Functional genome analysis of the intracellular lifestyle of *Pseudomonas aeruginosa* in phagocytes

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Abstract

The objectives of the present study were to identify genes that enable the opportunistic pathogen *P. aeruginosa* TB to survive intracellularly in polymorphonuclear granulocytes (PMNs). A signature-tagged mutant (STM) library made up of 2304 mutants was screened for intracellular survival in PMNs. Out of the identified 44 mutants, 35 mutants which have reduced survival significantly, while 9 mutants which survived even better as compared to the TB wild type. The genes identified encode elements of oxidative stress response, flagella and type IV pili biogenesis. Most of them are belong to the class of conserved hypotheticals of unknown function. Almost all genes that are needed by TB are present in the sequenced PAO1 core genome, only very few are strain-or clone-specific. This indicates that subtle shifts in the genetic repertoire are sufficient to cause a smooth transition from extracellular lifestyle to intracellular survival and growth in the major antipseudomonal host defense cell.

The genome wide transcriptional profile of *P. aeruginosa* against paraquat was performed using PAO1 GeneChips which uncovered one operon (PA0939–PA0942) encoding four proteins of previously unknown function being 20–200 fold up-regulated in all three analyzed *P. aeruginosa* strains TB, 892 and PAO1. It confers most resistance to paraquat-induced superoxide stress in addition to constitutively highly expressed genes *sodB*, *katA* and *ahpC.* The transcriptional profile of the three *P. aeruginosa* strains against hydrogen peroxide stress identified significantly more number of genes up-regulated in TB as compared to 892 and PAO1.

The GeneChip experiments with the *P. aeruginosa* TB mutants harboring transposon insertions in strain specific genes revealed that there were virtually no interference with the expression of the PAO1 defined core genome under standard growth conditions. Murine infection experiments indicated that the analyzed mutants are even more virulent than the TB wild type.

The transcriptome data together with murine infection model described in this study could be useful for experiments to dissect the intracellular survival mechanisms in *P. aeruginosa* TB and the biochemical function of the strain specific genes.

Key-words: *Pseudomonas aeruginosa*, Polymorphonuclear granulocytes, GeneChip

Zusammenfassung

Das Ziel dieser Arbeit war die Identifizierung von Genen, die es dem opportunistischen Pathogen *P. aeruginosa* TB ermöglichen, intrazellulär in polymorphonukleären Granulozyten (PMNs) zu überleben. 2304 Transposonmutanten einer STM- Bibliothek wurden auf ihre intrazelluläre Überlebensfähigkeit in PMNs untersucht. Hierbei wurden 44 Mutanten mit deutlichen Veränderungen des Phänotyps identifiziert. 35 hatten eine deutlich verringerte intrazelluläre Überlebensfähigkeit, wogegen 9 Mutanten sogar besser überlebten als der TB-Wildtyp. Die Funktionen der identifizierten Gene beinhalten die Abwehr von oxidativem Stress, den Aufbau von Flagellen und Typ IV Pili. Die meisten der identifizierten Gene haben aber bisher eine unbekannte Funktion. Es war auffällig, daß die meisten Gene, die von *P. aeruginosa* TB zum intrazellulären Überleben benötigt werden, aus dem sequenzierten PAO1 – Genom bekannt sind und nur sehr wenige Gene Stamm- oder Klon- spezifisch sind. Dies ist ein Hinweis darauf, daß nur geringste Veränderungen im genetischen Hintergrund ausreichend sind, um einen Übergang von einer extrazellulären Lebensweise zu einer intrazellulären auszulösen, bei der die *P. aeruginosa* Bakterien sogar in der Lage sind, sich in der wichtigsten Abwehrzelle des Menschen gegenüber *Pseudomonas*-Infektionen zu vermehren.

In PAO1 GeneChips wurde das genomweite Transkriptionsprofil von *P. aeruginosa* nach Exposition mit Paraquat analysiert. Ein Operon aus vier Genen (PA0939−PA0942) mit bisher unbekannter Funktion wurde in den drei analysierten Stämmen TB, 892 und TB 20-200fach stärker exprimiert. Es verleiht neben den konstitutiv hoch exprimierten Gene *sodB*, *katA* und *ahpC* den größten Anteil an Resistenz gegen Paraquat-induzierten Superoxid Stress. Das transkriptionelle Profil der drei *P. aeruginosa* Stämme während Wasserstoffperoxid-induziertem Stress zeigte signifikant mehr hochregulierte Gene bei TB als bei 892 und PAO1.

GeneChip Experimente mit zwei *P. aeruginosa* TB Mutanten, die Transposon-Insertionen in non-PAO1 Genen beherbergen, zeigten praktisch keine Interferenz mit der globalen Expression des PAO1 Kerngenoms unter Standard-Wachstumsbedingungen. Die Produkte dieser beiden Gene schützen *P. aeruginosa* TB vor dem bakteriziden Zugriff durch PMNs,, attenuieren aber gleichzeitig die Virulenz im Mäuseinfektionsversuch.

Die Transkriptom-Daten können zusammen mit den in dieser Studie beschriebenen Mausinfektions-Versuchen nützlich für gezielte Experimente sein, um die intrazellulären Überlebensmechanismen von *P. aeruginosa* TB sowie die biochemischen Funktionen der Stamm-spezifischen Gene aufzuklären.

Schlüsselwörter: *Pseudomonas aeruginosa*, polymorphnukleäre Granulozyten, GeneChip

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1. INTRODUCTION

1.1. Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative polarly flagellated rod which is a member of the γ-proteobacteria group (Olsen *et al.*, 1994), that is noted for its environmental versatility. Unlike many environmental bacteria, *P. aeruginosa* has a remarkable capacity to cause disease in susceptible hosts. It has the ability to adapt to and thrive in many ecological niches, from water and soil to plant and animal tissues (Hardalo and Edberg, 1997). The bacterium is capable of utilizing a wide range of organic compounds as food sources, thus giving it an exceptional ability to colonize ecological niches where nutrients are limited. *P. aeruginosa* can produce a number of toxic proteins which not only cause extensive tissue damage, but also interfere with the human immune system's defense mechanisms. These proteins range from potent toxins that enter and kill host cells at or near the site of colonization to degradative enzymes that permanently disrupt the cell membranes and connective tissues in various organs. 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).

Recently, Stover *et al.* (2000) has sequenced and published the complete genome of *P. aeruginosa* strain PAO1 (available on http://pseudomonas.com). The *P. aeruginosa* genome is markedly larger (6.3 million base pairs) than most of the 140 sequenced bacterial genomes. In fact, with 5,570 predicted open reading frames (ORFs), the genetic complexity of *P. aeruginosa* approaches 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). Consistent with its larger genome size and environmental adaptability, *P. aeruginosa* contains the highest proportion of regulatory genes observed for a bacterial genome and a large number of genes involved in the catabolism, transport and efflux of organic compounds as well as four potential chemotaxis systems (Stover *et al.*, 2000). These regulatory genes presumably modulate the diverse genetic and biochemical capabilities of this bacterium in changing environmental conditions.

1.1.1. *P. aeruginosa* and cystic fibrosis

P. aeruginosa typically causes disease only in individuals with impaired host defenses and is thus referred to as an opportunistic pathogen. Such compromised individuals include patients undergoing immunosuppressive therapies (e.g., cancer treatment), those receiving treatment for traumatic skin damage (burn wounds), those with human immunodeficiency virus infections, and those with cystic fibrosis (Govan and Deretic, 1996). CF arises from mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which interferes with chloride ion transport in CF patients. The gene defect results in a myriad of medical problems for the patient, the most prominent clinical feature being chronic pulmonary infection with *P. aeruginosa*, allows the basic pathologic process in CF to be designated as an infectious disease. Ultimately, 80 to 95 % of patients with CF succumb to respiratory failure brought on by chronic bacterial infection and concomitant airway inflammation. The discovery in 1989 of the genetic defect causing CF sparked an explosion of research efforts, which have led to a greater understanding of the molecular mechanisms underlying the various phenotypic manifestations of the disease. Yet the breadth of the link between mutant forms of the CF gene product, CFTR, and chronic bacterial respiratory infections, particularly by *P. aeruginosa*, remains elusive. Deciphering this link is critical, since this infection and the ensuing inflammation accounts for the majority of the morbidity and mortality in the disease. Chronic *P. aeruginosa* airway infection and the accompanying inflammatory response are clearly the major clinical problems for CF patients today. While antibiotic chemotherapy and chemoprophylaxis have reduced the morbidity and early mortality of CF patients from this infection, the intrinsic ability of *P. aeruginosa* to develop resistance to many commonly used antibiotics (Kobayashi *et al.*, 1972; Richmond *et al.*, 1971) probably underlies the

inability to eradicate *P. aeruginosa* from the CF patient's lung and ultimately allows this microbe to be highly problematic for these patients .

The abnormal composition of the airway secretions of the CF lung is frequently cited as the host factor that predisposes CF patients to chronic colonization by *P. aeruginosa*. One of the most striking and clinically important features of infection by *P. aeruginosa* is the tendency of this bacterium to change to a mucoid phenotype, probably initiating the chronic-infection stage of the disease. The mucoid phenotype results from bacterial production of a polysaccharide known as both alginate and mucoid exopolysaccharide (MEP) and plays an important role in bacterial evasion of the host immune response. The mucoid phenotype is often unstable, with a large percentage of isolates reverting to a nonmucoid phenotype during in vitro culture (Pugashetti *et al.*, 1982). This instability has been genetically mapped to changes in the one of the gene known as *mucA* (Boucher *et al.*, 1997). Production of MucA protein inhibits the activity of the alternative sigma factor (sigma E), which is required for the mucoid phenotype. Mutations in the *mucA* gene leading to a premature termination of the coding sequence are found in mucoid isolates (Schurr *et al.*, 1994). Accompanying the onset of alginate production is the initiation of the microcolony mode of growth within the lungs of CF patients, representing a bacterial biofilm composed of cells embedded within an alginate matrix (Costerton *et al.*, 1983). With the emergence of mucoid *P. aeruginosa* within the lungs, there are cell surface changes with respect to LPS phenotype (Fomsgaard *et al.*, 1988).

1.1.2. Pathogenesis of *P. aeruginosa* infections

P. aeruginosa is able to grow and survive in almost any environment, lives primarily in water, soil, and vegetation. It produces several cell associated and extracellular virulence factors (Van Delden and Iglewski, 1998).

1.1.2.1. Role of cell-associated virulence factors

To initiate infection, *P. aeruginosa* usually requires a substantial break in first-line defenses. Such a break can result from breach or bypass of normal cutaneous or mucosal barriers (e.g., trauma, surgery, serious burns, or indwelling devices), disruption of the protective balance of normal mucosal flora by broad-spectrum

antibiotics, or alteration of the immunological defense mechanisms (e.g., in chemotherapy-induced neutropenia, mucosal clearance defects from cystic fibrosis, AIDS, and diabetes mellitus). The first step in *P. aeruginosa* infections is the colonization of the altered epithelium. The pathogen colonizes the oropharynx of up to 6 % and is recovered from the feces of 3 % to 24 % of healthy persons (Pollack *et al.*, 1995). In contrast, up to 50 % of hospitalized patients are at high risk for *P. aeruginosa* colonization (Pollack *et al.*, 1995). Adherence of *P. aeruginosa* to epithelium is probably mediated by type 4 pili similar to those of *Neisseria gonorrhoeae* (de Bentzmann *et al.*, 1996). Several other nonpilus adhesins responsible for the binding to mucin have been described, but their role in the infection process remains unclear (Gilboa-Garber, 1996). Flagella, which are primarily responsible for motility, may also act as adhesins to epithelial cells (Feldman *et al.*, 1998). 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). Biofilm formation can take place on a variety of surfaces, such as medical instruments, leading to many types of nosocomial infections, and *P. aeruginosa* has been shown to persist in biofilm in the lungs of cystic fibrosis patients (Singh *et al.*, 2000). Biofilms are characterized by a complex, highly structured, bacterial organization (Costerton *et al.*, 1999). They are initiated by the attachment of a single planktonic cell on a surface. The chaperone-usher pathway has been identified in *P. aeruginosa* as one of the mechanisms that are involved in the attachment to the abiotic surfaces (Vallet *et al.*, 2001). The gene cluster was named as cup gene cluster. This suggest that multiple factors are available to *P. aeruginosa* to facilitate its binding to various surfaces and for interbacterial adhesion as well. The wide variety of such attachment mechanisms may reflect the complex needs of this organism during the colonization of widely diverse environmental niches.

