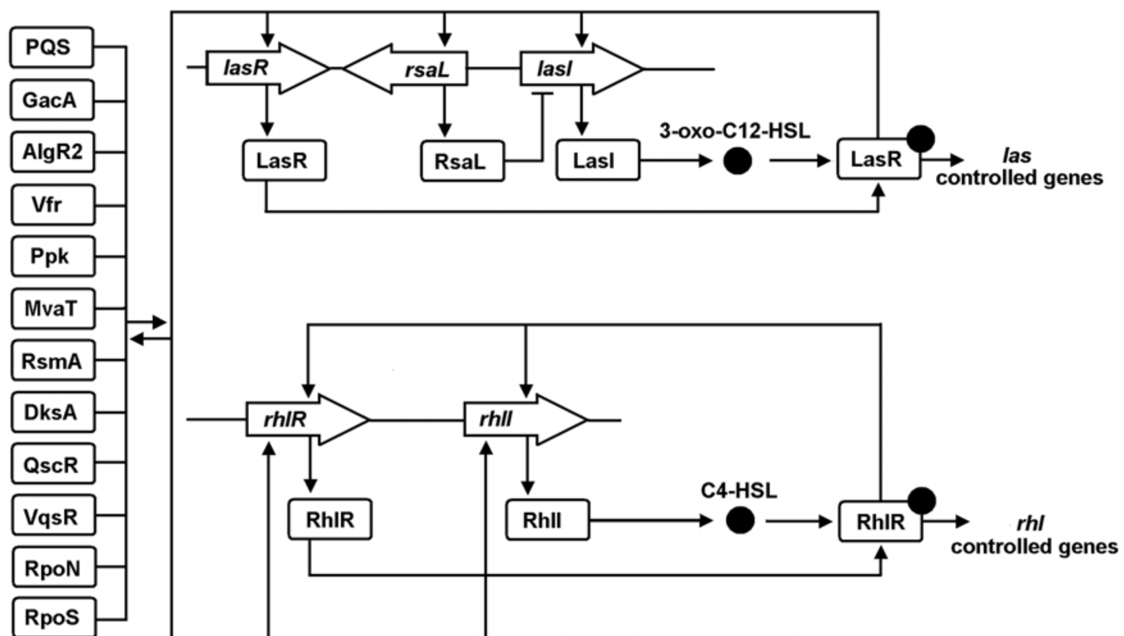


Fig. 4.2. Quorum sensing network in *P. aeruginosa*. Hierarchically arranged *las* and *rhl* systems are subject to modulation by a number of additional regulators, including VqsR, which represent a tool for the fine tuning of the quorum sensing in *P. aeruginosa*.

It would be interesting to further investigate the exact position of *vqsR* in the quorum sensing hierarchy and its delicate interplay with other key regulators of cell-to-cell communication in *P. aeruginosa*.

4.2. *gltR* and *47D7*

Disruption of *gltR* and *47D7* by Tn*5* transposon mutagenesis (Wiehlmann*,* 2001) caused decreased resistance to serum as well as the significant protease secretion deficiency of the *gltR* mutant and the complete loss of the protease secretion ability of the *47D7* mutant, thus suggesting an important role of these genes in *P. aeruginosa* virulence.

Therefore, *gltR* and *47D7* mutants were complemented *in trans* to ensure that their phenotypes were caused by the transposon inactivation of the respective genes. However, the complemented mutants maintaining the recombinant plasmids pME6010*gltR* and pME6010*47D7* did not show phenotypes that are typical for the wild type strain. Complementation *in trans* did neither restore the protease secretion ability nor the survival ability of the *gltR* mutant in the serum. Furthermore complementation *in trans* did not restore the protease secretion ability of the *47D7* mutant, thus providing evidence that in this case, like in the case of the *gltR* mutant, the observed phenotypes are most probably caused by a secondary genetic event elsewhere in the genome. Therefore the results presented in this work suggest that any further research on these two mutants would be meaningless.

4.3. *icsF*

The disruption of *icsF* altered the expression of a number of genes that are known to be implicated in the oxidative stress response of *P. aeruginosa*. As mentioned before, prior to phagocytosis of bacteria, the PMN begins to consume oxygen, a process known as oxidative burst, thus transforming the phagolysosomes into the harsh and microorganisms-killing compartments. Therefore bacteria have developed a variety of strategies to cope with oxidative stress in the intracellular compartments of PMNs. One of the most important from these strategies exploits the antioxidant enzymes (Staudinger *et al.,* 2002), but so far this type of intracellular survival mechanism was not reported for *P. aeruginosa*. In general, intracellular survival of *P. aeruginosa* in PMNs is very rare, because PMN-mediated phagocytosis represents one of the host’s most proficient antipseudomonal weapon (Döring *et al.*, 1995) and can be only seen in a minority of CF isolates. However, the investigated *P. aeruginosa* strain TB was shown to be capable of survival and replication in PMNs

irrespective of whether they were isolated from patients with CF or healthy donors (Tümmler, 1987).

The data presented in this work demonstrate that *icsF* is one of the key genes responsible for the regulation of the expression of oxidative stress genes. As oxidative stress response genes represent an effective tool to combat the harsh conditions in phagolysosomes, the disruption of *icsF* inevitably has a negative effect on the intracellular survival. The protein encoded by *icsF* (PA1572) has a large impact on the intracellular survival of *P. aeruginosa* in PMNs and hence the name *icsF*, (intracellular survival factor), was assigned to PA1572. Besides the intracellular survival, the disruption of *icsF* also affects cell-to-cell communication and overall virulence of *P. aeruginosa* against *C. elegans*.

It was shown, that the oxidative burst is caused by a membrane-associated NADPH oxidase (Rosen, 2004). Intriguingly, IcsF shares a strong homology with the NAD kinase from *E. coli.* Further investigation in this direction would help to reveal the exact enzymatic function of this protein and its dual role for the intracellular survival and quorum sensing of *P. aeruginosa*.

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6. Abbreviations

A	Absorbance
Aa	Amino acid
AHL	acylated homoserine lactones
bp	Base pair
approx.	approximately
^{o}C	Degree Celsius
cDNA	Complementary DNA
CDS	Coding sequence
CF	Cystic fibrosis
cfu	Colony forming units
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanosine triphosphate
DIG	Digoxigenin
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded DNA
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
et. al.,	*et alteri* (and others)
EtBr	Ethidium bromide
i.e.	That means
F	Farad
FCS	Fetal calf serum
x *g*	Centrifugal acceleration
h	Hour
I.E.	Injection units
Kb	1000 Base pairs
LB	Luria-Bertani
M	Molar
M	milli- (10^{-3}); Meter, Mass
Max.	Maximum
Mb	Megabases
µ	micro- (10^{-6})
min.	Minute
MOPS	Morpholinopropanesulfonic acid
mRNA	Messenger RNA
n	nano- (10^{-9})
dNTP	Deoxynucleotide triphosphate
OD	Optical Density
ORF	Open reading frame; possible gene
p	pico- (10^{-12})
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
QS	Quorum sensing
^{R,r}	Antibiotic resistance
RBS	Ribosome binding sequence
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature (23 ^{o}C)
s	Second
SDS	Sodium dodecyl sulfate
ssDNA	Single stranded DNA
t	Time
T	Temperature
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
TTSS	Type III secretion system
U	Unit (unit of enzymatic activity)
UV	Ultra violet
V	Volt
Vol.	Unit volumes
v/v	Percentage volume per total volume
w/v	Prcentage by weight per total volume

7. Appendices

Appendix I

Molecular weight standards used for DNA and RNA agarose gel electrophoresis in this work. The fragment sizes are given in base pairs.

λ DNA/BstE II	100 bp DNA ladder	0.16-1.77 Kb RNA ladder
8454	1517	1770
7242	1200	1520
6369	1000	1280
5686	900	780
4822	800	530
4324	700	400
3675	600	280
2323	517	155
1929	500	
1371	400	
1264	300	
702	200	
224	100	
117		

Appendix II

Differential transcription profile of the *vqsR* mutant grown in the ABC minimal medium.

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *vqsR* mutant in the ABC minimal medium			
PA0029		5.68	probable sulfate transporter
PA0038		6.37	hypothetical protein
PA0069		7.74	conserved hypothetical protein
PA0105[3]	*coxB*	31.50	cytochrome c oxidase, subunit II
PA0106[3]	*coxA*	54.70	cytochrome c oxidase, subunit I
PA0107[3]		49.55	conserved hypothetical protein
PA0108[3]	*colII*	46.18	cytochrome c oxidase, subunit III
PA0109[3]		6.16	hypothetical protein
PA0110		20.78	hypothetical protein
PA0111		41.62	hypothetical protein
PA0112		16.50	hypothetical protein
PA0113		15.05	probable cytochrome c oxidase assembly factor
PA0114		6.34	conserved hypothetical protein
PA0132[3]		10.79	beta-alanine--pyruvate transaminase
PA0134		7.22	probable guanine deaminase
PA0136		6.46	probable ATP-binding component of ABC transporter
PA0138		17.07	probable permease of ABC transporter
PA0140	*ahpF*	6.98	alkyl hydroperoxide reductase subunit F
PA0149		5.27	probable sigma-70 factor, ECF subfamily
PA0176[3]		5.80	probable chemotaxis transducer
PA0177		23.55	probable purine-binding chemotaxis protein
PA0178		5.98	probable two-component sensor
PA0179[3]		5.49	probable two-component response regulator
PA0207		8.66	probable transcriptional regulator
PA0209		7.21	conserved hypothetical protein
PA0215		7.10	probable transporter
PA0216		28.47	probable transporter
PA0227		7.21	probable CoA transferase, subunit B
PA0235	*pcaK*	5.66	4-hydroxybenzoate transporter PcaK
PA0238		7.11	hypothetical protein
PA0249		6.35	probable acetyltransferase
PA0250		6.65	conserved hypothetical protein
PA0251		17.03	hypothetical protein
PA0326		6.78	probable ATP-binding component of ABC transporter
PA0355[3]	*pfpI*	5.78	protease PfpI
PA0424	*mexR*	7.62	multidrug resistance operon repressor MexR
PA0435[3]		5.85	hypothetical protein
PA0439		12.22	probable oxidoreductase
PA0440		47.67	probable oxidoreductase
PA0441		74.30	dihydropyrimidinase
PA0443		11.77	probable transporter
PA0444		26.68	N-carbamoyl-beta-alanine amidohydrolase
PA0446		6.14	conserved hypothetical protein
PA0447[3]	*gcdH*	11.45	glutaryl-CoA dehydrogenase
PA0451		8.73	conserved hypothetical protein
PA0452		15.31	probable stomatin-like protein
PA0471		9.32	probable transmembrane sensor
PA0472		11.71	probable sigma-70 factor, ECF subfamily
PA0476		52.48	probable permease
PA0491		6.75	probable transcriptional regulator
PA0492		68.31	conserved hypothetical protein
PA0494		6.91	probable acyl-CoA carboxylase subunit
PA0499		28.37	probable pili assembly chaperone
PA0510[3]		14.04	probable uroporphyrin-III c-methyltransferase
PA0531		15.22	probable glutamine amidotransferase
PA0532		5.65	hypothetical protein
PA0543		7.90	hypothetical protein
PA0545		6.96	hypothetical protein
PA0585		33.31	hypothetical protein
PA0586[3]		14.20	conserved hypothetical protein
PA0587		14.10	conserved hypothetical protein
PA0588[3]		8.66	conserved hypothetical protein
PA0610	*prtN*	6.31	transcriptional regulator PrtN
PA0613		13.86	hypothetical protein
PA0670		13.36	hypothetical protein
PA0671		21.98	hypothetical protein
PA0672	*hemO*	5.75	heme oxygenase

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *vqsR* mutant in the ABC minimal medium			
PA0673		7.97	hypothetical protein
PA0680		7.25	probable type II secretion system protein
PA0718		11.60	hypothetical protein of bacteriophage Pf1
PA0722		5.78	hypothetical protein of bacteriophage Pf1
PA0726		9.81	hypothetical protein of bacteriophage Pf1
PA0737		52.08	hypothetical protein
PA0738		6.11	conserved hypothetical protein
PA0742		8.63	hypothetical protein
PA0743		13.33	probable 3-hydroxyisobutyrate dehydrogenase
PA0747		9.52	probable aldehyde dehydrogenase
PA0785		7.00	probable acyl carrier protein phosphodiesterase
PA0788		20.31	hypothetical protein
PA0816		7.20	probable transcriptional regulator
PA0821		6.75	hypothetical protein
PA0829		14.77	probable hydrolase
PA0845		15.23	conserved hypothetical protein
PA0865	*hpd*	417.72	4-hydroxyphenylpyruvate dioxygenase
PA0866	*aroP2*	25.84	aromatic amino acid transport protein AroP2
PA0872	*phhA*	21.65	phenylalanine-4-hydroxylase
PA0874		6.46	hypothetical protein
PA0875		11.84	conserved hypothetical protein
PA0882		7.30	hypothetical protein
PA0894		7.17	hypothetical protein
PA0907		9.52	hypothetical protein
PA0912		6.80	hypothetical protein
PA0931		38.93	siderophore receptor protein
PA0942		7.58	probable transcriptional regulator
PA1027		6.88	probable aldehyde dehydrogenase
PA1028		6.14	probable oxidoreductase
PA1029		8.72	hypothetical protein
PA1030		5.79	hypothetical protein
PA1135		8.62	conserved hypothetical protein
PA1136		5.20	probable transcriptional regulator
PA1137		5.41	probable oxidoreductase
PA1144		20.37	probable MFS transporter
PA1147		9.21	probable amino acid permease
PA1172	*napC*	14.43	cytochrome c-type protein NapC
PA1173[3]	*napB*	13.46	cytochrome c-type protein NapB precursor
PA1174	*napA*	12.01	periplasmic nitrate reductase protein NapA
PA1175[3]	*napD*	8.10	NapD protein of periplasmic nitrate reductase
PA1176[3]	*napF*	8.15	ferredoxin protein NapF
PA1177[3]	*napE*	6.11	periplasmic nitrate reductase protein NapE
PA1190		66.42	conserved hypothetical protein
PA1194		8.42	probable amino acid permease
PA1195		10.26	hypothetical protein
PA1231		8.08	conserved hypothetical protein
PA1240		19.08	probable enoyl-CoA hydratase/isomerase
PA1256		11.76	probable ATP-binding component of ABC transporter
PA1282		8.74	probable MFS transporter
PA1283		5.77	probable transcriptional regulator
PA1289[3]		7.00	hypothetical protein
PA1297		15.75	probable metal transporter
PA1300		9.81	probable sigma-70 factor, ECF subfamily
PA1301		7.34	probable transmembrane sensor
PA1313		15.14	probable MFS transporter
PA1328		11.55	probable transcriptional regulator
PA1332		24.21	hypothetical protein
PA1333		8.73	hypothetical protein
PA1348		9.93	hypothetical protein
PA1350		16.36	hypothetical protein
PA1352		8.22	conserved hypothetical protein
PA1485		7.88	probable amino acid permease
PA1497		9.10	probable transporter
PA1503		8.18	hypothetical protein
PA1513		5.97	hypothetical protein
PA1514		5.55	conserved hypothetical protein
PA1515	*alc*	5.76	allantoicase
PA1517		9.60	conserved hypothetical protein
PA1518		5.39	conserved hypothetical protein
PA1519		8.87	probable transporter
PA1522		5.49	hypothetical protein
PA1523	*xdhB*	11.56	xanthine dehydrogenase
PA1524	*xdhA*	12.01	xanthine dehydrogenase
PA1538		7.48	probable flavin-containing monooxygenase