1.1.2.2. Role of extracellular virulence factors

P. aeruginosa produces several extracellular products that after colonization can cause extensive tissue damage, bloodstream invasion, and dissemination. In vivo studies have shown that mutants defective in the production of exotoxin A, exoenzyme S, elastase, or alkaline protease are essential for minimum virulence of

P. aeruginosa; however, the relative contribution of a given factor may vary with the type of infection (Nicas and Iglewski, 1985). Many of these factors are controlled by regulatory systems involving cell-to-cell signaling. Exotoxin A is produced by most *P. aeruginosa* strains that cause clinical infections. Exotoxin A is responsible for local tissue damage, bacterial invasion (Lyczak *et al.*, 2000), and (possibly) immunosuppression (Vidal *et al.*, 1993). Purified exotoxin A is highly lethal for mice which supports its role as a major systemic virulence factor of *P. aeruginosa*. Exoenzyme S is responsible for direct tissue destruction in lung infection (Nicas *et al.*, 1985a) and may be important for bacterial dissemination (Nicas *et al.*, 1985b). Two hemolysins, phospholipase C and rhamnolipid, produced by *P. aeruginosa,* may act synergistically to breakdown lipids and lecithin. Both may contribute to tissue invasion by their cytotoxic effects. Rhamnolipid, a rhamnose-containing glycolipid biosurfactant, has a detergentlike structure and is believed to solubilize the phospholipids of lung surfactant, making them more accessible to cleavage by phospholipase C (Liu, 1974). Rhamnolipid also inhibits the mucociliary transport and ciliary function of human respiratory epithelium (Read *et al.*, 1992). Proteases are assumed to play a major role during acute *P. aeruginosa* infection. *P. aeruginosa* produces several proteases including LasB elastase, LasA elastase, and alkaline protease (Passador *et al.*, 1993). The ability of *P. aeruginosa* to destroy the protein elastin is a major virulence determinant during acute infection. Elastin is a major part of human lung tissue and is responsible for lung expansion and contraction. The concerted activity of two enzymes, LasB elastase and LasA elastase, is responsible for elastolytic activity (Galloway, 1991). Both LasB elastase and LasA elastase have been found in the sputum of CF patients during pulmonary exacerbation (Jaffar-Bandjee *et al.*, 1995). LasB elastase not only destroys tissue components but also interferes with host defense mechanisms (Galloway 1991; Hong and Ghebrehiwet, 1992). Studies in animal models show that mutants defective in LasB elastase production are less virulent than their parent strains (Nicas *et al.*, 1985b; Tamura *et al.*, 1992; Tang *et al.*, 1996), which supports the role of LasB elastase as a virulence factor.

1.2. Type III secretion system

Interaction of bacterial pathogens with host cells is particularly characterized by factors that are located on the bacterial surface or are secreted into the extracellular space. Although the secreted bacterial proteins are numerous and diverse and exhibit a wide variety of functions that include proteolysis, haemolysis, cytotoxicity, and protein phosphorylation and dephosphorylation, only a few pathways exist by which these proteins are transported from the bacterial cytoplasm to the extracellular space. Thus, four pathways of protein secretion (types I to IV) have been described in Gram-negative bacteria (Fath and Kolter, 1993; Finlay and Falkow, 1997; Salmond and Reeves, 1993). Type I, exemplified by the hemolysin secretion system of *Escherichia coli*, is a rather simple exporter that is based on only three proteins, one of which belongs to the ABC transporters. Type II is a very complex apparatus that extends the general secretary pathway and transfers fully folded enzymes or toxins from the periplasm to the extracellular medium, across the outer membrane. Type IV, another complex system that transfers pertussis toxin among others, is related to the apparatus of *Agrobacterium* spp. that transfers DNA to plant cells. Finally, type III, is a sophisticated apparatus that couples secretion with pathogenesis.

In bacteria that are pathogenic for animals, type III secretion systems allow extracellular bacteria adhering to the surface of a host cell to inject specialized proteins across the plasma membrane. This system probably also allows bacteria residing in vacuoles to inject proteins across the vacuolar membrane. The injected proteins subvert the functioning of the aggressed cell or destroy its communications, favoring the entry or survival of the invading bacteria. Type III secretion has been extensively studied in *Yersinia* spp. (Cornelis *et al.*, 1998), in *Salmonella* spp. (Galan and Collmer, 1999; Hensel *et al.*, 1997), in *Shigella* spp. (Van Nhieu *et al.*, 1997), and in enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (Frankel *et al.*, 1998; Goosney *et al.*, 1999; Jarvis *et al.*, 1995). It has also been described in *P. aeruginosa* (Yahr *et al.*, 1998), *Chlamydia trachomatis* and *Chlamydia pneumoniae* (Subtil *et al.*, 2000), *Bordetella bronchiseptica* (Yuk *et al.*, 1998), *Bordetella pertussis* (Kerr *et al.*, 1999) and in *Burkholderia pseudomallei* (Rainbow *et al.*, 2002). It is surprising that *Salmonella typhimurium* and *Yersinia* spp. have not only one type III system but two (Hensel *et al.*, 1998; Ochman *et al.*, 1996), presumably playing their role at different stages of the infection (figure 1.1).

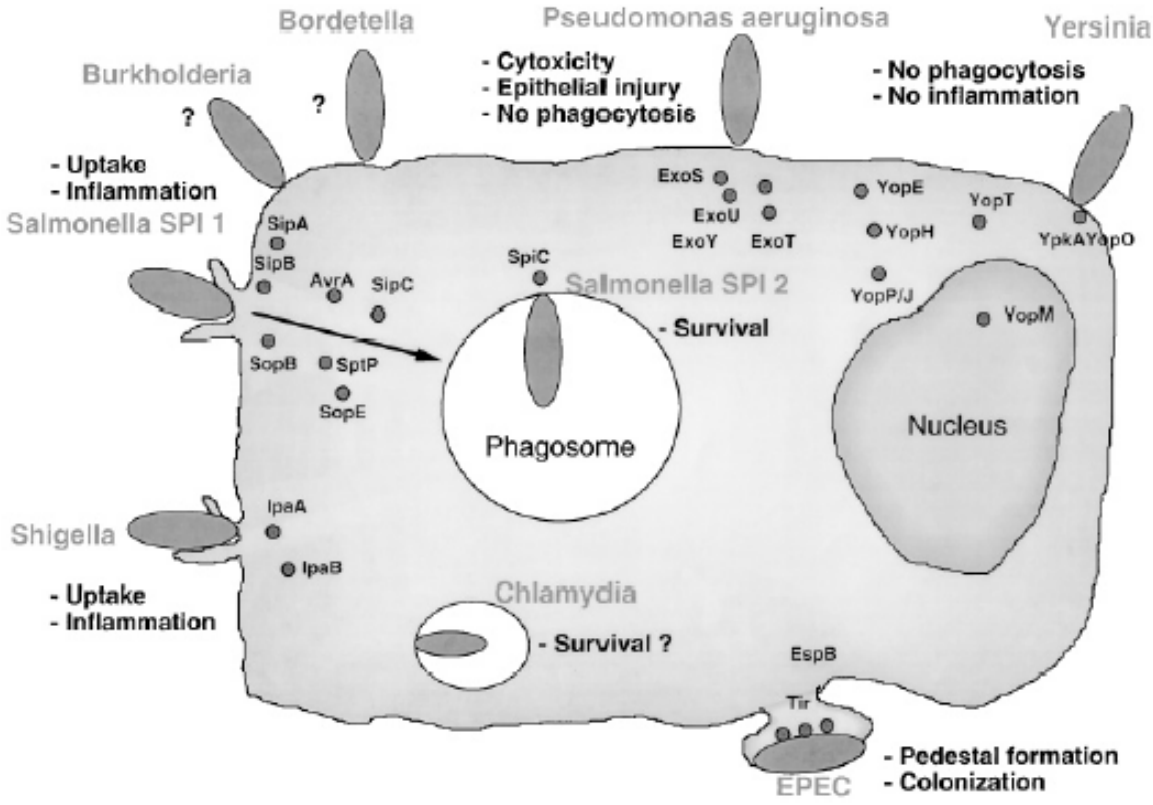


Fig. 1.1. Type III systems in animal pathogens. Illustrated are the various bacterial pathogens endowed with type III secretion, injecting effectors into the cytosol of a eukaryotic target cell (Cornelis and Gijsegem, 2000).

1.2.1. Proteins secreted by *P. aeruginosa* type III pathway

As mentioned above, *P. aeruginosa* secretes several proteins with high similarity to *Yersinia* type III secreted factors. The two related ADP-ribosyltransferases ExoS (453 aa) and ExoT (457 aa), which have 75% aa sequence identity, both carry an amino-terminal half similar to YopE, while the catalytic domain of the proteins resides in the carboxy terminus (Frank, 1997). Another secreted factor which is associated with epithelial cell damage is the 687−aa ExoU (Finck-Barbancon *et al.*, 1997). ExoU is coregulated with other type III secreted proteins and is highly similar in its first 6 aa to ExoS and ExoT, suggesting that ExoU might also be secreted via the type III secretion pathway. Furthermore, proteins which show 40 to 60% sequence identity to *Yersinia* LcrV (PcrV, 294 aa), YopB (PopB, 392 aa), YopD (PopD, 295 aa), and YopN (PopN, 288 aa) are secreted by *P. aeruginosa* (Yahr *et al.*, 1997) and may be involved in protein translocation by *P. aeruginosa* as their homologs are in *Yersinia* spp (Hueck, 1998).

1.2.2. *P. aeruginosa* type III secretion system genes

The chromosomal type III secretion system in *P. aeruginosa* has been discovered by characterization of ExoS secretion-deficient mutants. The part of the secretory apparatus which has been identified so far is encoded by 12 genes, 11 of which are arranged colinear to the *Yersinia yscB* to *yscL* genes. Except for their homologs in *Yersinia* spp., the *pscB*-, *pscE*-, and *pscGH*-encoded proteins do not have homologs in other type III secretion systems. In *P. aeruginosa*, the *exsD-pscB* to *pscL* genes are transcribed as a single operon (Yahr *et al.*, 1996). The *exsD*-encoded protein is unique to *P. aeruginosa*, but the preceding operon (*exsCBA*) (Yahr and Frank, 1994) encodes homologs of *Yersinia* VirG (ExsB) and VirF (ExsA). Like the *Yersinia* VirF, the ExsA protein is a positive regulatory factor of the *exsD–pscB* to *pscL* operon (Yahr and Frank, 1994), which belongs to the AraC family of transcriptional regulators (Frank and Iglewski, 1991). Yahr *et al.* (1997) reported that transposon insertions located immediately upstream of the *exsCBA* operon also led to an ExoS secretion-deficient phenotype, and by homology to *Yersinia* spp., it is likely that this region contains additional genes of the *P. aeruginosa* type III secretion system. A number of *P. aeruginosa* type III secretion genes homologous to respective *Yersinia* sp. genes have recently been described (Frank, 1997).

1.2.3. Transcriptional regulation in *P. aeruginosa*

As mentioned above, the *P. aeruginosa* type III secretion system is highly similar to the *Yersinia* system. Accordingly, the *P. aeruginosa* type III gene cluster contains a *trans*-regulatory locus encoding an AraC-type regulator (ExsA) which exhibits 56% sequence identity to *Yersinia* VirF. ExsA activates *P. aeruginosa* type III secretion gene transcription by specifically binding to promoters upstream of *pscN*, the *popN* to *pcrR* operon, the *exsD* to *pscB* to *pscL* operon, and the *exsC* to *exsA* operon. In addition, ExsA activates transcription of genes encoding the secreted proteins ExoS, ExoT, and ExoU (Frank, 1997; Hovey and Frank, 1995). As like in *Yersinia* spp., expression of *P. aeruginosa* secreted proteins is activated under low-Ca^{2+} conditions (Frank, 1997). The ExsA binding site has been determined for several promoters and comprises the core consensus sequence TNAAAANA, located 51 or 52 bp upstream of the transcriptional start site (Hovey and Frank, 1995).

1.3. Quorum sensing in Gram-negative bacteria

1.3.1. What is quorum sensing

Bacteria were considered for a long time to exist as independent single cells rather than collectively as co-ordinate members of an organized community. Emerging evidence demonstrate that bacteria can behave not only as individual cells, under appropriate conditions when their numbers have reached a critical value (approx. 10^7 cfu/ml in liquid medium), bacteria can modify and co-ordinate their behaviour to act uniformly as a multicellular group. The phenomenon is initiated when the bacterial cell density was achieved to a threshold value (that means a quorum has been reached), then the whole bacterial population responds to the high cell density via signal molecules by modifying its behaviour collectively for the synthesis of virulence factors which could not be executed by the individual cell. Hence the population of bacterial cells exhibit a new characteristic, which is dependent on cell density. Most Gram-negative bacteria can sense, integrate and process information from the environment via intercellular communication after a critical population has been achieved, the phenomenon was originally termed “autoinduction“, now most frequently called quorum-sensing (QS), a “symphony of bacterial voices“, and it can also be named “cell to cell communication“ (Van Delden and Iglewski, 1998). The vast majority of gram-negative quorum-sensing systems that have been studied thus far utilize *N*-acyl homoserine lactones (AHL) as signaling molecules. When in high enough concentration, these molecules can bind to and activate a transcriptional activator, or R protein, which in turn induces expression of target genes (figure 1.2).