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *vqsR* mutant in the ABC minimal medium			
PA1540		18.85	conserved hypothetical protein
PA1541		80.50	probable drug efflux transporter
PA1617		12.42	probable AMP-binding enzyme
PA1728		10.99	hypothetical protein
PA1761		12.14	hypothetical protein
PA1763		5.85	hypothetical protein
PA1785		22.11	conserved hypothetical protein
PA1786		6.57	conserved hypothetical protein
PA1851		10.19	hypothetical protein
PA1860		6.47	hypothetical protein
PA1874[3]		6.65	hypothetical protein
PA1875[3]		6.09	probable outer membrane protein precursor
PA1877		6.89	probable secretion protein
PA1885		6.76	conserved hypothetical protein
PA1911		7.01	probable transmembrane sensor
PA1912		6.33	probable sigma-70 factor, ECF subfamily
PA1920		7.35	conserved hypothetical protein
PA1930[3]		21.15	probable chemotaxis transducer
PA1931		5.35	probable ferredoxin
PA1946		5.37	binding protein component precursor of ABC ribose transporter
PA1977		5.39	hypothetical protein
PA1978[3]		5.30	probable transcriptional regulator
PA1992		6.22	probable two-component sensor
PA1997		6.31	probable AMP-binding enzyme
PA2009[3]	*hmgA*	7.76	homogentisate 1,2-dioxygenase
PA2014[3]		6.67	probable acyl-CoA carboxyltransferase beta chain
PA2015		5.96	probable acyl-CoA dehydrogenase
PA2016		11.00	probable transcriptional regulator
PA2017		7.59	hypothetical protein
PA2021		13.49	hypothetical protein
PA2024		27.89	probable ring-cleaving dioxygenase
PA2054	*cynR*	6.48	transcriptional regulator CynR
PA2094		6.13	probable transmembrane sensor
PA2120		6.01	hypothetical protein
PA2123		5.36	probable transcriptional regulator
PA2132		12.02	probable pili assembly chaperone
PA2137		19.34	hypothetical protein
PA2138		12.54	probable ATP-dependent DNA ligase
PA2145[3]		19.58	hypothetical protein
PA2146[3]		12.73	conserved hypothetical protein
PA2149		13.62	hypothetical protein
PA2151[3]		14.88	conserved hypothetical protein
PA2158[3]		7.02	probable alcohol dehydrogenase (Zn-dependent)
PA2163[3]		14.16	hypothetical protein
PA2166[3]		6.75	hypothetical protein
PA2174		10.06	hypothetical protein
PA2175		6.85	hypothetical protein
PA2176[3]		7.25	hypothetical protein
PA2187		7.58	hypothetical protein
PA2190[3]		26.47	conserved hypothetical protein
PA2210		13.82	probable MFS transporter
PA2211		15.21	conserved hypothetical protein
PA2247	*bkdA1*	46.12	2-oxoisovalerate dehydrogenase (alpha subunit)
PA2248	*bkdA2*	24.14	2-oxoisovalerate dehydrogenase (beta subunit)
PA2249	*bkdB*	15.94	branched-chain alpha-keto acid dehydrogenase
PA2250[3]	*lpdV*	13.07	lipoamide dehydrogenase-Val
PA2252		5.84	probable AGCS sodium/alanine/glycine symporter
PA2256	*pvcC*	5.26	pyoverdine biosynthesis protein PvcC
PA2282		5.97	hypothetical protein
PA2292		6.45	hypothetical protein
PA2313		5.29	hypothetical protein
PA2336		10.65	hypothetical protein
PA2341[3]		5.62	probable component of ABC maltose/mannitol transporter
PA2376		5.71	probable transcriptional regulator
PA2381		6.13	hypothetical protein
PA2414[3]		5.81	L-sorbosone dehydrogenase
PA2418		6.34	hypothetical protein
PA2422		8.23	hypothetical protein
PA2433[3]		17.96	hypothetical protein
PA2468		15.16	probable sigma-70 factor, ECF subfamily
PA2481		7.33	hypothetical protein
PA2482		7.94	probable cytochrome c
PA2485		14.01	hypothetical protein
PA2486		19.34	hypothetical protein

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *vqsR* mutant in the ABC minimal medium			
PA2504		7.96	hypothetical protein
PA2519	*xylS*	5.61	transcriptional regulator XylS
PA2523		20.07	probable two-component response regulator
PA2524		5.28	probable two-component sensor
PA2550		14.68	probable acyl-CoA dehydrogenase
PA2552[3]		6.86	probable acyl-CoA dehydrogenase
PA2553[3]		12.02	probable acyl-CoA thiolase
PA2554[3]		8.37	probable short-chain dehydrogenase
PA2572[3]		6.62	probable two-component response regulator
PA2573[3]		7.26	probable chemotaxis transducer
PA2574		13.94	conserved hypothetical protein
PA2576		5.20	hypothetical protein
PA2578		9.37	probable acetyltransferase
PA2590		6.21	hypothetical protein
PA2600		6.27	hypothetical protein
PA2618		9.94	hypothetical protein
PA2674		7.24	probable type II secretion system protein
PA2675		7.36	probable type II secretion system protein
PA2676		10.73	probable type II secretion system protein
PA2680		18.40	probable quinone oxidoreductase
PA2686	*pfeR*	9.72	two-component response regulator PfeR
PA2687	*pfeS*	6.25	two-component sensor PfeS
PA2688	*pfeA*	53.86	ferric enterobactin receptor precursor PfeA
PA2691		6.39	conserved hypothetical protein
PA2717		9.13	chloroperoxidase precursor
PA2719		6.22	hypothetical protein
PA2746		9.06	hypothetical protein
PA2754		6.47	conserved hypothetical protein
PA2759		39.19	hypothetical protein
PA2776		9.43	conserved hypothetical protein
PA2777		7.80	conserved hypothetical protein
PA2778		6.40	hypothetical protein
PA2779		14.65	hypothetical protein
PA2799		10.20	hypothetical protein
PA2835		21.82	probable MFS transporter
PA2862	*lipA*	8.92	lactonizing lipase precursor
PA2863	*lipH*	8.38	lipase modulator protein
PA2881		15.38	probable two-component response regulator
PA2883		14.09	hypothetical protein
PA2898		9.63	hypothetical protein
PA2899		5.96	probable transcriptional regulator
PA2910		6.53	conserved hypothetical protein
PA2916		19.26	hypothetical protein
PA2920		6.78	probable chemotaxis transducer
PA2935		6.25	hypothetical protein
PA2937		35.53	hypothetical protein
PA2938		21.52	probable transporter
PA3017		6.07	conserved hypothetical protein
PA3032[3]	*snr1*	7.51	cytochrome c Snr1
PA3044		5.97	probable two-component sensor
PA3045		6.03	probable two-component response regulator
PA3049	*rmf*	59.24	ribosome modulation factor
PA3067		12.42	probable transcriptional regulator
PA3089		13.39	hypothetical protein
PA3090		10.23	hypothetical protein
PA3174[3]		5.08	probable transcriptional regulator
PA3183[3]	*zwf*	5.30	glucose-6-phosphate 1-dehydrogenase
PA3194[3]	*edd*	5.57	phosphogluconate dehydratase
PA3195[3]	*gapA*	13.46	glyceraldehyde 3-phosphate dehydrogenase
PA3216		6.19	hypothetical protein
PA3231		17.84	hypothetical protein
PA3234[3]		5.89	probable sodium:solute symporter
PA3235[3]		13.76	conserved hypothetical protein
PA3237		40.51	hypothetical protein
PA3259		5.79	hypothetical protein
PA3273		23.86	hypothetical protein
PA3274		83.83	hypothetical protein
PA3277		5.31	probable short-chain dehydrogenase
PA3279	*oprP*	6.30	outer membrane porin OprP precursor
PA3323		6.34	conserved hypothetical protein
PA3324		13.12	probable short-chain dehydrogenase
PA3342		8.51	hypothetical protein
PA3343		6.60	hypothetical protein
PA3354		6.19	hypothetical protein

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *vqsR* mutant in the ABC minimal medium			
PA3362		5.13	hypothetical protein
PA3368		9.05	probable acetyltransferase
PA3369[3]		10.13	hypothetical protein
PA3370[3]		11.26	hypothetical protein
PA3371[3]		7.12	hypothetical protein
PA3383		13.85	binding protein component of ABC phosphonate transporter
PA3384	*phnC*	6.83	ATP-binding component of ABC phosphonate transporter
PA3386		8.24	conserved hypothetical protein
PA3390		5.77	hypothetical protein
PA3392[3]	*nosZ*	10.10	nitrous-oxide reductase precursor
PA3396[3]	*nosL*	8.61	NosL protein
PA3412		6.29	hypothetical protein
PA3415		64.25	probable dihydrolipoamide acetyltransferase
PA3416[3]		397.78	probable pyruvate dehydrogenase E1 component, beta chain
PA3417		74.11	probable pyruvate dehydrogenase E1 component, alpha subunit
PA3418[3]	*ldh*	24.06	leucine dehydrogenase
PA3421		15.64	conserved hypothetical protein
PA3422		83.72	hypothetical protein
PA3427		11.66	probable short-chain dehydrogenases
PA3428		28.79	hypothetical protein
PA3429		5.54	probable epoxide hydrolase
PA3430		9.45	probable aldolase
PA3432		10.35	hypothetical protein
PA3451		10.69	hypothetical protein
PA3546	*algX*	10.99	alginate biosynthesis protein AlgX
PA3568		8.74	probable acetyl-coa synthetase
PA3569	*mmsB*	6.59	3-hydroxyisobutyrate dehydrogenase
PA3570	*mmsA*	5.30	methylmalonate-semialdehyde dehydrogenase
PA3584	*glpD*	7.42	glycerol-3-phosphate dehydrogenase
PA3591		9.98	probable enoyl-CoA hydratase/isomerase
PA3630		11.46	probable transcriptional regulator
PA3688[3]		10.95	hypothetical protein
PA3692[3]		7.02	probable outer membrane protein precursor
PA3710		8.08	probable GMC-type oxidoreductase
PA3719		9.13	hypothetical protein
PA3720		13.44	hypothetical protein
PA3723		11.15	probable FMN oxidoreductase
PA3765		6.12	hypothetical protein
PA3865		11.72	probable amino acid binding protein
PA3872[3]	*narI*	6.71	respiratory nitrate reductase gamma chain
PA3875[3]	*narG*	11.26	respiratory nitrate reductase alpha chain
PA3899		7.92	probable sigma-70 factor, ECF subfamily
PA3900		6.71	probable transmembrane sensor
PA3919[3]		18.29	conserved hypothetical protein
PA3957		13.99	probable short-chain dehydrogenase
PA3986[3]		7.49	hypothetical protein
PA4027		7.23	hypothetical protein
PA4038		38.79	hypothetical protein
PA4039		8.03	hypothetical protein
PA4070		37.96	probable transcriptional regulator
PA4081		11.39	probable fimbrial protein
PA4084	*cupB3*	20.06	probable fimbrial biogenesis usher protein
PA4093		5.59	hypothetical protein
PA4094		8.30	probable transcriptional regulator
PA4096		5.26	probable MFS transporter
PA4097		6.08	probable alcohol dehydrogenase (Zn-dependent)
PA4104		6.65	conserved hypothetical protein
PA4108		6.70	hypothetical protein
PA4111		7.01	hypothetical protein
PA4115		6.04	conserved hypothetical protein
PA4120		6.07	probable transcriptional regulator
PA4121		7.31	conserved hypothetical protein
PA4124	*hpcB*	14.12	homoprotocatechuate 2,3-dioxygenase
PA4126		5.50	probable MFS transporter
PA4146		5.96	hypothetical protein
PA4147	*acoR*	15.15	transcriptional regulator AcoR
PA4149		5.68	conserved hypothetical protein
PA4152		6.94	probable hydrolase
PA4158	*fepC*	20.26	ferric enterobactin transport protein FepC
PA4160	*fepD*	10.09	ferric enterobactin transport protein FepD
PA4173		8.77	conserved hypothetical protein
PA4185		6.67	probable transcriptional regulator
PA4205		41.01	hypothetical protein
PA4221	*fptA*	27.22	Fe(III)-pyochelin receptor precursor