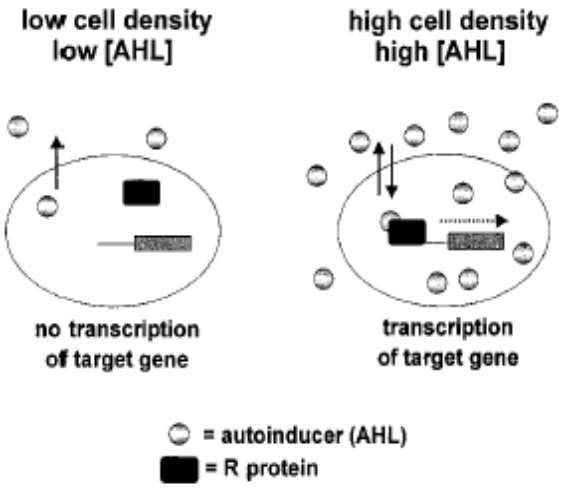


Fig.1.2. Quorum sensing in gram-negative organisms involves 2 regulatory components: the transcriptional activator protein (R protein) and the AI molecule. Accumulation of AI occurs until a threshold level is reached. Then AI binds to and activates the R protein, which in turn induces gene expression. The R protein consists of two domains: the N terminus of the protein that interacts with AI and the C terminus that is involved in DNA binding. Typically, gram-negative AI molecules are *N*-acyl-HSLs; however, other types of signal molecules do exist (Van Delden and Iglweski, 1998).

In the past decade quorum sensing circuits have been identified in over 25 species of Gram-negative bacteria (Bassler, 1999; de Kievit and Iglewski, 2000; Fuqua *et al.*, 1996; Parsek and Greenberg, 2000). In every case except those of *Vibrio harveyi* and *Myxococcus xanthus* the quorum sensing circuits identified in Gram-negative bacteria resemble the canonical quorum sensing circuit of the symbiotic bacterium *Vibrio fischeri*. Specifically, these Gram-negative bacterial quorum sensing circuits contain, at a minimum, homologues of two *V. fischeri* regulatory proteins called LuxI and LuxR. The LuxI-like proteins are responsible for the biosynthesis of a specific acylated homoserine lactone signaling molecule (HSL) known as an autoinducer. The LuxR-like proteins bind cognate HSL autoinducers that have achieved a critical threshold concentration, and the LuxR-autoinducer complexes also activate target gene transcription (Engebrecht and Silverman, 1987). Using this quorum sensing mechanism, Gram-negative bacteria can efficiently couple gene expression to fluctuations in cell-population density. Among the 25 species of bacteria that mediate quorum sensing by means of a LuxI/LuxR-type circuit, the *V. fischeri*, *P. aeruginosa*, *Agrobacterium tumefaciens*, and *Erwinia carotovora* systems are the best understood.

1.3.2. Quorum sensing systems in *P. aeruginosa*

In the opportunistic human pathogen *P. aeruginosa*, a hierarchical LuxI/LuxR circuit regulates quorum sensing. Two pairs of LuxI/LuxR homologues, LasI/LasR (Passador *et al.*, 1993) and RhlI/RhlR (Brint and Ohman, 1995), exist in *P. aeruginosa*. Both LasI and RhlI are autoinducer syntheses that catalyze the formation of the HSL autoinducers *N*-(3-oxododecanoyl)-homoserine lactone (Pearson *et al.*, 1994) and *N*-(butyryl)-homoserine lactone (Pearson *et al.*, 1995), respectively. The two regulatory circuits act in tandem to control the expression of a number of *P. aeruginosa* virulence factors. The *P. aeruginosa* quorum sensing circuit functions as follows. At high cell density, LasR binds to its cognate HSL autoinducer, and together they bind at promoter elements immediately preceding the genes encoding a number of secreted virulence factors that are responsible for host tissue destruction during initiation of the infection process. These pathogenicity determinants include elastase, encoded by *lasB*; a protease encoded by *lasA*; exotoxinA, encoded by *toxA*; and alkaline phosphatase, which is encoded by the *aprA* gene (Parsek *et al.*, 1999; Miller and Bassler, 2001). Analogous to the *V. fischeri* LuxI/LuxR circuit, LasR bound to

autoinducer also activates *lasI* expression, which establishes a positive feedback loop (Seed *et al.*, 1995). The LasR-autoinducer complex also activates the expression of the second quorum sensing system of *P. aeruginosa* (Ochsner and Reiser, 1995). Specifically, expression of *rhlR* is induced. RhlR binds the autoinducer produced by RhlI; this complex induces the expression of two genes that are also under the control of the LasI/LasR system, *lasB* and *aprA*. Additionally, the RhlR-autoinducer complex activates a second class of specific target genes. These genes include *rpoS*, which encodes the stationary phase sigma factor; *rhlAB*, which encodes rhamnosyltransferase and is involved in the synthesis of the biosurfactant/hemolysin rhamnolipid; genes involved in pyocyanin antibiotic synthesis; the *lecA* gene, which encodes a cytotoxic lectin; and the *rhlI* gene (Miller and Bassler, 2001). Again, similar to LasI/LasR and LuxI/LuxR, activation of *rhlI* establishes an autoregulatory loop. As mentioned above, the LasR-autoinducer complex activates *rhlR* expression to initiate the second signaling cascade. However, the LasR-dependent autoinducer, *N*-(3-oxododecanoyl)-homoserine lactone, also prevents the binding of the RhlI-dependent autoinducer, *N*-(butyryl)-homoserine lactone, to its cognate regulator RhlR (Pesci *et al.*, 1997). Presumably, this second level of control of RhlI/RhlR autoinduction by the LasI/LasR system ensures that the two systems initiate their cascades sequentially and in the appropriate order.

A novel, additional autoinducer has recently been demonstrated to be involved in quorum sensing in *P. aeruginosa*. This signal is noteworthy because it is not of the homoserine lactone class. Rather, it is 2-heptyl-3-hydroxy-4-quinolone (denoted PQS for *Pseudomonas* quinolone signal) (Pesci *et al.*, 1999). PQS partially controls the expression of the elastase gene *lasB* in conjunction with the Las and Rhl quorum sensing systems. The expression of PQS requires LasR, and PQS in turn induces transcription of *rhlI*. These data indicate that PQS is an additional link between the Las and Rhl circuits. The notion is that PQS initiates the Rhl cascade by allowing the production of the RhlI-directed autoinducer only after establishment of the LasI/LasR signaling cascade. Following is the model (figure 1.3) showing quorum sensing circuit and its regulation in *P. aeruginosa*.

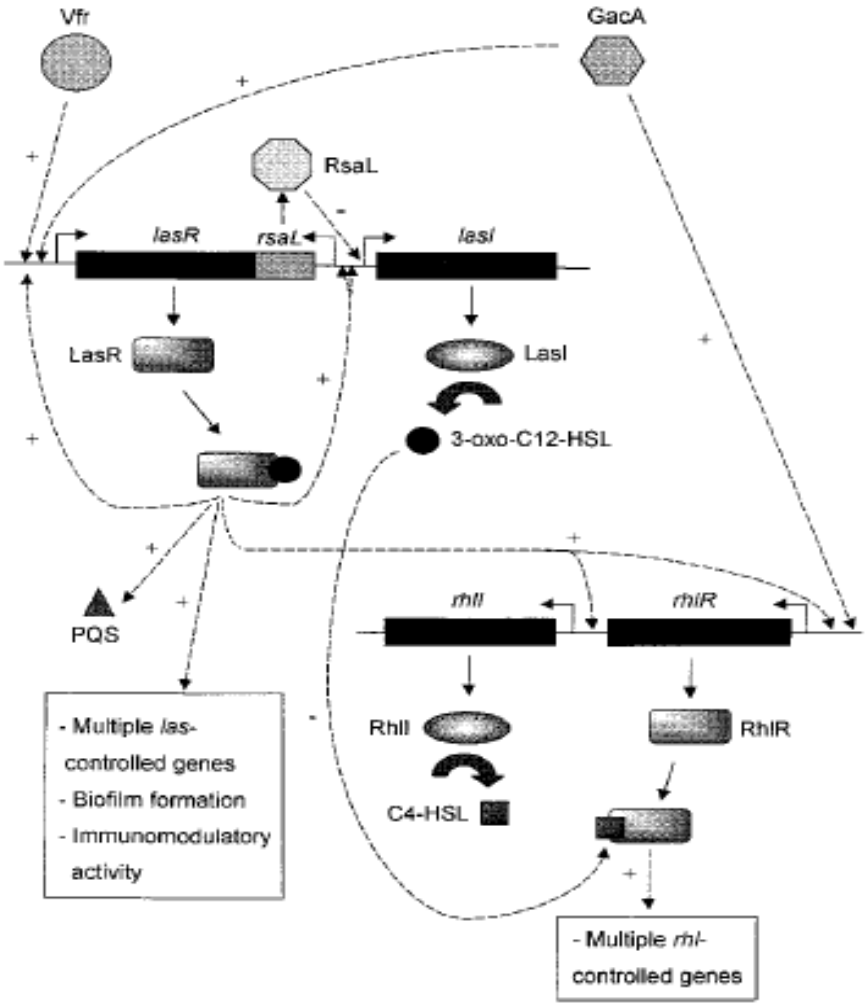


Fig. 1.3. The quorum-sensing circuitry of *P. aeruginosa* is illustrated. Expression of the *lasR* gene is subject to at least two levels of control: the global regulators Vfr and GacA and the *las* quorum-sensing system, which regulates expression of both *lasR* and *lasI*. The latter creates an autoinduction feedback loop. Regulation of the *rhl* system is similar to *las* in that GacA affects expression of *rhlR*, and the *rhlR* and *rhlI* genes are controlled to some degree by the *las* system. Interestingly, the *las* quorum-sensing system was shown to elicit an additional level of control over the *rhl* system; the *las* signal molecule, 3-oxo-C_{12}-HSL, can act posttranslationally to block RhlR activation by C_4-HSL. The *las* and *rhl* quorum-sensing systems regulate expression of numerous genes that contribute to the virulence of *P. aeruginosa*. In addition, the *las* signal molecule, 3-oxo-C_{12}-HSL, is required for biofilm differentiation and exhibits immunomodulatory activity (de Kievit and Iglewski, 2000).

1.4. Intracellular lifestyle of bacterial pathogens

The majority of bacteria (including many bacterial pathogens) that are phagocytosed by macrophages and polymorphonuclear leukocytes (PMNs) are killed. PMN-mediated phagocytosis and killing is the most important host defense mechanism against *P. aeruginosa* (Döring *et al.*, 1995). The PMN is a small cell, about 9-10 µm

in diameter, and is the most abundant leukocyte. It is referred to as a first line of defense, as it is the first defensive cell type to be recruited to a site of inflammation (König *et al.*, 1992). It is present in the bloodstream as well as attached to the epithelium, especially close to inflammation sites (Smith, 2000). Furthermore, PMN can enter tissues upon stimulation and move towards an inflammation site (Lala *et al.*, 1992). PMN possess an extensive array of antibacterial weaponary, both within its granules and by generating reactive oxygen species (Elsbach, 1998; Gudmundson and Agerberth, 1999). Prior to adherence to the target micro-organism, the phagocyte begins to consume oxygen (oxidative burst). In the phagolysosome, mainly superoxide anions are formed, which spontaneously or catalytically dismutate into hydrogen peroxide and dioxygen (Superoxide dismutase, SOD). Hydrogen peroxide is only slightly cytotoxic but due to its chemical properties can also act within the bacterium, for example inflicting damages upon the DNA after permeation of the bacterial cell wall.