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *vqsR* mutant in the ABC minimal medium			
PA4227	*pchR*	19.31	transcriptional regulator PchR
PA4229	*pchC*	14.90	pyochelin biosynthetic protein PchC
PA4231	*pchA*	30.45	salicylate biosynthesis isochorismate synthase
PA4289		5.54	probable transporter
PA4290		5.87	probable chemotaxis transducer
PA4293[3]		44.23	probable two-component sensor
PA4294[3]		10.75	hypothetical protein
PA4296[3]		7.61	probable two-component response regulator
PA4298[3]		12.21	hypothetical protein
PA4299[3]		12.56	hypothetical protein
PA4300[3]		13.66	hypothetical protein
PA4301		10.57	hypothetical protein
PA4302[3]		19.26	probable type II secretion system protein
PA4303[3]		9.40	hypothetical protein
PA4304[3]		25.06	probable type II secretion system protein
PA4305[3]		12.11	hypothetical protein
PA4306[3]		23.64	hypothetical protein
PA4311[3]		5.79	conserved hypothetical protein
PA4344		8.45	probable hydrolase
PA4350		7.05	conserved hypothetical protein
PA4364		20.94	hypothetical protein
PA4365		11.94	probable transporter
PA4469		8.08	hypothetical protein
PA4470	*fumC1*	13.34	fumarate hydratase
PA4471		145.86	hypothetical protein
PA4507		10.64	hypothetical protein
PA4523		31.54	hypothetical protein
PA4540		31.97	hypothetical protein
PA4541		14.90	hypothetical protein
PA4570		18.32	hypothetical protein
PA4573		12.01	hypothetical protein
PA4575		13.63	hypothetical protein
PA4596		5.90	probable transcriptional regulator
PA4608		7.99	hypothetical protein
PA4612		5.66	conserved hypothetical protein
PA4613		9.88	catalase
PA4616		5.41	probable c4-dicarboxylate-binding protein
PA4623		6.86	hypothetical protein
PA4630		14.88	hypothetical protein
PA4641		7.51	still frameshift hypothetical protein
PA4648[3]		11.94	hypothetical protein
PA4649[3]		8.82	hypothetical protein
PA4650[3]		6.64	hypothetical protein
PA4651[3]		6.00	probable pili assembly chaperone
PA4653		7.35	hypothetical protein
PA4654		34.62	probable MFS transporter
PA4658		9.05	hypothetical protein
PA4659		5.52	probable transcriptional regulator
PA4680		6.09	hypothetical protein
PA4702		10.24	hypothetical protein
PA4703[3]		7.27	hypothetical protein
PA4704		5.68	hypothetical protein
PA4709		6.53	probable hemin degrading factor
PA4711		6.04	hypothetical protein
PA4810	*fdnI*	5.37	nitrate-inducible formate dehydrogenase, gamma subunit
PA4811	*fdnH*	13.11	nitrate-inducible formate dehydrogenase, beta subunit
PA4812	*fdnG*	6.62	formate dehydrogenase-O, major subunit
PA4844		6.79	probable chemotaxis transducer
PA4859		5.28	probable permease of ABC transporter
PA4861		20.01	probable ATP-binding component of ABC transporter
PA4877		8.44	hypothetical protein
PA4879		5.42	conserved hypothetical protein
PA4881		9.77	hypothetical protein
PA4883		13.01	hypothetical protein
PA4892	*ureF*	5.79	urease accessory protein UreF
PA4895		9.62	probable transmembrane sensor
PA4896		25.59	probable sigma-70 factor, ECF subfamily
PA4897		5.40	hypothetical protein
PA4898		24.92	probable porin
PA4908		8.38	hypothetical protein
PA4910		5.80	probable ATP-binding component of ABC transporter
PA4912		6.49	probable ABC branched chain amino acid transporter
PA4913		10.75	probable binding protein component of ABC transporter
PA4914		13.24	probable transcriptional regulator

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *vqsR* mutant in the ABC minimal medium			
PA4915		11.19	probable chemotaxis transducer
PA4977		5.13	probable acetolactate synthase large subunit
PA4978		7.45	hypothetical protein
PA4995		7.30	probable acyl-CoA dehydrogenase
PA5020		5.94	probable acyl-CoA dehydrogenase
PA5023		5.20	conserved hypothetical protein
PA5085		5.03	probable transcriptional regulator
PA5087		12.73	hypothetical protein
PA5098	*hutH*	5.13	histidine ammonia-lyase
PA5100	*hutU*	6.71	urocanase
PA5106		12.39	conserved hypothetical protein
PA5313		32.15	probable pyridoxal-dependent aminotransferase
PA5314		5.51	hypothetical protein
PA5325		18.87	hypothetical protein
PA5328		6.54	probable cytochrome c(mono-heme type)
PA5352[3]		12.20	conserved hypothetical protein
PA5353[3]	*glcF*	12.03	glycolate oxidase subunit GlcF
PA5354[3]	*glcE*	11.50	glycolate oxidase subunit GlcE
PA5355[3]	*glcD*	9.01	glycolate oxidase subunit GlcD
PA5372	*betA*	7.22	choline dehydrogenase
PA5379	*sdaB*	5.69	L-serine dehydratase
PA5384		9.75	probable lipolytic enzyme
PA5385		7.13	hypothetical protein
PA5391		6.94	hypothetical protein
PA5418	*soxA*	16.85	sarcosine oxidase alpha subunit
PA5419	*soxG*	13.89	sarcosine oxidase gamma subunit
PA5431		5.18	probable transcriptional regulator
PA5446		12.35	hypothetical protein
PA5473		6.74	conserved hypothetical protein
PA5510		5.41	probable transporter
PA5522		15.83	probable glutamine synthetase
PA5523		8.59	probable aminotransferase
PA5531	*tonB*	9.07	TonB protein
PA5546		13.94	conserved hypothetical protein
Genes downregulated in the *vqsR* mutant in the ABC minimal medium			
PA0046		19.50	hypothetical protein
PA0070		5.28	hypothetical protein
PA0263[3]	*hcpC*	6.33	secreted protein Hcp
PA0281	*cysW*	10.59	sulfate transport protein CysW
PA0283	*sbp*	5.59	sulfate-binding protein precursor
PA0284		13.24	hypothetical protein
PA0291	*oprE*	6.65	outer membrane porin OprE precursor
PA0390	*metX*	7.23	homoserine O-acetyltransferase
PA0423		7.94	conserved hypothetical protein
PA0456		5.25	probable cold-shock protein
PA0546	*metK*	11.54	methionine adenosyltransferase
PA0547		10.05	probable transcriptional regulator
PA0548	*tktA*	12.98	transketolase
PA0552	*pgk*	14.96	phosphoglycerate kinase
PA0579	*rpsU*	18.19	30S ribosomal protein S21
PA0594	*surA*	5.30	peptidyl-prolyl cis-trans isomerase SurA
PA0595	*ostA*	7.34	organic solvent tolerance protein OstA precursor
PA0654	*speD*	6.40	S-adenosylmethionine decarboxylase proenzyme
PA0668	*tyrZ*	7.61	tyrosyl-tRNA synthetase 2
PA0751		14.96	conserved hypothetical protein
PA0752		16.98	conserved hypothetical protein
PA0753		15.94	hypothetical protein
PA0754		12.71	hypothetical protein
PA0755		19.18	probable porin
PA0779		6.52	probable ATP-dependent protease
PA0837	*slyD*	5.29	peptidyl-prolyl cis-trans isomerase SlyD
PA0856		8.31	hypothetical protein
PA0857	*bolA*	5.06	morphogene protein BolA
PA0904	*lysC*	9.67	aspartate kinase alpha and beta chain
PA0956	*proS*	6.90	prolyl-tRNA synthetase
PA0963	*aspS*	8.89	aspartyl-tRNA synthetase
PA0964		5.50	conserved hypothetical protein
PA0973	*oprL*	7.92	outer membrane protein OprL precursor
PA0974		6.57	conserved hypothetical protein
PA0996[3]	*pqsA*	15.70	probable coenzyme A ligase
PA0997[3]	*pqsB*	23.17	hypothetical protein
PA0998[3]	*pqsC*	55.21	hypothetical protein
PA0999[3]	*pqsD*	12.18	3-oxoacyl-[acyl-carrier-protein] synthase III
PA1000[3]	*pqsE*	11.98	hypothetical protein

ORF[1]	Gene name	Fold change[2]	Protein description
Genes downregulated in the *vqsR* mutant in the ABC minimal medium			
PA1001[3]	*phnA*	15.77	anthranilate synthase component I
PA1002[3]	*phnB*	10.96	anthranilate synthase component II
PA1010	*dapA*	5.80	dihydrodipicolinate synthase
PA1011		6.17	hypothetical protein
PA1013	*purC*	5.87	phosphoribosylaminoimidazole-succinocarboxamide synthase
PA1155	*nrdB*	6.99	ribonucleoside reductase, small chain
PA1159		18.80	probable cold-shock protein
PA1170		7.51	conserved hypothetical protein
PA1250[3]	*aprI*	8.58	alkaline proteinase inhibitor AprI
PA1431[3]	*rsaL*	17.17	regulatory protein RsaL
PA1481	*ccmG*	6.62	cytochrome C biogenesis protein CcmG
PA1552		9.73	probable cytochrome c
PA1553		11.75	probable cytochrome c oxidase subunit
PA1554		10.66	probable cytochrome oxidase subunit (cbb3-type)
PA1574		14.98	conserved hypothetical protein
PA1581	*sdhC*	8.47	succinate dehydrogenase (C subunit)
PA1582	*sdhD*	8.88	succinate dehydrogenase (D subunit)
PA1583	*sdhA*	21.89	succinate dehydrogenase (A subunit)
PA1584	*sdhB*	10.96	succinate dehydrogenase (B subunit)
PA1585	*sucA*	6.33	2-oxoglutarate dehydrogenase (E1 subunit)
PA1586	*sucB*	6.61	dihydrolipoamide succinyltransferase (E2 subunit)
PA1587	*lpdG*	6.07	lipoamide dehydrogenase-glc
PA1588	*sucC*	15.31	succinyl-CoA synthetase beta chain
PA1589	*sucD*	12.71	succinyl-CoA synthetase alpha chain
PA1596	*hptG*	10.37	heat shock protein HtpG
PA1609	*fabB*	5.25	beta-ketoacyl-ACP synthase I
PA1610	*fabA*	7.53	beta-hydroxydecanoyl-ACP dehydrase
PA1657[3]		24.31	conserved hypothetical protein
PA1658[3]		18.65	conserved hypothetical protein
PA1659[3]		5.63	hypothetical protein
PA1664[3]		5.88	hypothetical protein
PA1674	*folE2*	6.59	GTP cyclohydrolase I precursor
PA1750		9.63	phospho-2-dehydro-3-deoxyheptonate aldolase
PA1776		5.79	probable sigma-70 factor, ECF subfamily
PA1787	*acnB*	11.38	aconitate hydratase 2
PA1800	*tig*	28.46	trigger factor
PA1812	*mltD*	7.23	membrane-bound lytic murein transglycosylase D precursor
PA1837		8.47	hypothetical protein
PA1838	*cysI*	6.41	sulfite reductase
PA1969		5.99	hypothetical protein
PA2193[3]	*hcnA*	13.66	hydrogen cyanide synthase HcnA
PA2204		19.18	probable binding protein component of ABC transporter
PA2441		18.86	hypothetical protein
PA2619	*infA*	8.90	initiation factor
PA2629	*purB*	23.91	adenylosuccinate lyase
PA2740	*pheS*	5.37	phenylalanyl-tRNA synthetase, alpha-subunit
PA2741	*rplT*	5.31	50S ribosomal protein L20
PA2742	*rpmI*	5.22	50S ribosomal protein L35
PA2760		9.26	probable outer membrane protein precursor
PA2800		5.23	conserved hypothetical protein
PA2851	*efp*	23.23	translation elongation factor P
PA2950		10.45	hypothetical protein
PA2951	*etfA*	6.40	electron transfer flavoprotein alpha-subunit
PA2966	*acpP*	5.87	acyl carrier protein
PA2967	*fabG*	6.83	3-oxoacyl-[acyl-carrier-protein] reductase
PA2968	*fabD*	5.45	malonyl-CoA-[acyl-carrier-protein] transacylase
PA2970	*rpmF*	24.06	50S ribosomal protein L32
PA2971		13.20	conserved hypothetical protein
PA2993		8.34	conserved hypothetical protein
PA3000	*aroP1*	13.62	aromatic amino acid transport protein AroP1
PA3001		11.18	probable glyceraldehyde-3-phosphate dehydrogenase
PA3019		9.32	probable ATP-binding component of ABC transporter
PA3126	*IbpA*	13.09	heat-shock protein IbpA
PA3162	*rpsA*	13.76	30S ribosomal protein S1
PA3262		12.65	probable peptidyl-prolyl cis-trans isomerase, FkbP-type
PA3313		6.04	hypothetical protein
PA3326[3]		6.00	probable Clp-family ATP-dependent protease
PA3329[3]		8.09	hypothetical protein
PA3330[3]		9.89	probable short chain dehydrogenase
PA3331[3]		5.40	cytochrome P450
PA3332[3]		6.17	conserved hypothetical protein
PA3334[3]		7.58	probable acyl carrier protein
PA3397	*für*	64.55	ferredoxin--NADP+ reductase
PA3441		29.77	probable molybdopterin-binding protein

ORF[1]	Gene name	Fold change[2]	Protein description
Genes downregulated in the *vqsR* mutant in the ABC minimal medium			
PA3480		5.39	probable deoxycytidine triphosphate deaminase
PA3525	*argG*	20.15	argininosuccinate synthase
PA3529		5.52	probable peroxidase
PA3537	*argF*	5.52	ornithine carbamoyltransferase, anabolic
PA3621	*fdxA*	6.17	ferredoxin I
PA3635	*eno*	12.68	enolase
PA3645	*fabZ*	5.84	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase
PA3646	*lpxD*	5.30	UDP-3-O-[3-hydroxylauroyl] glucosamine N-acyltransferase
PA3647		5.47	probable outer membrane protein precursor
PA3648		6.96	probable outer membrane protein precursor
PA3653	*frr*	7.55	ribosome recycling factor
PA3655	*tsf*	14.79	elongation factor Ts
PA3656	*rpsB*	16.10	30S ribosomal protein S2
PA3686	*adk*	11.21	adenylate kinase
PA3700	*lysS*	9.61	lysyl-tRNA synthetase
PA3742	*rplS*	14.01	50S ribosomal protein L19
PA3743	*trmD*	20.14	tRNA (guanine-N1)-methyltransferase
PA3744	*rimM*	19.92	16S rRNA processing protein
PA3745	*rpsP*	40.89	30S ribosomal protein S16
PA3746	*ffh*	17.95	signal recognition particle protein Ffh
PA3770	*guaB*	7.81	inosine-5'-monophosphate dehydrogenase
PA3801		6.20	conserved hypothetical protein
PA3803	*gcpE*	5.74	probable isoprenoid biosynthetic protein GcpE
PA3806		5.69	conserved hypothetical protein
PA3807		12.40	nucleoside diphosphate kinase
PA3820	*secF*	6.97	secretion protein SecF
PA3821	*secD*	5.21	secretion protein SecD
PA3822		9.43	conserved hypothetical protein
PA3827		5.81	conserved hypothetical protein
PA3834	*valS*	7.90	valyl-tRNA synthetase
PA3861	*rhlB*	5.48	ATP-dependent RNA helicase RhlB
PA3904[3]		24.94	hypothetical protein
PA3905[3]		18.37	hypothetical protein
PA3906[3]		6.00	hypothetical protein
PA3907[3]		5.87	hypothetical protein
PA3908[3]		6.09	hypothetical protein
PA3931		7.57	conserved hypothetical protein
PA3940		9.35	probable DNA binding protein
PA3992		8.29	hypothetical protein
PA4031	*ppa*	26.46	inorganic pyrophosphatase
PA4129[3]		5.89	hypothetical protein
PA4131[3]		8.49	probable iron-sulfur protein
PA4133[3]		8.45	cytochrome c oxidase subunit (cbb3-type)
PA4134[3]		9.65	hypothetical protein
PA4139[3]		78.04	hypothetical protein
PA4140		16.50	hypothetical protein
PA4141[3]		22.36	hypothetical protein
PA4237	*rplQ*	8.26	50S ribosomal protein L17
PA4238	*rpoA*	16.61	DNA-directed RNA polymerase alpha chain
PA4239	*rpsD*	22.65	30S ribosomal protein S4
PA4240	*rpsK*	19.46	30S ribosomal protein S11
PA4241	*rpsM*	27.92	30S ribosomal protein S13
PA4242[3]	*rpmJ*	21.92	50S ribosomal protein L36
PA4243[3]	*secY*	15.54	secretion protein SecY
PA4244	*rplO*	28.66	50S ribosomal protein L15
PA4245	*rpmD*	39.48	50S ribosomal protein L30
PA4246	*rpsE*	35.21	30S ribosomal protein S5
PA4247	*rplR*	22.19	50S ribosomal protein L18
PA4248	*rplF*	20.89	50S ribosomal protein L6
PA4249	*rpsH*	14.57	30S ribosomal protein S8
PA4250	*rpsN*	11.22	30S ribosomal protein S14
PA4251	*rplE*	9.33	50S ribosomal protein L5
PA4252	*rplX*	19.18	50S ribosomal protein L24
PA4253	*rplN*	13.10	50S ribosomal protein L14
PA4254	*rpsQ*	26.99	30S ribosomal protein S17
PA4255	*rpmC*	27.32	50S ribosomal protein L29
PA4256	*rplP*	19.82	50S ribosomal protein L16
PA4257	*rpsC*	22.08	30S ribosomal protein S3
PA4258	*rplV*	19.46	50S ribosomal protein L22
PA4259	*rpsS*	15.91	30S ribosomal protein S19
PA4260	*rplB*	12.55	50S ribosomal protein L2
PA4261	*rplW*	43.24	50S ribosomal protein L23
PA4262	*rplD*	30.54	50S ribosomal protein L4
PA4263	*rplC*	17.52	50S ribosomal protein L3

ORF[1]	Gene name	Fold change[2]	Protein description
Genes downregulated in the *vqsR* mutant in the ABC minimal medium			
PA4264	*rpsJ*	16.56	30S ribosomal protein S10
PA4265	*tufA*	10.78	elongation factor Tu
PA4266	*fusA1*	17.17	elongation factor G
PA4267	*rpsG*	10.58	30S ribosomal protein S7
PA4268	*rpsL*	24.02	30S ribosomal protein S12
PA4269	*rpoC*	8.16	DNA-directed RNA polymerase beta* chain
PA4270	*rpoB*	11.02	DNA-directed RNA polymerase beta chain
PA4271	*rplL*	15.19	50S ribosomal protein L7 / L12
PA4272	*rplJ*	33.78	50S ribosomal protein L10
PA4273	*rplA*	22.87	50S ribosomal protein L1
PA4274	*rplK*	24.31	50S ribosomal protein L11
PA4275	*nusG*	6.57	transcription antitermination protein NusG
PA4276	*secE*	9.85	secretion protein SecE
PA4385	*groEL*	26.65	GroEL protein
PA4386	*groES*	19.39	GroES protein
PA4429		14.57	probable cytochrome c1 precursor
PA4430		10.04	probable cytochrome b
PA4431		9.07	probable iron-sulfur protein
PA4432	*rpsI*	33.79	30S ribosomal protein S9
PA4433	*rplM*	46.85	50S ribosomal protein L13
PA4442	*cysN*	13.73	ATP sulfurylase GTP-binding subunit/APS kinase
PA4443	*cysD*	13.91	ATP sulfurylase small subunit
PA4449	*hisG*	5.11	ATP-phosphoribosyltransferase
PA4450	*murA*	6.28	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
PA4451		11.00	conserved hypothetical protein
PA4481	*mreB*	7.23	rod shape-determining protein MreB
PA4482	*gatC*	12.40	Glu-tRNA(Gln) amidotransferase subunit C
PA4563	*rpsT*	35.87	30S ribosomal protein S20
PA4567	*rpmA*	6.13	50S ribosomal protein L27
PA4568	*rplU*	13.44	50S ribosomal protein L21
PA4569	*ispB*	5.62	octaprenyl-diphosphate synthase
PA4572	*flkB*	5.38	peptidyl-prolyl cis-trans isomerase FklB
PA4602	*glyA3*	12.07	serine hydroxymethyltransferase
PA4670	*prs*	8.10	ribose-phosphate pyrophosphokinase
PA4671		18.59	probable ribosomal protein L25
PA4672		5.22	peptidyl-tRNA hydrolase
PA4694	*ilvC*	17.54	ketol-acid reductoisomerase
PA4695	*ilvH*	11.86	acetolactate synthase isozyme III small subunit
PA4696	*ilvI*	6.21	acetolactate synthase large subunit
PA4723	*dksA*	7.88	suppressor protein DksA
PA4740	*pnp*	9.86	polyribonucleotide nucleotidyltransferase
PA4741	*rpsO*	17.80	30S ribosomal protein S15
PA4744	*infB*	6.74	translation initiation factor IF-2
PA4746		5.32	conserved hypothetical protein
PA4747	*secG*	7.84	secretion protein SecG
PA4748	*tpiA*	10.84	triosephosphate isomerase
PA4761	*dnaK*	6.01	DnaK protein
PA4762	*grpE*	8.36	heat shock protein GrpE
PA4765	*omlA*	7.37	outer membrane lipoprotein OmlA
PA4768	*smpB*	18.40	SmpB protein
PA4846	*aroQ1*	6.06	3-dehydroquinate dehydratase
PA4847	*arccB*	9.29	biotin carboxyl carrier protein (BCCP)
PA4848	*accC*	7.96	biotin carboxylase
PA4932	*rplI*	7.97	50S ribosomal protein L9
PA4933		29.71	hypothetical protein
PA4934	*rpsR*	24.37	30S ribosomal protein S18
PA4935	*rpsF*	37.87	30S ribosomal protein S6
PA4938	*purA*	6.17	adenylosuccinate synthetase
PA5046		8.90	malic enzyme
PA5049	*rpmE*	9.72	50S ribosomal protein L31
PA5053	*hslV*	6.30	heat shock protein HslV
PA5054	*hslU*	10.85	heat shock protein HslU
PA5076		12.81	probable binding protein component of ABC transporter
PA5117	*typA*	6.13	regulatory protein TypA
PA5119	*glnA*	6.13	glutamine synthetase
PA5128	*secB*	10.76	secretion protein SecB
PA5129	*grx*	9.40	glutaredoxin
PA5130		5.48	conserved hypothetical protein
PA5192	*pckA*	8.04	phosphoenolpyruvate carboxykinase
PA5300	*cycB*	7.59	cytochrome c5
PA5315	*rpmG*	10.77	50S ribosomal protein L33
PA5316	*rpmB*	42.33	50S ribosomal protein L28
PA5339		7.51	conserved hypothetical protein
PA5468		15.29	probable citrate transporter

ORF[1]	Gene name	Fold change[2]	Protein description
Genes downregulated in the *vqsR* mutant in the ABC minimal medium			
PA5469		11.81	conserved hypothetical protein
PA5479	*gltP*	5.66	proton-glutamate symporter
PA5490	*cc4*	6.28	cytochrome c4 precursor
PA5491		7.42	probable cytochrome
PA5505		8.95	probable TonB-dependent receptor
PA5553	*atpC*	7.47	ATP synthase epsilon chain
PA5554	*atpD*	18.09	ATP synthase beta chain
PA5555	*atpG*	34.95	ATP synthase gamma chain
PA5556	*atpA*	23.67	ATP synthase alpha chain
PA5557	*atpH*	38.17	ATP synthase delta chain
PA5558	*atpF*	20.27	ATP synthase B chain
PA5559	*atpE*	25.74	atp synthase C chain
PA5560	*atpB*	11.81	ATP synthase A chain
PA5569	*rnpA*	16.72	ribonuclease P protein component
PA5570	*rpmH*	34.85	50S ribosomal protein L34

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons. Only genes with 5-fold and higher differential expression are shown.

[3] Quorum sensing regulated genes (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003).

Appendix III

Differential transcription profile of the *icsF* mutant grown in the presence of PMNs.

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *icsF* mutant grown in the presence of PMNs			
PA0236		2.8	probable transcriptional regulator
PA0237		4.5	probable oxidoreductase
PA0340[3]		3.3	conserved hypothetical protein
PA0522[3]		4.8	hypothetical protein
PA0614[3]		3.6	hypothetical protein
PA0813		3.4	hypothetical protein
PA1127		3.7	probable oxidoreductase
PA1145		3.3	probable transcriptional regulator
PA1214		4.2	hypothetical protein
PA1321[3]	*cyoE*	4.4	cytochrome o ubiquinol oxidase protein CyoE
PA1435		3.3	probable RND efflux membrane fusion protein precursor
PA1467		4.0	hypothetical protein
PA1697		4.9	ATP synthase in type III secretion system
PA1723	*pscJ*	3.6	type III export protein PscJ
PA1910		3.4	probable tonB-dependent receptor protein
PA1911		2.8	probable transmembrane sensor
PA1928	*rimJ*	3.2	ribosomal protein alanine acetyltransferase
PA1952		4.7	hypothetical protein
PA1970[3]		8.7	hypothetical protein
PA1984[3]		4.5	probable aldehyde dehydrogenase
PA2022		5.5	probable nucleotide sugar dehydrogenase
PA2089		3.3	hypothetical protein
PA2169		3.3	hypothetical protein
PA2278	*arsB*	3.2	ArsB protein
PA2344	*mtlZ*	3.1	fructokinase
PA2385[3]	*pvdQ*	3.1	probable acylase
PA2390		3.0	probable ATP-binding/permease fusion ABC transporter
PA2394[3]	*pvdN*	3.1	probable aminotransferase
PA2486[3]		3.1	hypothetical protein
PA2488		3.8	probable transcriptional regulator
PA2506		3.5	hypothetical protein
PA2534		3.6	probable transcriptional regulator
PA2736[3]		3.7	hypothetical protein
PA2759		9.4	hypothetical protein

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *icsF* mutant grown in the presence of PMNs			
PA3229[3]		9.5	hypothetical protein
PA3293		8.3	hypothetical protein
PA3318		6.1	hypothetical protein
PA3384	*phnC*	3.3	ATP-binding component of ABC phosphonate transporter
PA3492		3.0	conserved hypothetical protein
PA3690		13.7	probable metal-transporting P-type ATPase
PA3774		2.9	probable acetylpolyamine aminohydrolase
PA3814[3]	*iscS*	3.9	L-cysteine desulfurase (pyridoxal phosphate-dependent)
PA3815		4.9	conserved hypothetical protein
PA3888[3]		5.0	probable permease of ABC transporter
PA3889[3]		3.1	probable binding protein component of ABC transporter
PA4160	*fepD*	3.4	ferric enterobactin transport protein FepD
PA4208	*opmD*	4.5	probable outer membrane protein precursor
PA4323[3]		2.8	hypothetical protein
PA4355		3.4	probable MFS transporter
PA4356[3]	*xenB*	4.8	xenobiotic reductase
PA4623[3]		14.9	hypothetical protein
PA4774		3.9	hypothetical protein
PA4783		5.4	conserved hypothetical protein
PA4881[3]		9.9	hypothetical protein
PA4888[3]		3.4	conserved hypothetical protein
PA5072		43.3	probable chemotaxis transducer
PA5407[3]		3.6	hypothetical protein
Genes downregulated in the *icsF* mutant grown in the presence of PMNs			
PA0122[3]		2.9	conserved hypothetical protein
PA0527	*dnr*	6.7	transcriptional regulator Dnr
PA0587[3]		2.8	conserved hypothetical protein
PA0761	*nadB*	3.9	L-aspartate oxidase
PA0820		2.9	hypothetical protein
PA0981[3]		18.7	hypothetical protein
PA1159		4.8	probable cold-shock protein
PA1177[3]	*napE*	3.5	periplasmic nitrate reductase protein NapE
PA1178	*oprH*	14.4	outer membrane protein H1 precursor
PA1179	*phoP*	6.2	two-component response regulator PhoP
PA1196		4.0	probable transcriptional regulator
PA1343		3.9	hypothetical protein
PA1344[3]		10.8	probable short-chain dehydrogenase
PA1557[3]		3.8	probable cytochrome oxidase subunit (cbb3-type)
PA1604[3]		3.3	hypothetical protein
PA1617		3.3	probable AMP-binding enzyme
PA1657[3]		3.1	conserved hypothetical protein
PA1664[3]		4.2	hypothetical protein
PA1769		3.0	conserved hypothetical protein
PA1789[3]		3.8	hypothetical protein
PA4211[3]	*phzB1*	2.9	probable phenazine biosynthesis protein
PA1914[3]		4.7	conserved hypothetical protein
PA2146[3]		3.8	conserved hypothetical protein
PA2193[3]	*hcnA*	3.1	hydrogen cyanide synthase HcnA
PA2381[3]		2.8	hypothetical protein
PA2622[3]	*cspD*	4.7	cold-shock protein CspD
PA3278[3]		3.5	hypothetical protein
PA3361[3]	*lecB*	2.8	hypothetical protein
PA3451[3]		3.7	hypothetical protein
PA3479[3]	*rhlA*	4.9	rhamnosyltransferase chain A
PA3520[3]		4.0	hypothetical protein
PA3600[3]		6.4	conserved hypothetical protein
PA3601[3]		8.0	conserved hypothetical protein
PA3615[3]		3.1	hypothetical protein
PA3724[3]	*lasB*	4.2	elastase LasB
PA3875[3]	*narG*	3.3	respiratory nitrate reductase alpha chain
PA4133[3]		3.1	cytochrome c oxidase subunit (cbb3-type)
PA4134[3]		6.3	hypothetical protein
PA4139		6.2	hypothetical protein
PA4141[3]		4.4	hypothetical protein
PA4221[3]	*ftpA*	2.9	Fe(III)-pyochelin receptor precursor
PA4226[3]	*pchE*	2.8	dihydroaeruginoic acid synthetase
PA4230[3]	*pchB*	3.5	salicylate biosynthesis protein PchB
PA4296[3]		2.7	probable two-component response regulator
PA4306[3]		3.9	hypothetical protein
PA4359[3]		3.6	conserved hypothetical protein
PA4377[3]		3.0	hypothetical protein
PA4611[3]		3.0	hypothetical protein
PA4648[3]		3.9	hypothetical protein
PA5170[3]	*arcD*	3.3	arginine/ornithine antiporter

ORF[1]	Gene name	Fold change[2]	Protein description
Genes downregulated in the *icsF* mutant grown in the presence of PMNs			
PA5436[3]		2.8	probable biotin carboxylase subunit of a transcarboxylase
PA5482[3]		3.7	hypothetical protein
PA5501	*znuB*	2.9	permease of ABC zinc transporter ZnuB

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons.