However, several pathogens have devised successful strategies that enable them to survive and replicate within potentially lethal, phagocytic host cells. Successful bacterial multiplication plays the pivotal role in causation of infection and disease. Thus, many intracellular bacterial pathogens have devised strategies to grow within host cells (intracellular multiplication). Bacteria use a variety of strategies to avoid engulfment and degradation by phagocytes and facilitate proliferation and spread among host tissues (Underhill and Ozinsky, 2002). Examples are the inhibition of phagocytosis by capsule formation or toxin-mediated cellular destruction and necrosis. In contrast, induction of apoptosis avoids the release of pro-inflammatory signals (Weinrauch and Zychlinsky, 1999). Host-induced apoptosis of lung epithelial cells during infection with *P. aeruginosa* plays an important role in reducing leukocyte infiltration and maintaining the essential function of the lung: the oxygenation of blood (Grassme *et al.*, 2000). In contrast, *Salmonella* and *Shigella* both actively stimulate pro-apoptotic pathways in order to paralyze phagocytic defense: SipB from *S. enterica* Typhimurium and the similar IpaB from *S. flexneri* are translocated *via* a type III secretion apparatus into the host cytosol. These proteins bind to caspase-1, which activates downstream caspases and induces apoptosis (Hersh *et al.*, 1999; Zychlinsky *et al.*, 1994). The observation that caspase-1–deficient mice are resistant to infection with wild-type *Salmonella* suggests that this mechanism may contribute to

the pathogenesis of this bacterium (Monack *et al.*, 2000). *Yersinia enterocolitica* YopP (like its homolog *Yersinia pseudotuberculosis* YopJ) can also inhibit anti-apoptotic signals *via* the repression of NF- B activation as well as stimulation of pro-apoptotic signals through LPS-mediated activation of the TLR4 pathway (Ruckdeschel *et al.*, 2002). *Y. enterocolitica* and Y. *pseudotuberculosis* can inhibit phagocytosis by the translocation of bacterial mediators that specifically disorganize the host cell cytoskeleton preventing bacterial uptake by macrophages and polymorphonuclear leukocytes (Black and Bliska, 2000; Fallman *et al.*, 1995). Once internalization has occurred, some bacteria such as the food-borne pathogen *Listeria monocytogenes*, which is responsible for serious infections in immunocompromised individuals: manage to survive, persist and even proliferate in host phagocytes. To avoid degradation in the phagolysosome, *L. monocytogenes* is able to escape into the host cell cytosol by means of a bacterial toxin, listeriolysin, which disrupts the endosomal membrane (Dramsi and Cossart, 2002). Other pathogens such as *Salmonella* are able to manipulate endosomal trafficking and recruit defense factors to the maturing vacuole (Eriksson *et al.*, 2000). *S. enterica Typhimurium*, for example, is able to reduce the recruitment of NADPH oxidase and inducible nitric oxide synthase (iNOS) to the vacuole through interference with vacuolar trafficking, thereby preventing oxygen radical production and bacterial killing in macrophages (Uchiya *et al.*, 1999; Vazquez-Torres *et al.*, 2000; Chakravortty *et al.*, 2002). The fact that many different *Salmonella* mutants that are able to down-regulate host iNOS activity could be isolated in a screen of macrophage-adapted bacteria suggests that *Salmonella* use several strategies for interfering with the host NO response (Eriksson *et al.*, 2000). And like many other bacteria, *Salmonella* is able to detoxify oxygen radicals enzymatically (Fang *et al.*, 1999). *M. tuberculosis* inhibits phagosomal maturation by depleting H^+ ATPase molecules from the vacuolar membrane (Sturgill-Koszycki *et al.*, 1994). This leads to reduced acidification and allows intracellular survival and growth.

Although the PMN is considered to be the host's most proficient antipseudomonal weapon, at least some *P. aeruginosa* isolates seem to be able to surmount even this defense mechanism. About 20% of tested CF clinical isolates have been shown to induce the oncosis of macrophages and PMNs as indicated by cellular and nuclear swelling, disintegration of the plasma membrane, and absence of DNA fragmentation

(Dacheux *et al.*, 2000). Cell death results from a pore-forming activity that requires the intact *pcrGVHpopBD* operon of the type III secretion system (Dacheux *et al.*, 2001). A *P. aeruginosa* strain that combats PMNs by other, type III-independent mechanisms has been detected (Tümmler, 1987). The highly virulent CF clinical isolate TB (TBCF10839) is able to constitutively survive and replicate in PMNs. TBCF10839 grows in human neutrophils irrespective of whether they were isolated from patients with CF or healthy donors (Tümmler, 1987). Intracellular survival in host defense cells is the typical lifestyle of a few obligatory pathogens but has so far not been attributed to the ubiquitous *P. aeruginosa*.

1.4.1. *P. aeruginosa* TB: Survival in PMN

The *P. aeruginosa* strain TB was isolated in 1983 from the sputum of a CF patient who had been rapidly deteriorating within three years after acquisition of airways with *P. aeruginosa* and who was severely ill at the time of strain isolation (Tümmler, 1987). After strain TBCF10839 had been eradicated from the patient's airways by high-dose antipseudomonal chemotherapy the patient recovered and since then has remained in a stable state. Macrorestriction fragment pattern analysis of sequential isolates over the last 18 years verified that the patient was not re-infected by the TBCF10839 clone after this episode (Tümmler, 1987). Strain TBCF10839 was clinically the most virulent strain amongst clone TB isolates which were frequently isolated from CF patients and burnt patients treated at the Medizinische Hochschule Hannover in the 80s (Tümmler *et al.*, 1991; Kiewitz and Tümmler, 2000). Phagocytosis assays with freshly isolated PMNs uncovered the putative reason for the virulence of strain TBCF10839. 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).

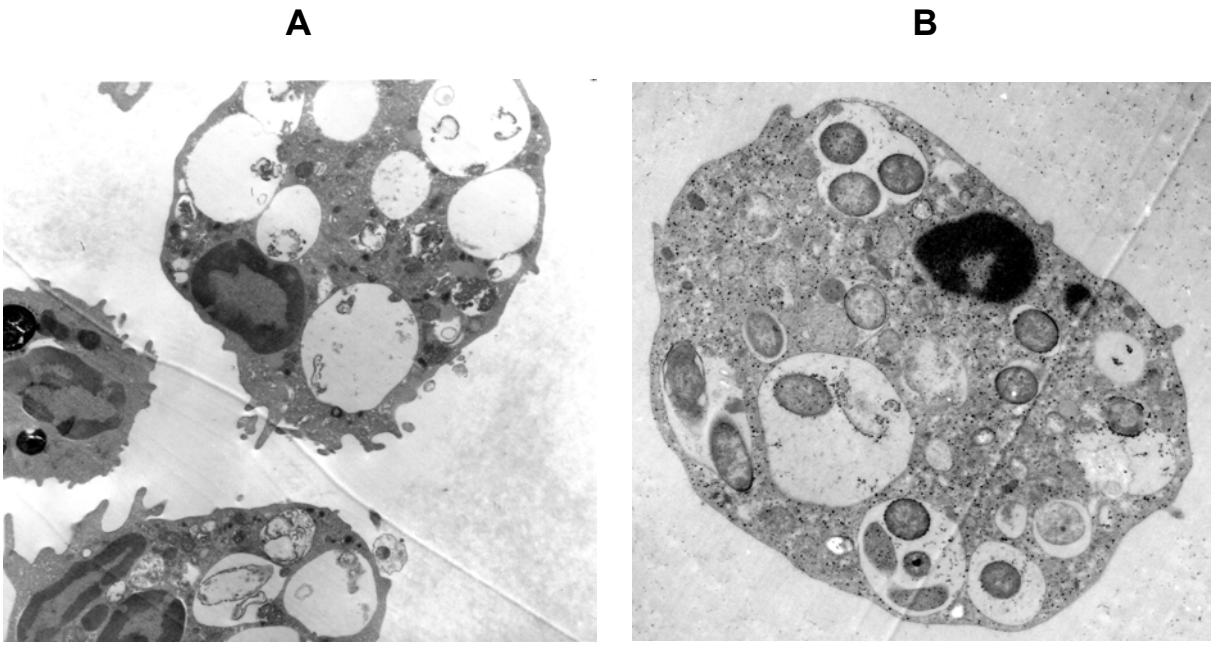


Fig. 1.4. Electron micrograph of *P. aeruginosa* (PAO1, or SG17M or C) (A) and TB (B) incubated with granulocytes. Bacteria were added at a multiplicity of infection (MOI) 20 and incubated at 37 ^{o}C for 30 min.

Electron microscopy revealed that after 30 minutes only cell debris remained from *P. aeruginosa* PAO1 in the phagolysosomes (figure 1.4A). In contrast, phagocytosed bacteria of strain TBCF10839 were still intact (figure 1.4B). Viable *P. aeruginosa* TBCF10839 and bacterial cell debris were often found in one and the same vacuole, thus implicating that the uptake of the bacteria by the PMN does not hinder the fusion of the phagosome with lysosomes (figure 1.5). TBCF10839 bacteria resided in the extracellular space and the cytosolic and lysosomal compartments of the PMNs. At higher magnification lysis of the phagolysosomal membrane was observed, thus allowing the bacteria direct access to the cytosol of the PMN. By 60 minutes and thereafter the TBCF10839 bacteria multiplied in the phagolysosomes (Wiehlmann, 2001). In summary, internalized *P. aeruginosa* TBCF10839 are far more resistant to the conditions in the PMN phagolysosome than common *P. aeruginosa* strains.

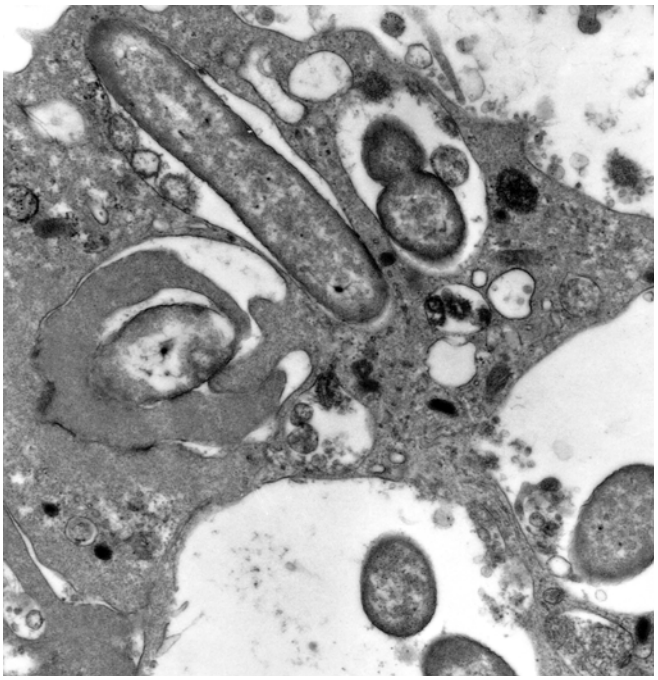


Fig. 1.5. Electron micrograph of *P. aeruginosa* TB (CF10839) incubated with granulocytes. Bacteria were added at a multiplicity of infection (MOI) 20 and incubated at 37 ^{o}C for 1 h.

Intracellular growth in PMNs is extremely rare amongst bacteria and has yet not been reported for *P. aeruginosa*, although a related type III-secretion dependent phenomenon in macrophages called oncosis has been detected in virulent clinical isolates (Dacheux *et al.*, 2001; Dacheux *et al.*, 2000). *P. aeruginosa* TBCF10839 is not only able to survive in PMN but also replicates after less than one hour. Intracellular pathogens typically undergo a lag phase, which is used for adaptation to the conditions in the phagosome as well as to eliminate the lytic processes of the phagocyting cell (Aldridge and Hughes, 2001; Ernst, 2000; Galan, 2001; Philpott *et al.*, 2001). Only after the completion of this phase, further cell divisions can be seen. *P. aeruginosa* TBCF10839 does not need such an adaptation. After 2 hours PMNs are starting to die, the bacteria are released and are growing fast in the nutrient-rich sludge. 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 micro-organism. The dominant-negative selection by signature-tagged mutagenesis (STM) (Hensel *et al.*, 1995) has been applied to unravel the etiology of this uncommon virulence trait (Wiehlmann *et al.*, 2002).

1.5. Methods for functional genome analysis

Microbial responses during the infection process cannot be discerned from in vitro studies alone. Host microenvironments are much more complex, and change as the infection proceeds due to inflammation, tissue breakdown, and immune responses of the host. Smith (1998) has distilled three implications from the fact that environmental conditions in vivo are not the same as those in vitro: (i) some candidate virulence determinants produced in vitro may not be produced during infection, (ii) some virulence determinants produced during infection may not be produced in vitro, and (iii) the repertoire of virulence determinants changes as the infection proceeds.

There are many fundamentally different methods for identifying in vivo expressed genes (Hautefort and Hinton, 2000; Heithoff *et al.*, 1997). All approaches benefit from the recent availability of microbial whole genome sequences, as often a short nucleotide sequence from any gene selected by these methods will suffice for database identification. The following methods have been used to investigate gene expression in various infections.

1.5.1. In vivo expression technology

In vivo expression technology (IVET) is a promoter trap strategy that selects bacterial promoters driving the expression of a gene required for growth within the host. Complementation in the animal demands elevated levels of gene expression compared to growth on laboratory medium (Mahan *et al.*, 1993; Mahan *et al.*, 1995). Modification of the selection/reporter system allows the study of bacterial gene expression in animals and within cultured cells. In addition, *lac* gene fusions have been used to identify co-ordinately regulated genes that are preferentially expressed during infection (Heithoff *et al.*, 1999a) as well as the regulatory parameters that govern their expression (Heithoff *et al.*, 1999b). The recombinase-based IVET system, RIVET (Camilli and Mekalanos, 1995), is designed to monitor spatial and temporal patterns of gene expression during infection. Because RIVET is independent of selection parameters such as innate auxotrophic requirements in the animal, bacterial genes can be identified that are weakly and/or transiently expressed during infection.

1.5.2. Differential fluorescence induction

Differential fluorescence induction (DFI) is a promoter trap strategy that takes advantage of the high throughput and semi-automation of fluorescence activated cell sorting (FACS) to measure intracellular fluorescence in individual bacterial cells (Cirillo *et al.*, 1998; Valdivia and Falkow, 1997). FACS can be used to identify either fluorescent bacteria or host cells containing fluorescent bacteria through the use of a green fluorescent protein (GFP) reporter. GFP facilitates real-time measurements of gene expression in vivo, including the analysis of large cell populations. Like RIVET, DFI screens are not influenced by selection parameters, and thus genes can be identified that are weakly and/or transiently expressed during infection. Moreover, DFI has identified genes encoding elusive functions involved in bacterial persistence within host granulomas (Ramakrishnan *et al.*, 2000). DFI may be best exploited in an approach that integrates high throughput screening and genomics (Strauss and Falkow, 1997).