[3] Oxidative stress response genes.

Appendix IV

Differential transcription profile of the *icsF* mutant grown in the presence of H_2O_2.

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA0007[3]		25.4	hypothetical protein
PA0025[3]	*aroE*	3.5	shikimate dehydrogenase
PA0027[3]		3.2	hypothetical protein
PA0028[3]		2.5	hypothetical protein
PA0050[3]		5.7	hypothetical protein
PA0052[3]		11.4	hypothetical protein
PA0105[3]	*coxB*	10.5	cytochrome c oxidase, subunit II
PA0106[3]	*coxA*	5.7	cytochrome c oxidase, subunit I
PA0107[3]		7.2	conserved hypothetical protein
PA0108[3]	*colII*	8.4	cytochrome c oxidase, subunit III
PA0109[3]		11.4	hypothetical protein
PA0122[3]		27.6	conserved hypothetical protein
PA0141[3]		7.1	conserved hypothetical protein
PA0143[3]		3.0	probable nucleoside hydrolase
PA0144[3]		7.6	hypothetical protein
PA0173[3]		38.3	probable methylesterase
PA0175[3]		12.7	probable chemotaxis protein methyltransferase
PA0176[3]		31.2	probable chemotaxis transducer
PA0177[3]		14.3	probable purine-binding chemotaxis protein
PA0178[3]		19.9	probable two-component sensor
PA0179[3]		28.2	probable two-component response regulator
PA0180[3]		6.0	probable chemotaxis transducer
PA0195[3]	*pntA*	3.4	still frameshift pyridine nucleotide transhydrog. alpha subunit
PA0200[3]		2.7	hypothetical protein
PA0209		4.1	conserved hypothetical protein
PA0210	*mdcC*	4.4	malonate decarboxylase delta subunit
PA0213[3]		10.6	hypothetical protein
PA0269[3]		2.8	conserved hypothetical protein
PA0270[3]		4.1	hypothetical protein
PA0271[3]		3.0	hypothetical protein
PA0387		2.7	conserved hypothetical protein
PA0413		2.7	still frameshift prob. component of chemotact. signal transd. syst.
PA0427	*oprM*	2.9	outer membrane protein OprM precursor
PA0441[3]	*dht*	16.8	dihydropyrimidinase
PA0443		22.3	probable transporter
PA0444[3]		9.9	N-carbamoyl-beta-alanine amidohydrolase
PA0446[3]		3.8	conserved hypothetical protein
PA0447[3]	*gcdH*	7.2	glutaryl-CoA dehydrogenase
PA0451		6.3	conserved hypothetical protein
PA0459[3]		21.2	probable ClpA/B protease ATP binding subunit
PA0460[3]		10.2	hypothetical protein
PA0468		3.6	hypothetical protein
PA0484[3]		5.8	conserved hypothetical protein
PA0492[3]		4.5	conserved hypothetical protein
PA0505[3]		3.3	hypothetical protein
PA0509[3]	*nirN*	67.8	probable c-type cytochrome
PA0510[3]		414.9	probable uroporphyrin-III c-methyltransferase

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA0511[3]	*nirJ*	78.4	heme d1 biosynthesis protein NirJ
PA0512[3]		111.0	conserved hypothetical protein
PA0513[3]		138.1	probable transcriptional regulator
PA0514[3]	*nirL*	224.0	heme d1 biosynthesis protein NirL
PA0515[3]		1153.9	probable transcriptional regulator
PA0516[3]	*nirF*	132.8	heme d1 biosynthesis protein NirF
PA0517[3]	*nirC*	198.9	probable c-type cytochrome precursor
PA0518[3]	*nirM*	148.6	cytochrome c-551 precursor
PA0519[3]	*nirS*	45.5	nitrite reductase precursor
PA0520[3]	*nirQ*	33.1	regulatory protein NirQ
PA0521[3]		28.7	probable cytochrome c oxidase subunit
PA0522[3]		10.6	hypothetical protein
PA0523[3]	*norC*	175.4	nitric-oxide reductase subunit C
PA0524[3]	*norB*	342.3	nitric-oxide reductase subunit B
PA0525[3]		116.3	probable dinitrification protein NorD
PA0526[3]		7.9	hypothetical protein
PA0534		27.2	conserved hypothetical protein
PA0543[3]		2.9	hypothetical protein
PA0567[3]		6.9	conserved hypothetical protein
PA0585[3]		7.3	hypothetical protein
PA0586[3]		38.6	conserved hypothetical protein
PA0587[3]		22.4	conserved hypothetical protein
PA0588[3]		16.8	conserved hypothetical protein
PA0704[3]		4.6	probable amidase
PA0709[3]		3.4	hypothetical protein
PA0710	*gloA2*	4.8	lactoylglutathione lyase
PA0713[3]		8.7	hypothetical protein
PA0714		14.7	hypothetical protein
PA0737[3]		3.3	hypothetical protein
PA0743[3]		14.7	probable 3-hydroxyisobutyrate dehydrogenase
PA0763[3]	*mucA*	3.3	anti-sigma factor MucA
PA0788[3]		8.8	hypothetical protein
PA0792	*prpD*	3.0	propionate catabolic protein PrpD
PA0798	*pmtA*	4.2	probable methyltransferase
PA0803[3]		3.7	hypothetical protein
PA0810[3]		2.8	probable haloacid dehalogenase
PA0835[3]		8.4	phosphate acetyltransferase
PA0836[3]		3.5	probable acetate kinase
PA0840[3]		4.1	probable oxidoreductase
PA0852[3]	*cpbD*	4.6	chitin-binding protein CbpD precursor
PA0853[3]		2.5	probable oxidoreductase
PA0854[3]		3.4	fumarate hydratase
PA0861[3]		5.7	hypothetical protein
PA0865	*hpd*	2.5	4-hydroxyphenylpyruvate dioxygenase
PA0870	*phhC*	3.1	aromatic amino acid aminotransferase
PA0872[3]	*phhA*	2.6	phenylalanine-4-hydroxylase
PA0918[3]		7.3	cytochrome b561
PA1003[3]		3.0	probable transcriptional regulator
PA1016		2.8	hypothetical protein
PA1041[3]		11.7	probable outer membrane protein precursor
PA1061		2.5	conserved hypothetical protein
PA1062		2.7	hypothetical protein
PA1065[3]		5.7	conserved hypothetical protein
PA1077[3]	*flgB*	4.6	flagellar basal-body rod protein FlgB
PA1078[3]	*flgC*	4.6	flagellar basal-body rod protein FlgC
PA1079	*flgD*	4.6	flagellar basal-body rod modification protein FlgD
PA1080[3]	*flgE*	4.7	flagellar hook protein FlgE
PA1082[3]	*flgG*	4.2	flagellar basal-body rod protein FlgG
PA1083[3]	*flgH*	3.7	flagellar L-ring protein precursor FlgH
PA1085[3]	*flgJ*	3.0	flagellar protein FlgJ
PA1087[3]	*flgL*	4.2	flagellar hook-associated protein type 3 FlgL
PA1088[3]		4.5	hypothetical protein
PA1089[3]		3.7	conserved hypothetical protein
PA1091		2.9	hypothetical protein
PA1092[3]	*fliC*	5.2	flagellin type B
PA1093[3]		13.8	hypothetical protein
PA1094[3]	*fliD*	5.1	flagellar capping protein FliD
PA1095[3]		4.2	hypothetical protein
PA1096[3]		5.4	hypothetical protein
PA1098[3]	*fleS*	2.9	two-component sensor
PA1100[3]	*fliE*	3.3	flagellar hook-basal body complex protein FliE
PA1114[3]		2.9	hypothetical protein
PA1118[3]		3.6	hypothetical protein
PA1121[3]		3.5	conserved hypothetical protein

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA1123[3]		13.0	hypothetical protein
PA1131[3]		4.0	probable MFS transporter
PA1137		4.0	probable oxidoreductase
PA1166[3]		4.7	hypothetical protein
PA1172[3]	*napC*	20.1	cytochrome c-type protein NapC
PA1173[3]	*napB*	16.5	cytochrome c-type protein NapB precursor
PA1174[3]	*napA*	23.1	periplasmic nitrate reductase protein NapA
PA1175[3]	*napD*	13.4	NapD protein of periplasmic nitrate reductase
PA1176[3]	*napF*	23.6	ferredoxin protein NapF
PA1177[3]	*napE*	22.8	periplasmic nitrate reductase protein NapE
PA1246	*aprD*	4.6	alkaline protease secretion protein AprD
PA1247	*aprE*	4.1	alkaline protease secretion protein AprE
PA1289[3]		12.3	hypothetical protein
PA1324[3]		3.4	hypothetical protein
PA1327[3]		4.4	probable protease
PA1333		2.8	hypothetical protein
PA1348[3]		3.6	hypothetical protein
PA1349[3]		3.2	conserved hypothetical protein
PA1414[3]		5.3	hypothetical protein
PA1415[3]		3.1	hypothetical protein
PA1429[3]		3.8	probable cation-transporting P-type ATPase
PA1431[3]	*rsaL*	4.4	regulatory protein RsaL
PA1441		4.5	hypothetical protein
PA1453[3]	*flhF*	3.3	flagellar biosynthesis protein FlhF
PA1457[3]	*cheZ*	4.0	chemotaxis protein CheZ
PA1458[3]		3.3	probable two-component sensor
PA1459[3]		5.7	probable methyltransferase
PA1460[3]		3.6	probable chemotaxis transmembrane proton channel
PA1463		4.0	hypothetical protein
PA1464[3]		4.0	probable purine-binding chemotaxis protein
PA1465[3]		3.9	hypothetical protein
PA1470[3]		3.6	probable short-chain dehydrogenase
PA1471		2.6	hypothetical protein
PA1473[3]		4.3	hypothetical protein
PA1474[3]		3.5	hypothetical protein
PA1521[3]		2.7	probable guanine deaminase
PA1522[3]		4.2	hypothetical protein
PA1523[3]	*xdhB*	7.4	xanthine dehydrogenase
PA1540		5.1	conserved hypothetical protein
PA1541		7.7	probable drug efflux transporter
PA1545[3]		5.1	hypothetical protein
PA1547		2.6	hypothetical protein
PA1549		3.7	probable cation-transporting P-type ATPase
PA1550[3]		3.2	hypothetical protein
PA1551[3]		3.2	probable ferredoxin
PA1555[3]		10.9	probable cytochrome c
PA1556[3]		7.0	probable cytochrome c oxidase subunit
PA1561[3]	*aer*	8.6	aerotaxis receptor Aer
PA1562[3]	*acnA*	6.8	aconitate hydratase 1
PA1601		3.3	probable aldehyde dehydrogenase
PA1641[3]		3.6	hypothetical protein
PA1656[3]		5.0	hypothetical protein
PA1679[3]		6.0	hypothetical protein
PA1728[3]		7.9	hypothetical protein
PA1729		3.4	conserved hypothetical protein
PA1731[3]		3.7	conserved hypothetical protein
PA1732[3]		9.9	conserved hypothetical protein
PA1733[3]		4.8	conserved hypothetical protein
PA1745[3]		6.6	hypothetical protein
PA1746[3]		10.4	hypothetical protein
PA1753[3]		5.5	conserved hypothetical protein
PA1760[3]		3.3	probable transcriptional regulator
PA1761[3]		4.0	hypothetical protein
PA1784[3]		13.0	hypothetical protein
PA1789[3]		3.5	hypothetical protein
PA1828		2.6	probable short-chain dehydrogenase
PA1833[3]		2.6	probable oxidoreductase
PA1860[3]		7.9	hypothetical protein
PA1869		2.6	probable acyl carrier protein
PA1871[3]	*lasA*	12.5	LasA protease precursor
PA1880[3]		4.5	probable oxidoreductase
PA1881[3]		6.2	probable oxidoreductase
PA4211[3]	*phzB2*	3.5	probable phenazine biosynthesis protein
PA1901[3]	*phzC*	27.7	phenazine biosynthesis protein PhzC

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA1905	*phzG2*	3.2	probable pyridoxamine 5'-phosphate oxidase
PA1930[3]		18.2	probable chemotaxis transducer
PA1931[3]		3.8	probable ferredoxin
PA1967[3]		2.8	hypothetical protein
PA2003	*bdhA*	4.3	3-hydroxybutyrate dehydrogenase
PA2004		2.5	conserved hypothetical protein
PA2007[3]	*maiA*	13.0	maleylacetoacetate isomerase
PA2008[3]	*fahA*	8.5	fumarylacetoacetase
PA2009[3]	*hmgA*	5.4	homogentisate 1,2-dioxygenase
PA2011		2.9	hydroxymethylglutaryl-CoA lyase
PA2013		2.8	probable enoyl-CoA hydratase/isomerase
PA2014		3.8	probable acyl-CoA carboxyltransferase beta chain
PA2015		2.8	probable acyl-CoA dehydrogenase
PA2016		3.8	probable transcriptional regulator
PA2017		2.6	hypothetical protein
PA2024[3]		19.5	probable ring-cleaving dioxygenase
PA2067[3]		3.1	probable hydrolase
PA2071[3]	*fusA2*	4.9	elongation factor G
PA2075		3.6	hypothetical protein
PA2119[3]		3.6	alcohol dehydrogenase (Zn-dependent)
PA2126[3]		4.0	conserved hypothetical protein
PA2137[3]		8.8	hypothetical protein
PA2146[3]		4.8	conserved hypothetical protein
PA2174[3]		8.6	hypothetical protein
PA2183[3]		12.3	hypothetical protein
PA2190[3]		4.5	conserved hypothetical protein
PA2193[3]	*hcnA*	4.8	hydrogen cyanide synthase HcnA
PA2194[3]	*hcnB*	10.6	hydrogen cyanide synthase HcnB
PA2195[3]	*hcnC*	18.4	hydrogen cyanide synthase HcnC
PA2197		3.3	conserved hypothetical protein
PA2231[3]		3.2	probable glycosyl transferase
PA2247[3]	*bkdA1*	5.4	2-oxoisovalerate dehydrogenase (alpha subunit)
PA2248[3]	*bkdA2*	6.7	2-oxoisovalerate dehydrogenase (beta subunit)
PA2249[3]	*bkdB*	6.1	branched-chain alpha-keto acid dehydrog. (lipoamide comp.)
PA2250[3]	*lpdV*	5.1	lipoamide dehydrogenase-Val
PA2345		2.4	conserved hypothetical protein
PA2364[3]		4.3	hypothetical protein
PA2365[3]		4.6	conserved hypothetical protein
PA2375[3]		16.6	hypothetical protein
PA2381[3]		6.9	hypothetical protein
PA2414[3]		11.7	L-sorbosone dehydrogenase
PA2423[3]		4.3	hypothetical protein
PA2433[3]		4.0	hypothetical protein
PA2504[3]		7.9	hypothetical protein
PA2550[3]		5.1	probable acyl-CoA dehydrogenase
PA2552		10.8	probable acyl-CoA dehydrogenase
PA2553		19.0	probable acyl-CoA thiolase
PA2554		8.9	probable short-chain dehydrogenase
PA2555		4.3	probable AMP-binding enzyme
PA2561		2.7	probable chemotaxis transducer
PA2562[3]		7.4	hypothetical protein
PA2567[3]		4.0	hypothetical protein
PA2571[3]		17.4	probable two-component sensor
PA2572[3]		10.4	probable two-component response regulator
PA2573[3]		14.4	probable chemotaxis transducer
PA2588[3]		4.1	probable transcriptional regulator
PA2591[3]		3.0	probable transcriptional regulator
PA2592[3]		2.8	probable periplasmic spermidine/putrescine-binding protein
PA2618[3]		5.7	hypothetical protein
PA2633[3]		3.5	hypothetical protein
PA2654[3]		5.0	probable chemotaxis transducer
PA2662[3]		104.1	conserved hypothetical protein
PA2663[3]		35.2	hypothetical protein
PA2664[3]	*fhp*	22.4	Flavohemoprotein
PA2722[3]		3.2	hypothetical protein
PA2746[3]		16.8	hypothetical protein
PA2753[3]		4.0	hypothetical protein
PA2754[3]		3.7	conserved hypothetical protein
PA2759		3.8	hypothetical protein
PA2771[3]		2.9	conserved hypothetical protein
PA2778[3]		3.7	hypothetical protein
PA2779[3]		9.3	hypothetical protein
PA2788[3]		28.0	probable chemotaxis transducer
PA2790		3.3	hypothetical protein

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA2814[3]		3.0	hypothetical protein
PA2815[3]		4.1	probable acyl-CoA dehydrogenase
PA2841		3.5	probable enoyl-CoA hydratase/isomerase
PA2920[3]		10.0	probable chemotaxis transducer
PA2937[3]		22.1	hypothetical protein
PA2939[3]		3.8	probable aminopeptidase
PA3017[3]		4.2	conserved hypothetical protein
PA3032[3]	*snr1*	2.7	cytochrome c Snr1
PA3040[3]		6.3	conserved hypothetical protein
PA3041[3]		9.7	hypothetical protein
PA3042[3]		6.4	hypothetical protein
PA3049[3]	*rmf*	5.9	ribosome modulation factor
PA3089[3]		9.1	hypothetical protein
PA3103[3]	*xcpR*	2.8	general secretion pathway protein E
PA3104[3]	*xcpP*	3.0	secretion protein XcpP
PA3105	*xcpQ*	2.8	general secretion pathway protein D
PA3119		2.5	conserved hypothetical protein
PA3123[3]		2.4	conserved hypothetical protein
PA3195	*gapA*	3.5	glyceraldehyde 3-phosphate dehydrogenase
PA3216[3]		3.7	hypothetical protein
PA3225[3]		3.9	probable transcriptional regulator
PA3226[3]		3.8	probable hydrolase
PA3228[3]		2.8	probable ATP-binding/permease fusion ABC transporter
PA3277[3]		3.7	probable short-chain dehydrogenase
PA3307[3]		6.8	hypothetical protein
PA3309[3]		7.1	conserved hypothetical protein
PA3311[3]		23.0	conserved hypothetical protein
PA3325		4.9	conserved hypothetical protein
PA3326[3]		5.2	probable Clp-family ATP-dependent protease
PA3337[3]	*rfaD*	3.5	ADP-L-glycero-D-mannoheptose 6-epimerase
PA3346[3]		7.4	probable two-component response regulator
PA3347[3]		6.3	hypothetical protein
PA3348[3]		3.8	probable chemotaxis protein methyltransferase
PA3349[3]		4.6	probable chemotaxis protein
PA3351[3]		4.4	hypothetical protein
PA3352[3]		4.5	hypothetical protein
PA3353		2.8	hypothetical protein
PA3354[3]		4.0	hypothetical protein
PA3361[3]		52.4	hypothetical protein
PA3362[3]		5.5	hypothetical protein
PA3363[3]	*amiR*	6.0	aliphatic amidase regulator
PA3364[3]	*amiC*	4.2	aliphatic amidase expression-regulating protein
PA3365		4.3	probable chaperone
PA3366[3]	*amiE*	3.6	aliphatic amidase
PA3369		3.0	hypothetical protein
PA3385		4.3	hypothetical protein
PA3391[3]	*nosR*	126.8	regulatory protein NosR
PA3392[3]	*nosZ*	219.9	nitrous-oxide reductase precursor
PA3393[3]	*nosD*	56.8	NosD protein
PA3394[3]	*nosF*	54.2	NosF protein
PA3395[3]	*nosY*	39.0	NosY protein
PA3396[3]	*nosL*	59.4	NosL protein
PA3415[3]		26.8	probable dihydrolipoamide acetyltransferase
PA3416[3]		70.8	probable pyruvate dehydrogenase E1 component, beta chain
PA3417[3]		22.3	probable pyruvate dehydrogenase E1 component, alpha subunit
PA3418[3]	*ldh*	39.0	leucine dehydrogenase
PA3427[3]		4.8	probable short-chain dehydrogenases
PA3428[3]		3.1	hypothetical protein
PA3429		3.0	probable epoxide hydrolase
PA3430[3]		8.0	probable aldolase
PA3431[3]		7.1	conserved hypothetical protein
PA3451[3]		4.8	hypothetical protein
PA3461[3]		7.0	conserved hypothetical protein
PA3465[3]		6.2	conserved hypothetical protein
PA3477[3]	*rhlR*	4.9	transcriptional regulator RhlR
PA3478[3]	*rhlB*	16.8	rhamnosyltransferase chain B
PA3479[3]	*rhlA*	20.3	rhamnosyltransferase chain A
PA3520[3]		16.1	hypothetical protein
PA3526[3]		7.0	probable outer membrane protein precursor
PA3572[3]		3.3	hypothetical protein
PA3576[3]		4.8	hypothetical protein
PA3581	*glpF*	2.6	glycerol uptake facilitator protein
PA3582[3]	*glpK*	3.6	glycerol kinase
PA3584[3]	*glpD*	3.5	glycerol-3-phosphate dehydrogenase

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA3600[3]		10.5	conserved hypothetical protein
PA3613[3]		4.9	hypothetical protein
PA3614[3]		3.0	hypothetical protein
PA3662		3.4	hypothetical protein
PA3688[3]		9.0	hypothetical protein
PA3691[3]		4.5	hypothetical protein
PA3692[3]		2.8	probable outer membrane protein precursor
PA3704	*wspE*	4.2	probable chemotaxis sensor/effector fusion protein
PA3708[3]		2.4	probable chemotaxis transducer
PA3712[3]		4.1	hypothetical protein
PA3723[3]		16.7	probable FMN oxidoreductase
PA3724[3]	*lasB*	7.1	elastase LasB
PA3784[3]		3.0	hypothetical protein
PA3785[3]		2.7	conserved hypothetical protein
PA3790	*oprC*	2.8	outer membrane protein OprC
PA3796[3]		3.0	hypothetical protein
PA3819[3]		3.3	conserved hypothetical protein
PA3844[3]		2.4	hypothetical protein
PA3846[3]		3.5	hypothetical protein
PA3872[3]	*narI*	12.9	respiratory nitrate reductase gamma chain
PA3873[3]	*narJ*	15.8	respiratory nitrate reductase delta chain
PA3874[3]	*narH*	14.1	respiratory nitrate reductase beta chain
PA3875[3]	*narG*	30.8	respiratory nitrate reductase alpha chain
PA3876[3]	*narK2*	5.5	nitrite extrusion protein 2
PA3879[3]	*narL*	2.9	two-component response regulator NarL
PA3880[3]		7.5	conserved hypothetical protein
PA3881		2.9	hypothetical protein
PA3911[3]		11.6	conserved hypothetical protein
PA3912[3]		11.9	conserved hypothetical protein
PA3913[3]		6.1	probable protease
PA3919[3]		2.8	conserved hypothetical protein
PA3921[3]		3.5	probable transcriptional regulator
PA3930	*cioA*	2.6	cyanide insensitive terminal oxidase
PA3945[3]		15.1	conserved hypothetical protein
PA3957[3]		12.2	probable short-chain dehydrogenase
PA3971[3]		7.7	hypothetical protein
PA3972[3]		12.7	probable acyl-CoA dehydrogenase
PA3973[3]		12.0	probable transcriptional regulator
PA3986[3]		10.2	hypothetical protein
PA4017[3]		3.6	conserved hypothetical protein
PA4027		2.9	hypothetical protein
PA4049[3]		2.7	hypothetical protein
PA4063		3.1	hypothetical protein
PA4067[3]	*oprG*	3.2	outer membrane protein OprG precursor
PA4078[3]		6.3	probable nonribosomal peptide synthetase
PA4108[3]		4.6	hypothetical protein
PA4111		3.6	hypothetical protein
PA4112[3]		6.3	probable sensor/response regulator hybrid
PA4117[3]		6.9	probable bacteriophytochrome
PA4137		10.6	probable porin
PA4175[3]	*prpL*	3.9	probable endoproteinase Arg-C precursor
PA4181		3.0	hypothetical protein
PA4196[3]		2.5	probable two-component response regulator
PA4209[3]		3.2	probable O-methyltransferase
PA4293[3]		4.0	probable two-component sensor
PA4294[3]		5.1	hypothetical protein
PA4296[3]		5.8	probable two-component response regulator
PA4299[3]		3.8	hypothetical protein
PA4300[3]		3.3	hypothetical protein
PA4302[3]		3.1	probable type II secretion system protein
PA4303[3]		3.0	hypothetical protein
PA4304[3]		7.4	probable type II secretion system protein
PA4305[3]		5.8	hypothetical protein
PA4306[3]		6.8	hypothetical protein
PA4309[3]	*pctA*	13.7	chemotactic transducer PctA
PA4310[3]	*pctB*	3.3	chemotactic transducer PctB
PA4311[3]		9.5	conserved hypothetical protein
PA4326[3]		5.9	hypothetical protein
PA4349[3]		7.5	hypothetical protein
PA4351		3.7	probable acyltransferase
PA4357[3]		5.4	conserved hypothetical protein
PA4358[3]		7.4	probable ferrous iron transport protein
PA4359[3]		5.0	conserved hypothetical protein
PA4362[3]		3.3	hypothetical protein

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA4364		14.0	hypothetical protein
PA4365		16.1	probable transporter
PA4472[3]	*pmbA*	4.0	PmbA protein
PA4474		10.4	conserved hypothetical protein
PA4493		2.6	probable two-component response regulator
PA4506[3]		2.4	probable ATP-binding component of ABC dipeptide transporter
PA4520		3.9	probable chemotaxis transducer
PA4523[3]		10.4	hypothetical protein
PA4573[3]		14.8	hypothetical protein
PA4575[3]		2.8	hypothetical protein
PA4577[3]		7.3	hypothetical protein
PA4587[3]	*ccpR*	9.7	cytochrome c551 peroxidase precursor
PA4596[3]		10.9	probable transcriptional regulator
PA4607[3]		13.1	hypothetical protein
PA4608[3]		5.5	hypothetical protein
PA4610[3]		13.1	hypothetical protein
PA4620		3.5	hypothetical protein
PA4621		2.5	probable oxidoreductase
PA4623		5.9	hypothetical protein
PA4633[3]		11.8	probable chemotaxis transducer
PA4641[3]		6.4	still frameshift hypothetical protein
PA4648[3]		3.6	hypothetical protein
PA4657[3]		5.6	hypothetical protein
PA4677[3]		3.3	hypothetical protein
PA4702[3]		5.6	hypothetical protein
PA4703[3]		8.2	hypothetical protein
PA4713[3]		3.1	hypothetical protein
PA4714[3]		2.9	conserved hypothetical protein
PA4717[3]		2.5	conserved hypothetical protein
PA4738[3]		3.3	conserved hypothetical protein
PA4739[3]		4.6	conserved hypothetical protein
PA4778[3]		4.4	probable transcriptional regulator
PA4781[3]		6.7	probable two-component response regulator
PA4809	*fdhE*	2.6	FdhE protein
PA4810[3]	*fdnI*	4.8	nitrate-inducible formate dehydrogenase, gamma subunit
PA4811[3]	*fdnH*	6.8	nitrate-inducible formate dehydrogenase, beta subunit
PA4812[3]	*fdnG*	4.6	formate dehydrogenase-O, major subunit
PA4825	*mgtA*	4.9	Mg(2+) transport ATPase, P-type 2
PA4843		2.6	probable two-component response regulator
PA4876[3]	*osmE*	3.0	osmotically inducible lipoprotein OsmE
PA4878		3.5	probable transcriptional regulator
PA4880[3]		3.5	probable bacterioferritin
PA4915[3]		15.7	probable chemotaxis transducer
PA4916[3]		7.1	hypothetical protein
PA4917[3]		4.2	hypothetical protein
PA4925[3]		3.6	conserved hypothetical protein
PA4929[3]		7.4	hypothetical protein
PA5020[3]		2.5	probable acyl-CoA dehydrogenase
PA5027[3]		3.4	hypothetical protein
PA5052		2.6	hypothetical protein
PA5056[3]	*phaC1*	3.1	poly(3-hydroxyalkanoic acid) synthase 1
PA5057[3]	*phaD*	3.0	poly(3-hydroxyalkanoic acid) depolymerase
PA5058[3]	*phaC2*	5.2	poly(3-hydroxyalkanoic acid) synthase 2
PA5060[3]	*phaF*	3.2	polyhydroxyalkanoate synthesis protein PhaF
PA5091[3]	*hutG*	3.2	N-formylglutamate amidohydrolase
PA5101[3]		5.5	hypothetical protein
PA5170[3]	*arcD*	4.3	arginine/ornithine antiporter
PA5171[3]	*arcA*	30.9	arginine deiminase
PA5172[3]	*arcB*	78.3	ornithine carbamoyltransferase, catabolic
PA5173[3]	*arcC*	104.6	carbamate kinase
PA5213[3]	*gcvP1*	2.7	glycine cleavage system protein P1
PA5220[3]		2.4	hypothetical protein
PA5230[3]		13.1	probable permease of ABC transporter
PA5231[3]		4.6	probable ATP-binding/permease fusion ABC transporter
PA5232		3.6	conserved hypothetical protein
PA5242	*ppk*	3.2	polyphosphate kinase
PA5272	*cyaA*	2.5	adenylate cyclase
PA5359[3]		9.4	hypothetical protein
PA5374	*betI*	2.6	transcriptional regulator BetI
PA5395[3]		4.2	conserved hypothetical protein
PA5424[3]		3.8	conserved hypothetical protein
PA5427[3]	*adhA*	8.9	alcohol dehydrogenase
PA5435[3]		10.5	probable transcarboxylase subunit
PA5436[3]		8.8	probable biotin carboxylase subunit of a transcarboxylase