1.5.3. Signature tagged mutagenesis

Signature tagged mutagenesis (STM) is a negative selection strategy in which an animal host is infected with a pool of sequence-tagged insertion mutants. Mutations represented in the initial inoculum but not recovered from the host are required for infection (Hensel *et al.*, 1995). The major advantage of STM is that it directly identifies genes involved in virulence rather than indirectly by in vivo expression. Like RIVET and DFI, it does not depend on selection parameters and can be used to identify genes that are expressed transiently or at low levels. STM is relatively easy to adapt to many host-pathogen systems and, unlike IVET methods, it is not dependent on preferential transcription in host tissues. Rather, its principal operating strategy is to identify functions required for survival within the host. However, mutants that are slow-growing, inviable, contain mutations in genes encoding redundant functions, or that can be complemented in a mixed population (e.g. by secreted factors), may be underrepresented.

Recently, Lehoux *et al.* (2002) adapted PCR-based signature-tagged mutagenesis to *P. aeruginosa* and a collection of 1056 mutants was screened in a chronic lung infection rat model. STM is also used successfully in identification of factors involved in virulence/colonization in many pathogenic bacteria, such as *Staphylococcus*

aureus (Coulter *et al.*, 1998; Mei *et al.*, 1997; Schwan *et al.*, 1998), *Salmonella typhimurium* (Shea *et al.*, 1996), *Vibrio cholerae* (Chiang and Mekalanos, 1998), *Neisseria meningitidis* (Claus *et al.*, 1998), *Streptococcus pneumoniae* (Polissi *et al.*, 1998), *Legionella pneumophila* (Edelstein *et al.*, 1999), *Yersinia enterocolitica* (Darwin and Miller, 1999), *Proteus mirabilis* (Zhao *et al.*, 1999), *Mycobacterium tuberculosis* (Camacho *et al.*, 1999) *Brucella abortus* (Hong *et al.*, 2000), *Brucella melitensis* (Lestrate *et al.*, 2000), and *Streptococcus agalactiae* (Jones *et al.*, 2000) including *P. aeruginosa* (Lehoux *et al.*, 2002). The strength of STM is that it combines the power of insertional mutagenesis with *in vivo* negative selection of attenuated strains in an animal model. The presence or loss of each mutant from an animal model can be assessed by following the unique signature tags present on the insertional element. Thus, with STM, one can screen many different mutants in a single assay to identify those factors that are essential for intracellular survival in the host cells.

Modifications of the STM method include the use of pre-selected tagged transposons to construct the mutant library, which simplifies the screening significantly (Mei *et al*., 1997). STM screenings of a mutant library in more than one infection model have been performed (Coulter *et al*., 1998; Tsolis *et al*., 1999; Bispham *et al*., 2001; Lau *et al*., 2001). Such screenings provide valuable knowledge of whether the same virulence factors are involved in different types of infections. By use of the traditional STM methodology, screenings in more than one infection model are, however, very laborious.

1.5.4. Differential display

Differential Display (DD) is a subtractive hybridization strategy in which bacterial cDNAs from infected tissue are hybridized against a cDNA library constructed from laboratory-grown bacteria (Abu Kwaik and Pederson, 1996; Plum and Clarke-Curtiss, 1994). The resulting host-specific cDNAs are used as probes to isolate genes that are preferentially expressed in host tissues. DD can be used in pathogens lacking well-developed genetic tools, can be applied to two or more conditions simultaneously, and has the added advantage of being able to detect both up- and down-regulation of in vivo expressed genes. Disadvantages include the instability of bacterial mRNA for the construction of cDNA libraries, the low abundance of

messages from transiently expressed genes, and the difficulty in isolation of sufficient high-quality mRNA from small populations of bacteria in vivo. PCR-based modifications may allow representation of low-abundance mRNAs, but the concomitant concern is the generation of false-positives.

1.5.5. DNA microarray technology

A main characteristic of pathogenic micro-organisms is their ability to cause tissue damage and consequently disease in their host. For decades, scientists have used a wide variety of methods to study how micro-organisms interact with the host to cause damage and the mechanisms by which the host protects itself from micro-organisms. This information has been invaluable in the design of safe and effective diagnostics, therapeutics and vaccines. We are currently in the midst of an explosive increase in the availability of genomic sequence information, with 144 genomes having been fully sequenced and published, and more than 344 prokaryotic genomes are in the process of being sequenced (http://www.tigr.org/tdb/). The availability of the complete sequence of both host and pathogen is promising in developing novel insights into the host-pathogen relationship. However, extracting biological knowledge from sequence data is the essential challenge of this post-genome era. DNA microarray (GeneChip) based approaches have gained rapid acceptance in a variety of fields for studying the roles of genes in the pathogenesis of infectious disease.

The theory and background of microarray technology, as well as the technology itself, have been described in detail elsewhere (Ferea and Brown, 1999; Lipshutz *et al.*, 1999). In brief, a DNA microarray is a microscopic chequerboard representing thousands of different DNA sequences. There are several methods of producing microarrays, but the two most commonly used techniques: spotted glass slide microarray and high-density oligonucleotide array technology. 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 polymerase chain reaction (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). Both types of DNA microarrays are used to measure the relative abundance of DNA or RNA in order to compare genomes or gene expression profiles.

Microarray-based approaches have several advantages over other systems that have been used previously to study pathogens and their interaction with hosts, such as those that measure the expression of a small number of genes in individual experiments. The major advantage of microarrays is their ability to measure simultaneously the presence of tens of thousands of different nucleic acid sequences. 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.

Microarray technology has been used in variety of applications such as to study the genetic polymorphism of pathogens (Fitzgerald *et al.*, 2003; Kato-Maeda *et al*., 2001; Salama *et al*., 2000; Troesch *et al*., 1999; Hacia and Collins, 1999; Gingeras *et al*., 1998), host-pathogen interactions (Ren *et al.*, 2003; Brooks *et al.*, 2003; Ng *et al.*, 2003; Boyce *et al.*, 2002; Schoolnik, 2002; Detweiler *et al*., 2001; Eckmann *et al*., 2000; Cummings and Relman, 2000; Manger and Relman, 2000), the gene expression profiles of pathogens (Minagawa *et al.*, 2003; Lobner-Olesen, *et al.*, 2003; Schembri *et al.*, 2003; Phadtare *et al.*, 2002; de Saizieu *et al*., 2000; Eckmann *et al*., 2000; Tao *et al*., 1999) and drug target identification (Anzick and Trent, 2002; Debouck and Goodfellow, 1999; Braxton and Bedilion, 1998).

1.6. *P. aeruginosa* pathogenicity in murine infection model

A pathogenesis model in which both the pathogen and the host are genetically tractable would greatly facilitate our understanding of some of the universal mechanisms underlying host-pathogen interactions. In 1979, Cash *et al.* enmeshed different concentration of *P. aeruginosa* in agar beads and installed the inocula intratracheally into the lungs of normal rats in order to mimic the chronic *P.*

aeruginosa infection in CF. The immobilized bacteria were not cleared and proliferated within 3 days, and high dose of bacteria was still persisted in the lungs after 35 days (Cash *et al.*, 1979). The histopathologic and serologic changes were similar to what observed in the chronic lungs of CF patients, i.e. Inflammation reactions with many PMNs surrounding a bead containing bacteria and small microcolonies formed at the periphery of the bead, and strong antibodies against *P. aeruginosa* could be detected in the serum (Cash *et al.*, 1979). Since then, several chronic models of lung *P. aeruginosa* infection have been established in animal species including rat (Pedersen *et al.*, 1990), guinea pig (Pennington *et al.*, 1981), cats (Thomassen *et al.*, 1984), inbred mice (Morissette *et al.*, 1995; Stevenson *et al.*, 1995), outbreed mice (Starke *et al.*, 1987) and monkeys (Cheung *et al.*, 1992; Cheung *et al.*,1993). *P. aeruginosa* must be entrapped into immobilizing agents such as agar, agarose or seaweed alginate to limit bacterial clearance in order to achieve a chronic infection lasting for a longer period of time.

Several groups have tried to use different variants of transgenic CF mice for the study of *P. aeruginosa* lung infection since the CFTR gene was found in 1989, and first CF mouse model was generated by Snouwaert *et al.* (1992) and Clarke *et al.* (1992). Rahme *et al*. (1995) introduced a multihost system by showing that several strains of the human opportunistic pathogen *P. aeruginosa* cause disease in both plants and animals. *P. aeruginosa* virulence has been tested in several pathogenicity models such as *Arabidopsis* leaves (Rahme *et al.*, 1995), wax moth caterpillar *Galleria mellonella* (Jander *et al.*, 2000), mouse full-skin-thickness burn model (Rahme *et al.*, 1995), and nematode *Caenorhabditis elegans* (Mahajan-Miklos *et al.*, 1999).

1.7. Objectives of the present investigation

One of the challenges of the future is the functional analysis of putative ORFs identified in genome sequencing projects and their integration into regulatory networks and metabolic pathways. This thesis applies the up-to-date technology of functional genomics to unravel virulence factors and pathogenicity mechanisms of the opportunistic pathogen *P. aeruginosa*.

Phagocytosis and lysis of *P. aeruginosa* by PMNs is the most important antipseudomonal host defense mechanism in man. The supervisor's laboratory identified one of the most virulent *P. aeruginosa* strains known to date. This strain TB (TBCF10839) from the lung of a cystic fibrosis patient (Miethke, 1985) survives and multiplies both in the phagolysosomal compartment and the cytosol of polymorphonuclear granulocytes. A minitransposon Tn*5* library of non-auxotrophic isogenic mutants has been constructed in the laboratory to identify the encoded phenotypic traits that are responsible for the transition from the typical extracellular lifestyle of the ubiquitous *P. aeruginosa* to intracellular growth in the most hostile environment the bacterium can encounter. The transposon mutants carry specific single-copy oligonucleotides. These tags allow to test numerous mutants simultaneously for changes in phenotype compared to the wild type strain. Hence, multiple mutants each carrying a different signature tag can be tested for differential survival in PMNs. A previous Ph. D. student of the group constructed this so-called STM library of strain TB and screened one third of the mutants for differential survival in PMNs (Wiehlmann *et al.*, 2002).

It is the major objective of the thesis to search within the remaining pool of two-thirds of non-screened mutants for key genes that are essential for intracellular growth of *P. aeruginosa* TB and thereafter to characterize the role of the inactivated gene for virulence and lifestyle by whole-genome gene expression profiling and specific phenotypic assays. In other words, the objectives of the present investigation are to identify genes which contribute for the survival and growth of *P. aeruginosa* TB in PMNs and to understand the genetic mechanism as how *P. aeruginosa* TB can become an intracellular pathogen.

For this, the following tasks need to be accomplished:

1. Screening of the STM library for mutants with differential intracellular survival in granulocytes and quorum-sensing mediated virulence.
2. Identification of the transposon inactivated genes by sequencing.
3. Phenotypic comparison of wild type strain and isogenic mutant in various bioassays such as sensitivity towards hydrogen peroxide, cytotoxicity and quorum sensing.

4. Whole genome gene expression profiling (transcriptome) of the subset of mutants carrying the transposon in regulatory genes or novel genes that are not present in the completely sequenced genetic reference strain PAO1, under conditions mimicking differential aspects of the PMN habitat and standard growth conditions.