ORF[1]	Gene name	Fold change[2]	Protein description
Genes upregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA5446		2.9	hypothetical protein
PA5460[3]		6.9	hypothetical protein
PA5475[3]		5.9	hypothetical protein
PA5482[3]		4.6	hypothetical protein
PA5495	*thrB*	3.1	homoserine kinase
PA5496[3]		3.6	hypothetical protein
PA5497[3]		3.8	hypothetical protein
PA5498		3.3	probable adhesin
PA5499[3]	*np20*	6.1	transcriptional regulator np20
PA5500	*znuC*	3.0	zinc transport protein ZnuC
PA5531	*tonB*	5.1	TonB protein
Genes downregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA0046[3]		5.2	hypothetical protein
PA0047[3]		3.6	hypothetical protein
PA0069[3]		4.1	conserved hypothetical protein
PA0070[3]		6.8	hypothetical protein
PA0076		17.2	hypothetical protein
PA0077		3.6	hypothetical protein
PA0078[3]		5.1	hypothetical protein
PA0080		4.6	hypothetical protein
PA0082[3]		3.0	hypothetical protein
PA0083[3]		10.5	conserved hypothetical protein
PA0084[3]		8.1	conserved hypothetical protein
PA0085[3]		10.9	conserved hypothetical protein
PA0087[3]		13.0	hypothetical protein
PA0089[3]		9.9	hypothetical protein
PA0090[3]		4.7	probable ClpA/B-type chaperone
PA0091		3.3	conserved hypothetical protein
PA0093[3]		6.9	hypothetical protein
PA0094		3.9	hypothetical protein
PA0126		4.1	hypothetical protein
PA0140[3]	*ahpF*	25.7	alkyl hydroperoxide reductase subunit F
PA0165[3]		3.5	hypothetical protein
PA0167[3]		3.8	probable transcriptional regulator
PA0169[3]		15.1	hypothetical protein
PA0170[3]		8.2	hypothetical protein
PA0171[3]		12.6	hypothetical protein
PA0172[3]		2.8	hypothetical protein
PA0201[3]		5.3	hypothetical protein
PA0263	*hcpC*	4.8	secreted protein Hcp
PA0277		4.9	conserved hypothetical protein
PA0280[3]	*cysA*	13.7	sulfate transport protein CysA
PA0281[3]	*cysW*	29.9	sulfate transport protein CysW
PA0282[3]	*cysT*	9.4	sulfate transport protein CysT
PA0283[3]	*sbp*	5.5	sulfate-binding protein precursor
PA0284[3]		9.9	hypothetical protein
PA0293[3]	*aguA*	2.7	probable hydratase
PA0320		2.5	conserved hypothetical protein
PA0341[3]	*lgt*	3.5	prolipoprotein diacylglyceryl transferase
PA0350[3]	*folA*	3.2	dihydrofolate reductase
PA0363[3]	*coaD*	5.0	phosphopantetheine adenylyltransferase
PA0389[3]		2.7	hypothetical protein
PA0390[3]	*metX*	3.3	homoserine O-acetyltransferase
PA0421[3]		2.8	hypothetical protein
PA0449		2.7	hypothetical protein
PA0456		2.6	probable cold-shock protein
PA0461		2.4	conserved hypothetical protein
PA0538[3]	*dsbB*	14.2	disulfide bond formation protein
PA0563[3]		5.3	conserved hypothetical protein
PA0579[3]	*rpsU*	2.9	30S ribosomal protein S21
PA0582[3]	*folB*	2.4	dihydroneopterin aldolase
PA0593[3]	*pdxA*	3.7	pyridoxal phosphate biosynthetic protein PdxA
PA0594[3]	*surA*	3.0	peptidyl-prolyl cis-trans isomerase SurA
PA0603[3]		6.5	probable ATP-binding component of ABC transporter
PA0604[3]		4.9	probable binding protein component of ABC transporter
PA0605[3]		8.1	probable permease of ABC transporter
PA0606[3]		7.5	probable permease of ABC transporter
PA0610[3]	*prtN*	4.2	transcriptional regulator PrtN
PA0611[3]	*prtR*	3.6	transcriptional regulator PrtR
PA0612[3]		19.1	hypothetical protein
PA0613[3]		21.9	hypothetical protein
PA0614[3]		15.0	hypothetical protein
PA0615[3]		5.2	hypothetical protein
PA0616[3]		6.8	hypothetical protein

ORF[1]	Gene name	Fold change[2]	Protein description
Genes downregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA0617[3]		45.0	probable bacteriophage protein
PA0618[3]		6.6	probable bacteriophage protein
PA0619[3]		6.4	probable bacteriophage protein
PA0622[3]		14.0	probable bacteriophage protein
PA0623[3]		7.6	probable bacteriophage protein
PA0624[3]		8.8	hypothetical protein
PA0625[3]		13.8	hypothetical protein
PA0626[3]		6.0	hypothetical protein
PA0627[3]		9.3	conserved hypothetical protein
PA0628[3]		11.9	conserved hypothetical protein
PA0629[3]		12.2	conserved hypothetical protein
PA0630[3]		6.6	hypothetical protein
PA0631[3]		48.3	hypothetical protein
PA0654[3]	*sped*	7.4	S-adenosylmethionine decarboxylase proenzyme
PA0663[3]		2.6	hypothetical protein
PA0670[3]		3.6	hypothetical protein
PA0671[3]		2.7	hypothetical protein
PA0750[3]	*ung*	4.8	uracil-DNA glycosylase
PA0782[3]	*putA*	2.4	proline dehydrogenase PutA
PA0783[3]	*putP*	3.2	sodium/proline symporter PutP
PA0789[3]		3.5	probable amino acid permease
PA0802		2.7	hypothetical protein
PA0848[3]		17.3	probable alkyl hydroperoxide reductase
PA0849[3]	*trxB2*	13.2	thioredoxin reductase 2
PA0904[3]	*lysC*	4.8	aspartate kinase alpha and beta chain
PA0921[3]		2.6	hypothetical protein
PA0922[3]		5.6	hypothetical protein
PA0937[3]		3.0	conserved hypothetical protein
PA0961[3]		5.3	probable cold-shock protein
PA0964		2.6	conserved hypothetical protein
PA0968[3]		3.0	conserved hypothetical protein
PA0969[3]	*tolQ*	2.4	TolQ protein
PA0970	*tolR*	2.8	TolR protein
PA0975[3]		4.6	probable radical activating enzyme
PA0976[3]		8.0	conserved hypothetical protein
PA0979		2.5	conserved hypothetical protein
PA0985		88.9	pyocin S5
PA0996	*pqsA*	2.8	probable coenzyme A ligase
PA1006[3]		3.9	conserved hypothetical protein
PA1009[3]		3.9	hypothetical protein
PA1013[3]	*purC*	2.6	phosphoribosylaminoimidazole-succinocarboxamide synthase
PA1034		3.7	hypothetical protein
PA1035		3.7	hypothetical protein
PA1159		6.4	probable cold-shock protein
PA1183[3]	*dctA*	4.5	C4-dicarboxylate transport protein
PA1192		2.6	conserved hypothetical protein
PA1228[3]		17.0	hypothetical protein
PA1271[3]		2.7	probable tonB-dependent receptor
PA1274[3]		3.2	conserved hypothetical protein
PA1275[3]	*cobD*	2.9	cobalamin biosynthetic protein CobD
PA1276[3]	*cobC*	3.6	cobalamin biosynthetic protein CobC
PA1277[3]		4.8	cobyric acid synthase
PA1278[3]	*cobP*	3.0	cobinamide kinase
PA1281	*cobV*	3.0	cobalamin (5'-phosphate) synthase
PA1288[3]		3.0	probable outer membrane protein precursor
PA1293[3]		2.5	hypothetical protein
PA1295[3]		3.1	conserved hypothetical protein
PA1317[3]	*cyoA*	3.9	cytochrome o ubiquinol oxidase subunit II
PA1318[3]	*cyoB*	3.7	cytochrome o ubiquinol oxidase subunit I
PA1319[3]	*cyoC*	4.3	cytochrome o ubiquinol oxidase subunit III
PA1320[3]	*cyoD*	6.4	cytochrome o ubiquinol oxidase subunit IV
PA1321[3]	*cyoE*	2.6	cytochrome o ubiquinol oxidase protein CyoE
PA1377[3]		2.4	conserved hypothetical protein
PA1395		10.4	hypothetical protein
PA1466[3]		11.2	hypothetical protein
PA1493[3]	*cysP*	5.0	sulfate-binding protein of ABC transporter
PA1504[3]		4.5	probable transcriptional regulator
PA1533[3]		2.6	conserved hypothetical protein
PA1534[3]	*recR*	2.7	recombination protein RecR
PA1554[3]		2.6	probable cytochrome oxidase subunit (cbb3-type)
PA1719[3]	*pscF*	3.8	type III export protein PscF
PA1750[3]		3.9	phospho-2-dehydro-3-deoxyheptonate aldolase
PA1756	*cysH*	2.7	3'-phosphoadenosine-5'-phosphosulfate reductase
PA1757[3]	*thrH*	3.2	homoserine kinase

ORF[1]	Gene name	Fold change[2]	Protein description
Genes downregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA1766[3]		2.6	hypothetical protein
PA1767[3]		3.3	hypothetical protein
PA1771[3]		12.7	probable esterase/lipase
PA1790		13.5	hypothetical protein
PA1791		2.6	hypothetical protein
PA1792[3]		2.8	conserved hypothetical protein
PA1793	*ppiB*	4.2	peptidyl-prolyl cis-trans isomerase B
PA1805[3]	*ppiD*	2.8	peptidyl-prolyl cis-trans isomerase D
PA1806[3]	*fabI*	2.8	NADH-dependent enoyl-ACP reductase
PA1811[3]		5.9	probable solute-binding protein
PA1812[3]	*mltD*	2.7	membrane-bound lytic murein transglycosylase D precursor
PA1837[3]		7.0	hypothetical protein
PA1838[3]	*cysI*	5.0	sulfite reductase
PA1859		4.7	probable transcriptional regulator
PA1971[3]	*braZ*	4.2	branched chain amino acid transporter BraZ
PA2023[3]	*galU*	2.8	UTP--glucose-1-phosphate uridylyltransferase
PA2038[3]		2.5	hypothetical protein
PA2042[3]		2.6	probable transporter (membrane subunit)
PA2204[3]		21.2	probable binding protein component of ABC transporter
PA2230		3.0	hypothetical protein
PA2252[3]		3.1	probable AGCS sodium/alanine/glycine symporter
PA2253[3]	*ansA*	3.2	L-asparaginase I
PA2279	*arsC*	3.2	ArsC protein
PA2288[3]		2.9	hypothetical protein
PA2321		2.7	gluconokinase
PA2322[3]		5.0	gluconate permease
PA2327[3]		5.4	probable permease of ABC transporter
PA2328[3]		3.8	hypothetical protein
PA2329[3]		4.3	probable ATP-binding component of ABC transporter
PA2330[3]		2.8	hypothetical protein
PA2331[3]		9.2	hypothetical protein
PA2359		8.0	probable transcriptional regulator
PA2384[3]		2.7	hypothetical protein
PA2385[3]	*pvdQ*	2.6	probable acylase
PA2386[3]	*pvdA*	5.8	L-ornithine N5-oxygenase
PA2394[3]	*pvdN*	2.4	probable aminotransferase
PA2409[3]		5.6	probable permease of ABC transporter
PA2412[3]		13.2	conserved hypothetical protein
PA2441[3]		5.0	hypothetical protein
PA2453		4.3	hypothetical protein
PA2536		2.9	probable phosphatidate cytidylyltransferase
PA2539		9.3	conserved hypothetical protein
PA2542		2.7	conserved hypothetical protein
PA2543		7.1	conserved hypothetical protein
PA2545[3]	*xthA*	2.9	exodeoxyribonuclease III
PA2579		2.4	hypothetical protein
PA2581		4.1	hypothetical protein
PA2619	*infA*	2.7	initiation factor
PA2660[3]		2.4	hypothetical protein
PA2667		9.3	conserved hypothetical protein
PA2748[3]		3.3	probable methionine aminopeptidase
PA2757[3]		4.0	hypothetical protein
PA2760[3]		4.4	probable outer membrane protein precursor
PA2800		3.6	conserved hypothetical protein
PA2828[3]		3.1	probable aminotransferase
PA2843[3]		3.1	probable aldolase
PA2850[3]	*ohr*	3.5	organic hydroperoxide resistance protein
PA2851[3]	*efp*	4.1	translation elongation factor P
PA2876[3]	*pyrF*	2.8	orotidine 5'-phosphate decarboxylase
PA2905	*cobH*	2.6	precorrin isomerase CobH
PA2911[3]		11.4	probable TonB-dependent receptor
PA2929		4.5	hypothetical protein
PA2950[3]		3.8	hypothetical protein
PA2953		2.5	electron transfer flavoprotein-ubiquinone oxidoreductase
PA2957		3.5	probable transcriptional regulator
PA2966	*acpP*	3.1	acyl carrier protein
PA2970[3]	*rpmF*	2.5	50S ribosomal protein L32
PA2983		2.4	probable tolQ-type transport protein
PA2986[3]		3.0	conserved hypothetical protein
PA2994[3]	*nqrF*	2.8	Na+-translocating NADH:quinone oxidoreductase, subunit Nqr6
PA3007[3]	*lexA*	5.7	repressor protein LexA
PA3008[3]		4.2	hypothetical protein
PA3046[3]		2.4	conserved hypothetical protein
PA3134[3]	*gltX*	2.5	glutamyl-tRNA synthetase

ORF[1]	Gene name	Fold change[2]	Protein description
Genes downregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA3181		3.2	2-keto-3-deoxy-6-phosphogluconate aldolase
PA3237[3]		67.1	hypothetical protein
PA3243[3]	*minC*	4.7	cell division inhibitor MinC
PA3245[3]	*mine*	4.5	cell division topological specificity factor MinE
PA3262[3]		3.6	probable peptidyl-prolyl cis-trans isomerase, FkbP-type
PA3263[3]		3.4	conserved hypothetical protein
PA3266[3]	*capB*	3.1	cold acclimation protein B
PA3268[3]		2.8	probable TonB-dependent receptor
PA3280[3]	*oprO*	18.6	outer membrane porin OprO precursor
PA3284[3]		4.3	hypothetical protein
PA3287[3]		11.2	conserved hypothetical protein
PA3295[3]		3.2	probable HIT family protein
PA3313[3]		3.3	hypothetical protein
PA3397[3]	*fpr*	2.9	ferredoxin--NADP+ reductase
PA3402		3.9	hypothetical protein
PA3410		6.0	probable sigma-70 factor, ECF subfamily
PA3413[3]		3.8	conserved hypothetical protein
PA3414[3]		2.8	hypothetical protein
PA3438	*folE1*	2.4	GTP cyclohydrolase I precursor
PA3450[3]		4.8	probable antioxidant protein
PA3452[3]	*mqoA*	4.4	malate:quinone oxidoreductase
PA3480[3]		2.6	probable deoxycytidine triphosphate deaminase
PA3496		3.9	hypothetical protein
PA3539[3]		3.9	conserved hypothetical protein
PA3610[3]	*potD*	5.3	polyamine transport protein PotD
PA3611		2.5	hypothetical protein
PA3612		2.4	conserved hypothetical protein
PA3616[3]		3.4	conserved hypothetical protein
PA3620[3]	*mutS*	2.8	DNA mismatch repair protein MutS
PA3621	*fdxA*	4.2	ferredoxin I
PA3641[3]		5.0	probable amino acid permease
PA3642[3]	*rnhB*	4.9	ribonuclease HII
PA3644[3]	*lpxA*	2.4	UDP-N-acetylglucosamine acyltransferase
PA3645	*fabZ*	3.2	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase
PA3646[3]	*lpxD*	2.7	UDP-3-O-[3-hydroxylauroyl] glucosamine N-acyltransferase
PA3655[3]	*tsf*	3.2	elongation factor Ts
PA3675[3]		2.7	hypothetical protein
PA3686[3]	*adk*	2.9	adenylate kinase
PA3713[3]		3.9	hypothetical protein
PA3728[3]		4.2	hypothetical protein
PA3729[3]		3.5	conserved hypothetical protein
PA3730[3]		5.7	hypothetical protein
PA3731		2.7	conserved hypothetical protein
PA3732[3]		3.9	conserved hypothetical protein
PA3735[3]	*thrC*	2.7	threonine synthase
PA3737[3]	*dsbC*	2.8	thiol:disulfide interchange protein DsbC
PA3743[3]	*trmD*	2.5	tRNA (guanine-N1)-methyltransferase
PA3746[3]	*ffh*	2.8	signal recognition particle protein Ffh
PA3747[3]		2.8	conserved hypothetical protein
PA3798		3.3	probable aminotransferase
PA3820[3]	*secF*	3.1	secretion protein SecF
PA3821[3]	*secD*	2.4	secretion protein SecD
PA3822[3]		4.2	conserved hypothetical protein
PA3887[3]	*nhaP*	6.6	Na+/H+ antiporter NhaP
PA3931[3]		2.8	conserved hypothetical protein
PA3940		2.9	probable DNA binding protein
PA3967[3]		5.0	hypothetical protein
PA3984[3]	*int*	3.2	apolipoprotein N-acyltransferase
PA4002[3]	*rodA*	3.4	rod shape-determining protein
PA4031[3]	*ppa*	3.2	inorganic pyrophosphatase
PA4035[3]		3.4	hypothetical protein
PA4042[3]	*xseB*	3.9	exodeoxyribonuclease VII small subunit
PA4133[3]		4.2	cytochrome c oxidase subunit (cbb3-type)
PA4139		2.8	hypothetical protein
PA4140[3]		4.9	hypothetical protein
PA4218[3]		5.9	probable transporter
PA4219[3]		15.6	hypothetical protein
PA4220[3]		75.4	hypothetical protein
PA4221[3]	*fptA*	28.7	Fe(III)-pyochelin receptor precursor
PA4223[3]		7.4	probable ATP-binding component of ABC transporter
PA4224[3]	*pchG*	8.4	pyochelin biosynthetic protein PchG
PA4225[3]	*pchF*	9.8	pyochelin synthetase
PA4226[3]	*pchE*	35.9	dihydroaeruginoic acid synthetase
PA4228[3]	*pchD*	44.2	pyochelin biosynthesis protein PchD

ORF[1]	Gene name	Fold change[2]	Protein description
Genes downregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA4229[3]	*pchC*	20.6	pyochelin biosynthetic protein PchC
PA4230[3]	*pchB*	23.5	salicylate biosynthesis protein PchB
PA4231[3]	*pchA*	10.6	salicylate biosynthesis isochorismate synthase
PA4255[3]	*rpmC*	3.3	50S ribosomal protein L29
PA4271[3]	*rplL*	2.5	50S ribosomal protein L7 / L12
PA4314[3]	*purU1*	3.4	formyltetrahydrofolate deformylase
PA4317[3]		7.8	hypothetical protein
PA4318[3]		4.0	hypothetical protein
PA4319[3]		4.1	conserved hypothetical protein
PA4320[3]		2.7	hypothetical protein
PA4321[3]		2.9	hypothetical protein
PA4354[3]		6.2	conserved hypothetical protein
PA4390[3]		2.6	hypothetical protein
PA4428[3]	*sspA*	2.9	stringent starvation protein A
PA4432[3]	*rpsI*	2.8	30S ribosomal protein S9
PA4442[3]	*cysN*	11.9	ATP sulfurylase GTP-binding subunit/APS kinase
PA4443[3]	*cysD*	14.3	ATP sulfurylase small subunit
PA4455[3]		3.7	probable permease of ABC transporter
PA4456[3]		3.0	probable ATP-binding component of ABC transporter
PA4482	*gatC*	5.5	Glu-tRNA(Gln) amidotransferase subunit C
PA4512[3]	*lpxO1*	2.5	lipopolysaccharide biosynthetic protein LpxO1
PA4545	*comL*	3.1	competence protein ComL
PA4569[3]	*ispB*	3.2	octaprenyl-diphosphate synthase
PA4574[3]		2.5	conserved hypothetical protein
PA4602[3]	*glyA3*	2.8	serine hydroxymethyltransferase
PA4612[3]		3.3	conserved hypothetical protein
PA4613[3]	*katB*	65.8	catalase
PA4625		3.9	hypothetical protein
PA4628[3]	*lysP*	2.8	lysine-specific permease
PA4629[3]		4.3	hypothetical protein
PA4632[3]		4.2	hypothetical protein
PA4636[3]		2.8	hypothetical protein
PA4637[3]		3.4	hypothetical protein
PA4642[3]		6.4	hypothetical protein
PA4645[3]		3.6	probable purine/pyrimidine phosphoribosyl transferase
PA4668[3]		2.8	probable lipoprotein localization protein LolB
PA4669[3]	*ipk*	2.7	isopentenyl monophosphate kinase
PA4671[3]		2.6	probable ribosomal protein L25
PA4672[3]		3.1	peptidyl-tRNA hydrolase
PA4675[3]		2.9	probable TonB-dependent receptor
PA4685[3]		2.7	hypothetical protein
PA4693[3]	*pssA*	2.8	phosphatidylserine synthase
PA4695	*ilvH*	3.1	acetolactate synthase isozyme III small subunit
PA4723[3]	*dksA*	3.4	suppressor protein DksA
PA4729[3]	*panB*	2.5	3-methyl-2-oxobutanoate hydroxymethyltransferase
PA4730[3]	*panC*	2.6	pantoate--beta-alanine ligase
PA4741[3]	*rpsO*	3.2	30S ribosomal protein S15
PA4747	*secG*	3.1	secretion protein SecG
PA4763[3]	*recN*	2.7	DNA repair protein RecN
PA4770[3]	*lldP*	3.9	L-lactate permease
PA4801[3]		4.2	hypothetical protein
PA4846[3]	*aroQ1*	2.9	3-dehydroquinate dehydratase
PA4851[3]		2.5	hypothetical protein
PA4854[3]	*purH*	2.4	phosphoribosylaminoimidazolecarboxamide formyltransferase
PA4923[3]		3.1	conserved hypothetical protein
PA4933[3]		2.8	hypothetical protein
PA4967[3]	*parE*	2.9	topoisomerase IV subunit B
PA5001[3]		2.6	hypothetical protein
PA5021[3]		3.5	probable sodium/hydrogen antiporter
PA5024[3]		18.5	conserved hypothetical protein
PA5046[3]		2.7	malic enzyme
PA5049[3]	*rpmE*	8.9	50S ribosomal protein L31
PA5074[3]		2.8	probable ATP-binding component of ABC transporter
PA5075[3]		2.7	probable permease of ABC transporter
PA5076[3]		4.4	probable binding protein component of ABC transporter
PA5117[3]	*typA*	4.1	regulatory protein TypA
PA5121		2.5	hypothetical protein
PA5131	*pgm*	3.0	phosphoglycerate mutase
PA5136		8.4	hypothetical protein
PA5138		4.7	hypothetical protein
PA5154		3.1	probable permease of ABC transporter
PA5157[3]		3.4	probable transcriptional regulator
PA5181[3]		3.6	probable oxidoreductase
PA5192[3]	*pckA*	2.9	phosphoenolpyruvate carboxykinase

ORF[1]	Gene name	Fold change[2]	Protein description
Genes downregulated in the *icsF* mutant grown in the presence of H_2O_2			
PA5194[3]		5.4	hypothetical protein
PA5202[3]		4.9	hypothetical protein
PA5215	*gcvT1*	2.5	glycine-cleavage system protein T1
PA5240	*trxA*	2.6	thioredoxin
PA5250[3]		3.2	conserved hypothetical protein
PA5285		2.5	hypothetical protein
PA5286[3]		3.6	conserved hypothetical protein
PA5298[3]		3.3	xanthine phosphoribosyltransferase
PA5300	*cycB*	2.6	cytochrome c5
PA5308[3]	*lrp*	2.6	leucine-responsive regulatory protein
PA5315	*rpmG*	2.6	50S ribosomal protein L33
PA5351	*rubA1*	10.1	rubredoxin
PA5366	*pstB*	3.0	ATP-binding component of ABC phosphate transporter
PA5370[3]		3.7	probable MFS transporter
PA5402[3]		4.2	hypothetical protein
PA5403[3]		2.8	probable transcriptional regulator
PA5404[3]		8.0	hypothetical protein
PA5406[3]		3.0	hypothetical protein
PA5407[3]		3.3	hypothetical protein
PA5414[3]		3.1	hypothetical protein
PA5425[3]	*purK*	2.4	phosphoribosylaminoimidazole carboxylase
PA5441		3.6	hypothetical protein
PA5445[3]		3.1	probable coenzyme A transferase
PA5470		2.9	probable peptide chain release factor
PA5505		3.7	probable TonB-dependent receptor
PA5530[3]		14.5	probable MFS dicarboxylate transporter
PA5549[3]	*glmS*	4.4	glucosamine--fructose-6-phosphate aminotransferase
PA5550[3]		3.4	probable transcriptional regulator
PA5553[3]	*atpC*	3.8	ATP synthase epsilon chain
PA5560[3]	*atpB*	2.7	ATP synthase A chain
PA5564	*gidB*	2.7	glucose inhibited division protein B
PA5568[3]		3.0	conserved hypothetical protein

[1] PA numbers are from *Pseudomonas* genome project (www.pseudomonas.com).

[2] Numbers represent the arithmetic average of four independent GeneChip comparisons.

[3] Oxidative stress response genes.

Curriculum vitae

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Nationality	Slovak

Educational qualifications:

1983-1991	Primary school, Partizanske
1991-1995	Grammar school (A levels), Partizanske
1996-1999	B. Sc., Comenius University, Bratislava
1999-2001	M. Sc. (Diploma with Honours), Comenius University, Bratislava M. Sc. thesis: "Detection of the DNA damage and repair after influence of gamma-ray, UV radiation and Methylmethanesulphonate using PFGE in *Saccharomyces cerevisiae* and *Chlamydomonas reinhardtii*" Department of Molecular Genetics, Cancer Research Institute, Bratislava
2001-	Ph. D. in Biochemistry, University of Hannover Ph. D. thesis: "Global virulence regulators of *Pseudomonas aeruginosa*" Klinische Forschergruppe, Medizinische Hochschule Hannover, supervised by Prof. Dr. Dr. Burkhard Tümmler

Others:

1995-1996	Military services

Publications:

1. **Juhas, M.,** Wiehlmann, L., Huber, B., Jordan, D., Lauber, J., Salunkhe, P., Limpert, A., von Götz, F., Steinmetz I., Eberl, L. and Tümmler, B. (2004). Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. *Microbiology* (150): 831-841.

   Comments:

   a) New regulator linking quorum sensing and iron uptake in *Pseudomonas aeruginosa*. *Microbiology* (2004) 150: 752-756 2004.

   b) Control freak. *Nature Rev Micro* (2004) 2:356.

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