5. Comparison of the global transcriptional pattern of *P. aeruginosa* TB, its less virulent clonal variant 892 and the genetic reference strain PAO1 under paraquat mediated superoxide stress and hydrogen peroxide mediated oxidative stress.
6. Global impact of the identified genes on inflammation and pathogenicity by comparison of wild type TB and isogenic transposon mutants in a murine *P. aeruginosa* lung infection model.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Equipments

Balance	BP3100S	
	BP210 S	Sartorius
Centrifuge		Hettich Universal
Eppendorf centrifuge 5415C		Eppendorf
Eppendorf centrifuge 5417R		Eppendorf
Heating block DR-block DB-3		Techne
Hybridization oven 400 HY		Bachofer
Hybridization oven		Biometra
Incubator		Heraeus
Minifold I Vacuum blotter		Schleicher and Schuell
pH meter 766 Calimatic		Knick
Spectrophotometer U3000		Hitachi
Thermocycler		Landgraf
Thermomixer		Eppendorf
UV- Transilluminator		Bachofer
UV Stratalinker 1800		Stratagene
Vacuum concentrator		Bachofer
Voltage supply power pack 300		Bio-Rad

2.1.2. Consumables

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 poresize	Sartorius
Filter paper GB003	Schleicher and Schuell
Pasteurpipette	Sarstedt
Petri plates 9 cm Ø	Sarstedt
Pipette tips (1 ml, 200 μl, 10 μl)	Sarstedt
Plastic tubes (50 ml, 15 ml)	Greiner
Polaroid film 667	Polaroid
TLC plate	Merck
X-ray film	Kodak, AGFA

2.1.3. Chemicals and enzymes

Affymetrix	Enzo BioArray Terminal Labeling 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)
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 ViTeK	*Taq*-Polymerase + buffer system
Invitrogen	Bromophenol blue, Bovine serum albumin, Gentamicin, MOPS buffer, Random Primers, RNA-standard, Serva Blau G-250, SuperScript II Reverse Transcriptase, Xylene cyanol FF
Janssen, Denmark	Hypnorm
KVL, Denmark	Pentobarbital
Leo Pharma, Denmark	Heparin
Merck	Formaldehyde 35 %, Hydrogen peroxide (30 %), Liquemin (Heparin), Uranylacetate
Molecular Probes	Streptavidine-Phycoerythrine, SYBRGreen
MWG-Biotech	Oligodeoxynucleotides, Primers
New England Biolabs	DNA-ladder standards, Restriction enzymes + buffer system, T4-DNA-Ligase
Pierce Chemical	Streptavidine
Promega	Herring-Sperm DNA, RNase Inhibitor
Proton A/S, Norway	Protonal
Qiagen	Plasmid Spin Kit, Qia- Mini Prep plasmid kit, QIAquick Gel Extraction Kit, RNeasy Kit, RNase A
Roche Molecular Biochemicals	Alkaline Phosphatase, Anti-Dioxigenin AP fab fragment, DNA Labeling and Detection Kit, Klenow Polymerase I, Midazolam, RNase-free DNase I, Terminal Transferase + Buffer system, Blocking reagent
Roth	Acrylamide solution (Rotiphorese-Gel 40), Ethidium bromide, Phenol (Rotiphenol), DPEC
Schleicher & Schuell	Nitrocellulose membrane
Serva	Gentamicin
Sigma	Chelex-100 (iminodiacetic acid), Ethidium bromide, Proteinase K, paraquat, Propidium iodide, Tween 20, H_2SO_4

Tropix	CDP- Star, DEA
Vector Laboratories	Biotin anti-Streptavidine

2.1.4. Media and solutions

2.1.4.1. Media

(1) LB Medium:

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

LB-GM: LB medium with 25 μg/l Gentamicin

LB- Agar: LB medium was solidified by adding 15 g/l agar and autoclaved.

(2) 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
Casein	0.5 % (w/v)

M9- Agar: The M9 (10x) medium and agar (15 g/l water) were autoclaved separately. Casein was warmed in M9 medium and mixed with the melted agar when the temperature was about 50 ^oC.

(3) M8- Medium:

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
Aspartate	0.05 % (w/v)
Glucose	0.2 % (w/v)

M8- Agar: The M8 medium was supplemented with 0.5 % agar for the solidification.

(4) SOB:

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

after autoclaving, the following filter sterilized stock solutions were added

$MgCl_2$ (1 g/l)	10 mM
$MgSO_4$ (1.2 g/l)	10 mM

(5) SOC:

SOB + 20 mM Glucose (3.6 g/l)

(6) 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

All the components were separately sterilized and mixed with water at the above mentioned final concentration. Components A & C were autoclaved, while the component B was filter sterilized.

2.1.4.2. Solutions for DNA work

(1) 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

(2) 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

(3) 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

(4) TB Buffer:

PIPES (3 g/l)	10 mM
$CaCl_2$ (1.6 g/l)	15 mM
KCl (18.6 g/l)	250 mM

pH adjusted to 6.7 with KOH, then $MnCl_2$ (50 mM-9.58 g/l) was added, sterilized by filtration and stored at 4 ^{o}C.

(5) TE Buffer:

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

(6) 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

(7) 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

(8) Fixation solution:

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

(9) Colour solution for gel filtration:

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

(10) Blot buffer:

NaOH (16 g/l)	0.4 M

(11) Blot wash buffer:

$NaH_2PO_4 \cdot 2H_2O$ (7.8 g/l)	50 mM
pH	6.5 with NaOH

(12) Pre-hybridization buffer:

SDS	7 % (w/v)
$NaH_2PO_4 \cdot 2H_2O$ (78 g/l)	0.5 M
EDTA (0.38 g/l)	1 mM
pH	7.2
Blocking reagent	0.5 % (w/v)

Blocking reagent was dissolved by heating the solution to 50–70 ^oC.

(13) 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 + 0.5 % (w/v) Blocking reagent

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

(14) Wash solution:

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

(15) Sucrose solution:

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

(16) 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

2.1.4.3. Solutions for RNA work

(1) RNA Lysis buffer:

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

(2) 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

(3) 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

(4) RNA loading buffer:

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

(5) SSC buffer (20 x):

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

(6) 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

The Herring sperm DNA was fragmented by the ultrasound sonication treatment and then denatured (10 min at 95 ^{o}C) before adding it to the hybridization solution.

Denhardt's solution:

Ficoll (Type 400)	2 % (v/v)
Polyvinylpyrrolidine (PVP-40)	2 % (v/v)
Cattle serum albumin	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 + 0.5 % (w/v) Blocking reagent

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

(7) cDNA Reaction mixture:

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

(8) MES hybridization buffer:

MES	100 mM
NaCl (58 g/l)	1 M
EDTA (5.84)	20 mM
Tween 20	0.01 % (v/v)
(protected from light and stored at 2–8 ^{o}C)	

(9) Non-stringent buffer:

SSPE	6 x
Tween 20	0.01 % (v/v)
(filtered through a 0.2 µM filter)	

(10) Stringent wash buffer:

MES	100 mM
NaCl (5.8 g/l)	0.1 M
Tween 20	0.01 % (v/v)
(filtered through a 0.2 µM filter, shielded from light and stored at 2–8 ^{o}C)	

(11) 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)

(12) Antibody solution mix (secondary stain):

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)

(13) SAPE solution mix (Tertiary stain):

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

2.1.4.4. Solutions for animal experiments

(1) Alginate solution:

Protonal	11 mg/ml
NaCl	0.9 % (w/v)
(autoclaved)	

(2) Tris- calcium chloride buffer:

Tris-HCl (12 g/l)	0.1 M
$CaCl_2$. $2H_2O$ (14.7 g/l)	0.1 M
pH	7.0

(3) Hypnorm & Midazolam mixture:

Hypnorm	2.5 mg/ml
Midazolam	1.25 mg/ml

2.1.5. Bacterial cultures

2.1.5.1. *Escherichia coli* DH5α:

F $^-$,φ80, m80lacZΔM1S, Δ(lacYZA-argF)$_{U169}$, recA1, endA1, hsdR17(r_K^-;m_K^+), supE44,λ^-,thi$^-$, gyrA, relA1

2.1.5.2. *Pseudomonas aeruginosa*

The following *P. aeruginosa* strains were used during the present investigation.

TB:

Serotype:	4
Pyocin type:	1h
Phage lysotype:	F8, M4, PS2, PS24, PS31, 352, 46b/2, 1214, Col21,F7, F10, PS21, PS73, no plasmids (Tümmler, 1987).

892:

Serotype:	4
Pyocin type:	1h
Phage lysotype:	F8, M4, PS2, PS24, PS31 no plasmids, the strain 892 is a clonal variant of the strain TB (Tümmler *et al.*, 1991).

PAO1: wound isolate, genetic reference strain (Holloway *et al.*, 1994)

SG17M: clone C, environmental isolate (river) (Römling *et al.*, 1994)

CSGB8: clone C, CF airways isolate (Römling *et. al.*, 1994)

CHA: CF airways isolate (Dacheux *et. al.*, 2000)

2.1.5.3. *Pseudomonas putida*

KT2440: soil isolate, plasmid-free derivative of strain mt-2 (Bagdasarian *et al.*, 1981).

2.2. Methods

The following part represents the basic methods that were used to carry out this work.

2.2.1. Microbiological methods

2.2.1.1. Bacterial growth conditions

All bacterial cultures were grown in the LB medium unless and otherwise specified other growth conditions. For this, a single colony was inoculated in a glass tube containing 5 ml of LB medium with or without antibiotic depending on the experiment and incubated at 37 ^{o}C (250 rpm) for 12−16 h. If large volumes were required, 1−2 ml of bacteria were inoculated in Erlenmeyer flasks containing 400 ml LB medium and incubated at 37 ^{o}C (250 rpm) for 12−16 h.
LB agar with appropriate antibiotic was used as selection media.

Selection medium:

E.coli DH5α pTnMod-OGm	LB + 25 µg/ml Gentamicin
P. aeruginosa	LB + 30 µg/ml Gentamicin

2.2.1.2. Determination of bacterial cell density

The optical density (OD) for all the bacteria was measured on the photometer at a wavelength of 600 nm (OD_{600}). The correlation between optical density and cell density in terms of colony forming units (cfu) is given as follows.

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

2.2.1.3. Maintenance of bacterial cultures

All bacterial cultures were maintained in LB medium containing 15 % glycerol (v/v) and stored at −80 ^{o}C. LB medium with 2.5 % (w/v) agar was used to store cultures at 4 ^{o}C.

2.2.2. Separation of DNA

2.2.2.1. Agarose gel electrophoresis

The DNA was mixed with 1/5th volume of loading buffer and loaded on an 1 % (w/v) precasted agarose gel. 1x TBE buffer was used as running buffer and 2.0 V/cm (run for overnight) to 8.5 V/cm field strength was applied. The gel was run until the front line of the dye crossed 2/3rd of the total gel length. After the run, the agarose gel was stained with 0.5 µg/ml ethidium bromide for 30 min, destained in water for 30 min and

photographed on a UV transilluminator (312 nm). Lambda phage DNA digested with *Bst*EII was used as molecular size standard.

2.2.2.2. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used for the isolation of small DNA fragments. For this, the DNA fragments were loaded on a 10 % gel (19+1 acrylamide/bis-acrylamide) in TBE buffer and separated with a field strength of 6.5 V/cm. The gel was stained with 0.5 μg/ml ethidium bromide for 5 min and destained in water for 5–10 min and photographed on a UV transilluminator (312 nm). Longer destaining should be avoided due to substantial loss of the signal strength since the DNA fragments are very small (40 bp).

2.2.3. Transformation

2.2.3.1. Generation of transformation competent cells

Competent cells from *E. coli* DH5α were prepared according to Inoue *et al.* (1990). 10–12 colonies from LB agar plate were inoculated in a 1 l Erlenmeyer flask containing 250 ml of SOB medium and grown at 18–20 °C on a rotary shaker (200 rpm) up to an optical density of 0.6–0.8. After the appropriate cell density, flasks were chilled on ice and the cells were harvested by centrifugation (2500 x *g*, 10 min, 4 °C). The pellet was resuspended in 80 ml of ice-cold TB buffer. After 10 min incubation on ice, the cells were centrifuged again (2500 x *g*, 10 min, 4 °C) and the pellet completely resuspended in 20 ml of ice cold TB buffer and 1.4 ml of DMSO (approx.. 7 %, v/v) was added. The cells were incubated on ice for 10 min, aliquoted and immediately frozen in liquid nitrogen. The aliquots were stored at −80 °C.

2.2.3.2. Transformation by heat shock method

Transformation was carried out by heat shock method as described previously (Dagert and Ehrlich, 1979). Competent cells were thawed on ice, 20 μl cells were aliquoted in reaction tubes and 40–60 ng of DNA were added to the cells. After 30 min incubation on ice, the cells were heat shocked at 42 °C for 40 s and immediately placed on ice for 2 min. Then 80 μl of LB medium was added to the mixture and the cells were incubated at 37 °C for 1 h. After the incubation period, the whole mixture

was plated on LB agar containing an appropriate antibiotic and incubated at 37 °C. The colonies harboring plasmids were visible after 16–24 h of incubation.

2.2.4. DNA isolation

2.2.4.1. Isolation of genomic DNA from Gram-negative bacteria

The genomic DNA from *P. aeruginosa* was isolated by a method that is described for Gram-negative bacteria (Chen and Kuo, 1993). DNA isolated by this method was used as template in PCR reactions or for the preparation of Southern blots. The bacterial cells from 1.5 ml of overnight grown culture were harvested by centrifugation (14,000 x *g*, 3 min). The pellet was resuspended in 300 µl of Lysis buffer, 100 µl of 5 M NaCl were added and mixed thoroughly. Cell debris was separated by centrifugation (14,000 x *g*, 60 min, 4 °C). The supernatant was transferred in another eppendorf tube and the RNA was digested (37 °C, 30 min) by addition of 3 µl of RNase (10 mg/ml). To remove proteins and lipids, the DNA solution was successively treated with equal volumes of phenol, phenol:chloroform:isoamyl alcohol (25:24:1, v/v), and chloroform:isoamyl alcohol (24:1, v/v). The mixture was thoroughly mixed every time and phases were separated by centrifugation (14,000 x *g*, 15 min) at every step. DNA was precipitated by addition of equal volume of isopropanol to the supernatant and centrifuged (14,000 x *g*, 15 min). The pellet was washed with 70 % (v/v) ethanol, dried in a vacuum concentrator and resuspended in 50 µl TE buffer.

2.2.4.2. Isolation of plasmid DNA

The plasmid DNA from the transformed *E. coli* was isolated by the modified alkaline lysis method (Birnboim & Doly, 1979). 3 ml of the overnight grown culture in LB medium was centrifuged (5000 x *g*, 5 min) and resuspended in 300 µl solution I. Then 300 µl solution II was added and mixed properly by inverting the eppendorf tube. After 5 min incubation period, 300 µl solution III was added, thoroughly mixed and incubated on ice for 15 min. The precipitate was centrifuged (10,000 x *g*, 10 min, 4 °C) and the supernatant was transferred into a fresh eppendorf tube. To remove proteins and lipids, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) mixture was added to the above supernatant, thoroughly mixed and centrifuged (14000 x *g*, 2 min, 4 °C) for phase separation. The aqueous phase was pipetted into

a fresh eppendorf tube, chloroform:isoamyl alcohol (24:1, v/v) was added to the above solution, mixed thoroughly and centrifuged (10,000 x *g*, 10 min, 4 ^{o}C). After centrifugation, an equal volume of isopropanol was added to the supernatant for the precipitation of plasmid DNA and centrifuged (14,000 x *g*, 15 min, at room temperature). The pellet was washed with 70 % (v/v) ethanol and dried in a vacuum concentrator. The dried pellet was resuspended in 50 µl TE buffer and stored at −20 ^{o}C until further use.

For sequencing, the extra pure plasmid DNA was isolated by using Qiagen Mini and Maxi Prep kit according to the manufacture's procedure.

2.2.4.3. Quantitation of DNA and RNA

The nitrogenous bases in DNA or RNA molecules absorb UV light. Spectrophotometric measurement of UV absorbance by nucleic acids in solution is a simple and fast method of determining concentration, but is accurate only if the preparations are relatively free of contaminants such as proteins, phenol or particulate matter. The concentration of DNA and RNA was determined photometrically (Ausubel *et al.*, 1988; Sambrook *et al.*, 1989) by measuring the absorption at 260 nm in a quartz cuvette with 1 cm thickness. Double distilled water was used as a control. Based on extinction coefficients, an optical density (OD) of 1.0 at 260 nm, corresponds to approximately:

for double stranded DNA: 50 µg/ml
for single stranded DNA: 33 µg/ml
for RNA: 40 µg/ml

The ratio of OD_{260} / OD_{280} provides the estimate for purity of DNA. A typically pure preparation of DNA and RNA has a ratio of 1.8 and 2.0 respectively. The concentration of the DNA and RNA can be calculated from the following formula:

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

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

2.2.5. Polymerase chain reaction

Polymerase chain reaction (PCR) developed by Mullis *et al.* 1986, is one of the important standard technique used in molecular biology for the amplification of DNA (Mullis and Faloona, 1987). PCR is based on the features of the semi conservative DNA replication carried out in prokaryotic and eukaryotic cells by DNA polymerases. All the polymerases can catalyze the addition of bases to the 3' hydroxyl end and can extend a primer, if a template is provided. PCR is based on these principles and is used to amplify the segment of DNA that lies between two regions of known sequence. PCR technology has revolutionized the field of molecular biology and finds tremendous applications in gene isolation, genomic DNA amplification, biodiversity analysis, DNA fingerprinting and tagging of important genes.

This technique was used for the following purposes in this study.

- amplification of specific sequence of a transposons and *P. aeruginosa* gene for probe preparation for Southern and Northern hybridization.
- amplification of the signal sequences from PTnMod-OGm plasmid.

2.2.5.1. Construction of primers

A critical factor for yield and purity of the PCR product is the construction of correct primers. The following criteria were used to construct primers (Spangenberg, 1997).

- Length of the primer was kept to approximately 20 base pairs.
- Primer should end with at least two guanine/cytosine residues to ensure stable hybridization during PCR.
- Primer should not be complementary with itself or with other primer to form a hairpin structure above the T_m = 50 ^oC
- The melting temperature of the primer should be above 60 ^oC to avoid the non specific amplification. The melting temperature can be calculated from the rule of thumb T_m= 4 $^oC \cdot \Sigma$(GC) + 2 $^oC \cdot \Sigma$(AT). Primers from the one pair should exhibit the same melting point.

2.2.5.2. PCR reaction

The following protocol was used to amplify the DNA sequences.

2.5 µl	10x Buffer (InViTek)
2.5 µl	8 mM dNTP (2 mM for each nucleotide)

2.5 µl	5 µM Primer 1
2.5 µl	5 µM Primer 2
1.25 µl	DMSO
0.75 µl	50 mM $MgCl_2$
0.2 µl	*Taq*- DNA Polymerase 5 U/µl
10–200 ng	Template DNA (depending upon experiment)

Total volume was adjusted up to 25 µl with double distilled water.

DMSO acts as chaotropic reagent and enhances specificity of binding during annealing. The hybridization temperature is lowered and formation of secondary structures is also suppressed.

2.2.5.3. PCR parameters

During this study, PCR was used for the following applications in order to amplify the DNA sequence.

a) Amplification of specific signal sequences from pTnMod-OGM

For the individual changes of temperature, an interval (temperature ramp) of 10 s was programmed in each case. This PCR gave high yields over a concentration range of plasmids and the expected size of the product was 80 bp. Thus all the temperatures were run only for very short times. TAG-1 and TAG-2 were used as primers (for sequences, see appendix II) and pTnMod-OGM plasmid as template in the PCR reaction. Small reaction volumes and short cycle times were used at moderate temperatures. If necessary, the PCR product from many reactions of small volume (max. 25 µl), were pooled together.

b) Amplification of specific signal sequences from *P. aeruginosa*

For the individual changes of temperature, an interval (temperature ramp) of 10 s was programmed in each case. Primers TAG-1 and TAG-2 were used to amplify the signal sequence from *P. aeruginosa* genomic DNA. This PCR required the appropriate amount of template since small inaccuracy leads to substantial loss of product.

c) Amplification of genomic sequences from *P. aeruginosa*

The standard protocol with the change in temperature was used for the amplification of genomic sequences from *P. aeruginosa*. The primer sequences are given in the

appendix (II). The variable parameters of different PCR, depending upon intended purpose, are given below.

a)	b)	c)
300 s 96 °C	300 s 96 °C	300 s 96 °C
20 s 61 °C* ↑ 20 s 72 °C* 40 x 30 s 94 °C* ↓	20 s 58 °C* ↑ 20 s 72 °C* 40 x 30 s 94 °C* ↓	20 s variable ↑ 20 s 72 °C* 40 x 30 s 94 °C* ↓
20 s 61 °C* 40 s 72 °C*	20 s 58 °C* 40 s 72 °C*	20 s variable 40 s 72 °C*
∞ 10 °C	∞ 10 °C	∞ 10 °C
Primers: TAG-1; TAG-2	Primers: TAG-1; TAG-2	Primers: variable
DNA conc.: 5-100 ng	DNA conc.: 3-10 ng	DNA conc.: 10-100 ng
Volume: 25 µl	Volume: 25 µl	Volume: 25 µl

*: indicate the PCR program steps where changes in temperature (temperature ramp) were accomplished in 10 s. Program steps without * indicate that the change in temperature was as fast as possible and it was designed conditionally in the thermocycler.

2.2.6. Restriction digestion of DNA

The DNA was digested with the appropriate restriction enzyme according to the manufacturer's instructions. The restriction enzyme was also used for the digestion of specific signal sequences, which were amplified by PCR. These sequences which are relatively small in size were digested with *Hin*dIII where several microgram of DNA was used in each digestion. Signal sequences (5 µg) were digested with 250 U of restriction enzyme *Hin*dIII in a total volume of 250 µl and the reaction mixture was

incubated overnight. Because of the high multiplicity of the restriction sites, more than 50 U/μg signal sequence was added for a complete digestion of the DNA.

The unit definition of restriction enzyme is defined as the quantity of enzyme required for the digestion of 1 μg of DNA in 1 h in the assay buffer.

2.2.7. Plasmid rescue

Plasmid rescue (ligation of digested DNA) was performed to transfer the minitransposon with its flanking sequences as stable episomal plasmids into *E. coli*. The protocol by Dennis and Zylstra (1998) was modified as follows: 10 μg of genomic DNA from *P. aeruginosa* attenuated virulence transposon mutants was digested with 40 U of *Pst*I at 37 ^{o}C overnight in 40 μl restriction buffer. The restriction enzyme cut on either end of transposon. Digested DNA was purified by phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and then by chloroform:isoamyl alcohol (24:1, v/v) extractions and the DNA was precipitated by ethanol. The pellet obtained was dried in vacuum concentrator and resuspended in 25 μl of TE buffer. An aliquot of 500 ng digested genomic DNA was incubated with 1000 cohesive end ligation units of T4-DNA ligase for 6 h at 16 ^{o}C in a total volume of 25 μl ligase buffer. Forty to sixty ng of ligated DNA were transformed into highly competent *E. coli* DH5α (10^7 transformants/ng DNA) by the heat shock method (see 2.3.3.2) and plasmid harboring cells were selected on LB agar containing getamycin (30 μg/ml). These plasmids were sent to Qiagen (Hilden, Germany) for sequencing of the genomic insertion site of the transposon. Sequence of *P. aeruginosa* genomic DNA immediately adjacent to the transposon were determined annealing to both ends of Tn5.

2.2.8. Generation of DIG- labeled probe

2.2.8.1. Isolation of DNA fragments from agarose

The following two methods were used for the isolation of DNA from agarose gels.

1. Method

The DNA was isolated from agarose gel by the freeze and squeeze method developed by Walker (1984). This method has two disadvantages, first it is not applicable for large DNA fragments (5000 bp) and second, the DNA samples are

always contaminated with agarose. So this interferes with the ligation and sequencing reactions but it is applicable for DIG labeling.

After agarose gel electrophoresis, the gel was stained with ethidium bromide, destained and observed under the UV transilluminator. The DNA bands were located and cut out of the gel with the help of scalpel. The gel pieces were kept in the separate eppendorf tubes containing 3–4 layers of muslin cloth which acts as filter. The eppendorf tubes were kept at −80 ^{o}C for 2 h. Then the eppendorf tubes were thawed and the DNA solution was eluted by centrifugation (14,000 x *g*, 20 min, 4 ^{o}C). The DNA was precipitated by the addition of 0.5 volume (v/v) of sodium acetate (pH 7.0) and 3.5 volume (v/v) of ethanol and incubated at −80 ^{o}C for overnight. The DNA was pelleted by centrifugation (14,000 x *g*,15 min, 0 ^{o}C), washed, dried and the pellet was resuspended in small volume of TE buffer.

2. Method

The DNA was recovered by the method as described by Qiagen (Hilden, Germany). For this, DNA was separated by agarose gel electrophoresis, stained, destained and visualized under the UV transilluminator. Then the DNA fragments were excised from the agarose gel and weighed. 3 volumes of buffer QG was added to 1 volume of gel and incubated at 50 ^{o}C for 10 min or until the gel slice has completely dissolved. Then 1 gel volume of isopropanol was added to the sample and mixed by inverting the tube several times. This solution was applied to a MinElute column (Qiagen) and centrifuged for 1 min. The column was washed with 500 µl of buffer QG and then with 750 µl of buffer PE. The MinElute column was centrifuged for an additional 1 min. 10 µl of buffer EB was applied onto the center of the membrane and kept for 5 min, then the DNA was eluted by centrifugation for 1 min. All the centrifugation steps were carried out at more than 10,000 x *g* at room temperature. The DNA recovered in this way was used for sequencing or probe generation in Northern Blotting.

2.2.8.2. Separation of small DNA fragments

The *P. aeruginosa* transposon mutants contains the specific signal sequence (40 bp) in the center flanked by non-specific primer (20 bp) sequences on either sides. The following method was used for the separation of signal sequence from primer sequence. For this, sephadex G100 column was generated in a 3 ml syringe and

equilibrated with TE buffer. Then the column was centrifuged (1000 x *g*, 1 min) and the 80 bp *Hin*dIII digested sequence with dextran blue was loaded on the sephadex column. Then this column was centrifuged (1000 x *g*, 2 min). The 40 bp fragments with dextran blue were eluted & recovered in the fresh eppendorf tube at the bottom of column and the 20 bp fragments along with phenol red stayed in the column. The enriched 40 bp fragments were precipitated with ethanol, pellet dried and then resuspended in TE buffer. These fragments were used for probe generation. Unfortunately, the separation of the 40 bp fragments from 20 bp was not complete. About 5–10 times enrichment was achieved in one procedure by this method, this was sufficient for the subsequent probe preparation and hybridization.

2.2.8.3. Random primer labeling by DIG-DNA labeling kit

Random primer labeling, described by Feinberg and Vogelstein (1983) is the most widely used method for uniform labeling of DNA probes. The method utilizes the ability of *E.coli* DNA polymerase (Klenow fragment) to synthesize a new strand complementary to a template DNA strand starting from a free 3' hydroxyl end in order to label both the strands simultaneously. Klenow polymerase cannot start synthesis without primers, therefore, a set of primers was added to the single stranded template. Random primer labeling leads to a highly efficient reaction giving good levels of incorporation even with small amount of template DNA. With 1 pg DNA per assay approx. 10 % of the nucleotides are incorporated into about 250 ng of newly synthesized labeled DNA within 1 h and approx. 30 % of the nucleotides into about 750 ng after 20 h. The labeled DNA by this method was used as probe in Southern and Northern hybridizations.

The DNA was labeled by using the kit supplied by Roche Molecular Biochemicals.

1 The DNA was denatured in a boiling water bath for 10 min and immediately chilled on ice.
2 The following components were added

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

3 Mix gently by pipetting, then spin briefly in a microcentifuge and incubate the reaction mixture at 37 ^{o}C for overnight.

The labeled DNA was purified by a gel filtration procedure. For this the sephadex G50 column was prepared in 1 ml syringe until top and equilibrated with TE buffer. Then this column was dried by centrifugation (1000 x *g*, 45 s) to remove the excess buffer. The coloring solution (8 µl) and 72 µl of TE buffer was added in the labeled DNA mixture so that the final volume achieved was 100 µl. Then this mixture was loaded on the column and centrifuged (1000 x *g*, 30 s). The labeled/marked DNA fragments with dextran blue were eluted & recovered in the fresh eppendorf tube which was kept at the bottom of column during centrifugation. The DNA labeled in this way was used immediately or stored at −20 ^{o}C until use.

2.2.8.4. DIG- 3' end labeling of oligonucleotides

The transposon mutants of *P. aeruginosa* TB harbor a specific 40 bp sequence. This sequence should be labeled/marked during the procedure for the screening of these mutants for intracellular survival in polymorphonuclear granulocytes. For this, the reagents for a Dig- Oligonucleotide 3' end labeling kit was used. DIG ddUTP (Digoxigenin-11-2', 3' dideoxyuridine 5' triphosphate) was incorporated into the single stranded DNA by Terminal transferase. This DIG ddUTP can serve as recognizable antigen in the immunobiological detection. The manufacturer recommended this procedure for the labeling of oligonucleotides with a length of 25−100 bps. For the longer fragments, the signal intensity can decrease. The cleaned specific signal sequences (see 2.2.8.2) were denatured at 95 ^{o}C for 5 min and then immediately chilled on ice. At least 1 µg of denatured DNA was mixed with the other components of the labeling kit as described by the manufacturer. Then this mixture was incubated at 37 ^{o}C for 4−8 hours and used directly as a probe without any further treatment in the pre-hybridization buffer.

2.2.9. DNA fixation and hybridization

2.2.9.1. Southern Transfer

This method was developed by E. M. Southern in 1975. It involves transfer of single stranded DNA fragments from an agarose gel to a solid support, usually a nylon

membrane where the fragments are immobilized. The nylon membrane has a high binding capacity, physically strengthened DNA fragments can bind rapidly by UV cross linking or baking, making it ideal for DNA blotting applications. The transfer and immobilization of the DNA fragments separated by gel electrophoresis can be achieved by different methods like capillary blotting or vacuum blotting. Capillary blotting is the most commonly used approach. In this procedure, the agarose gel is mounted on a porous support which dips into a reservoir containing transfer buffer. The membrane is sandwiched between the gel and a stack of filter papers which serves to draw transfer buffer through the gel by capillary action. The DNA molecules are carried out of the gel by the buffer flow and immobilized on the membrane.

The procedure for Southern blotting is described below.

1. The agarose gel was destained in sufficient amount of distilled water at room temperature with gentle shaking.
2. Water was removed and the gel was denatured in 0.4 M NaOH for 30 min at room temperature with constant shaking. The alkali treatment denatures the fragments prior to transfer ensuring that they are in single stranded state, latter accessible for labeling.
3. A Whatman filter paper bridge was prepared on the blotting apparatus and the gel was kept upside down on the Whatman filter paper. Prewetted nylon (Hybond N^+) membrane with the same size of gel was kept without air bubbles in between gel and membrane. The 0.4 M NaOH solution was added in the bath.
4. The Whatman filter paper with the size of the gel was placed on top of the membrane. A stack of ordinary toilette paper with the same size as the gel was kept on the Whatman filter paper. The glass plate with the weight of approximately 500 g was kept on the top of the toilet papers.
5. The transfer of DNA was allowed for about 16–24 hours.
6. The membrane was removed, washed with 50 mM phosphate buffer for 5 min and dried at room temperature for 20 min and then at 65 ^{o}C.
7. DNA on the membrane was cross-linked in UV stratalinker with the program autocrosslink on both sides of the membrane. The blot was stored at room temperature till further use.

2.2.9.2. Dot- blot preparation

The dot blots were used during the selection experiments carried out for the screening of STM library for intracellular survival in PMNs. The labeled DNA obtained from the signal sequences was hybridized specifically on these dot blots. For this, the signal sequences of the donor plasmids were amplified by PCR (see 2.2.5.3a). The PCR product (80 µl) from each signal sequence was mixed with 40 µl of 3 M NaOH and 280 µl of TE buffer and denatured at 65 ^{o}C for 30 min. After cooling to ambient temperature, 400 µl of 2 M ammonium acetate was mixed and after short incubation period, this denatured DNA solution was aliquoted in a 96 well plate. The DNA solution (95 µl) was applied to the Minifold-DOT-vacuum-blot- equipment with the help of a multi-channel pipette. The DNA was sucked and immobilized on the blot/nylon membrane (Hybond N^{+}) by the vacuum generated in the equipment. The dot blots were rinsed with the 1 M ammonium acetate solution and dried at room temperature. DNA on the dot blots was cross-linked in UV stratalinker with the program autocross link on both sides of the blot and was stored at room temperature till further use.

2.2.9.3. Southern and dot blot hybridization

All the hybridization methods are based on the ability of denatured DNA to anneal to complementary strand at a temperature below their T_m (Church & Gilbert, 1984). Southern hybridization (Southern, 1975) involves a reaction between denatured DNA immobilized on the nylon membrane and single stranded DNA probe. It is a function of a temperature, time, salt concentration, G+C content, probe length and hybridization volume. All the hybridization protocols are designed to get intense signals and reduced background for a particular system. Prior to hybridization, prehybridization is carried out in which potential binding sites for the probe other than complementary DNA are blocked to avoid background hybridization to the membrane. The dot blots were also treated in a similar way which were used during the screening for intracellular survival in PMNs.

The following protocol was used for the Southern and dot blot hybridization except that the hybridization temperature for the 3' labeled 40 bp sequence was 65 ^{o}C.

1. The required volume of prehybridization buffer was preheated to 68 ^{o}C (65 ^{o}C for 40 bp sequence) and the membrane/blot was kept in a large glass tubes

(Biometra) or in 50 ml plastic Falcon tubes. The membrane was kept in the tube with the DNA adhered side inward in the tube. Prehybridization buffer (10 ml/100 cm^2) was added in the tube and prehybridized at 68 ^oC (65 ^oC for 40 bp sequence) for at least 2–3 hours with constant shaking.

2. The Dig labeled probe was added in 10 ml total volume of prehybridization buffer and denatured by boiling for 10 min then added in the tube containing blot.
3. The blot was hybridized for 16–24 hours at 68 ^oC (65 ^oC for 40 bp sequence) with gentle shaking in the hybridization oven.
4. After hybridization, the hybridization buffer was poured in a sterile tube and the probe was saved for future use at –20 ^oC.
5. The blots were rinsed twice with wash solution by adding in the glass tube and shaking and the solution discarded.
6. Then 30–40 ml of wash solution was added in the tube and incubated at 68 ^oC (65 ^oC for 40 bp sequence) for 30 min in the hybridization oven. The wash solution was discarded and the same procedure was repeated for a second time.

2.2.9.4. Immunological detection of the hybridized blot

After hybridization, the hybrids were detected by incubation with an anti-fluorescein alkaline phosphatase conjugate. The DIG labeled DNA fragments show fluorescence with the help of an anti Dioxigenin (Fab fragment). The Alkaline Phosphatase catalyzes a chemo-luminescence reaction with CDP Star$^{(TM)}$, whose light intensity is constant over several hours. The detection was performed as previously described by Allefs *et al.* (1990). In briefly, the protocol is described below.

1. Following the stringency washes, the blot was equilibrated in buffer I for 5 min. Then it was saturated in buffer II for 30 min to remove non specific signals by incubation on a shaker with shaking.
2. Blot was incubated with buffer II containing 5,000 fold (v/v) dilution of Anti-fluorescein-AP conjugate (4 ml/100 cm^2) with gentle agitation at room temperature for 30 min.
3. Unbound conjugate was removed by washing for 3 x 15 min in buffer I.
4. Then the blot was equilibrated in freshly filtered buffer III for 2–5 min, then incubated with CDP star solution (1:500, v/v) in filtered buffer III for 5–10 min.
5. After incubation, the excess buffer III was drained off from the blots and wrapped in plastic foil. After 15–30 min incubation i.e. the maximum chemo-luminescence

was reached (Bronstein *et al.*, 1989). The X-ray film was kept on the blot in dark and developed. Different exposures were taken depending upon the signal intensity. This produced signals with constant intensity over several hours.

2.2.9.5. Washing of Southern and dot-blots

The labeled and hybridized membranes can be used several times after washing (stripping) under alkaline conditions. After washing, the membrane did not show any signals from the previously labeled and bound DANN, because the DIG dUTP is not stable in an alkaline environment and disintegrates by the release of dioxigenins. For washing, the membrane was incubated twice with wash solution for 30 min under strong shaking conditions. Then the membrane was neutralized by keeping it in sodium phosphate buffer (pH 6.5) for about 15 min. Then it was wrapped in plastic foil and stored at −20 ^{o}C until the further use.

2.3. Screening of the STM library for intracellular survival in PMNs

The following procedure was used for the screening of *P. aeruginosa* TB transposon mutant library for intracellular survival in PMNs.

2.3.1. Growth of transposon mutants

A library of *P. aeruginosa* TB signature tagged mini Tn5 OGm transposon mutants was constructed by Lutz Wiehlmann (Ph. D. thesis, 2001) as described previously (Hensel *et al.*, 1995). The STM library was maintained as glycerol stocks in 96 well plates at −80 ^{o}C. Each transposon mutant inoculated from frozen glycerol stock, was grown individually in a micro titer plate containing 200 µl of LB medium and incubated at 37 ^{o}C for 16 h. Culture from 48 mutants with different preselected signature tags were collected, mixed properly and considered as one pool.

2.3.2. Isolation of granulocytes from human blood

PMNs were isolated from freshly obtained blood of a healthy donor. Approximately 10 ml of fresh blood (with 100 I.U. heparin; 100 µl Liquemin) were used for each experiment. The blood was added in the glass tube containing 5 ml of 10 % hydroxyethyl starch (HES) and incubated for 45−60 min at room temperature. Most of

the erythrocytes were separated by sedimentation and the supernatant was added into another fresh plastic tube with a pasteur pipette. 2 ml of lymphocyte separation medium (a Ficoll solution of density = 1.077 g/ml in isotonic saline solution) was carefully added at the bottom of the blood plasma with a pasteur pipette and separated by centrifugation (3000 x *g*, 15 min). The lymphocytes and the macrophages accumulated at the phase boundary and the granulocytes and erythrocytes were pelleted. The pellet was resuspended in 1 ml eukaryotic RPMI1640 media and stored on ice.

2.3.3. Determination of granulocyte concentration

The erythrocytes were lysed from granulocytes by resuspending the pellet in 1 ml of autoclaved sterilized double distilled water and incubated for 10 s, then 4 ml of 5x PBS buffer was added immediately to avoid the lysis of granulocytes. Then this solution was centrifuged (3000 x *g*, 5 min) and the granulocyte pellet was resuspended in 1 ml RMI1640 medium and kept on ice. The granulocyte cell number was determined by putting a aliquot in a Neubauer chamber and granulocytes were counted from 10 squares at different locations.
Following correlation was observed:

1 Granulocyte/large square = 2.5 x 10^5 granulocytes/ml

Concentration of granulocytes from 10 ml blood was in the range of 1 x 10^7 – 1.5 x 10^7 cells/ml.

2.3.4. Phagocytosis assay in granulocytes

The mutants from one pool were screened for intracellular survival in a phagocytosis assay that were performed in duplicate. Approximately 1 x 10^7 freshly isolated granulocytes were added in the RPMI1640 medium containing AB- blood serum at a final concentration of 10 % (v/v) and 10 fold excess of bacteria (1 x 10^8) (determined by spectrophotometry, 0.6 OD = 10^9 cfu/ml) added in the 14 ml plastic tube. The relationship between bacteria and granulocytes was in the range of 10:1 to 20:1 (Miethke, 1985). The final volume was adjusted to 2 ml with the RPMI1640 medium and this was considered as input pool (selection). The same amount of bacteria were added in the control experiment except PMNs which was called as output pool

(without selection). Addition of serum was essential since the granulocytes need a functional complement system for the recognition of bacteria. Phagocytosis could not take place without the opsonization of bacteria. The serum with a standard complement system, from different blood donors (irrespective of blood groups) was collected. Approximately 300 ml serum was frozen at −80 ^{o}C before beginning of the experiment and same serum was used in all the experiments.

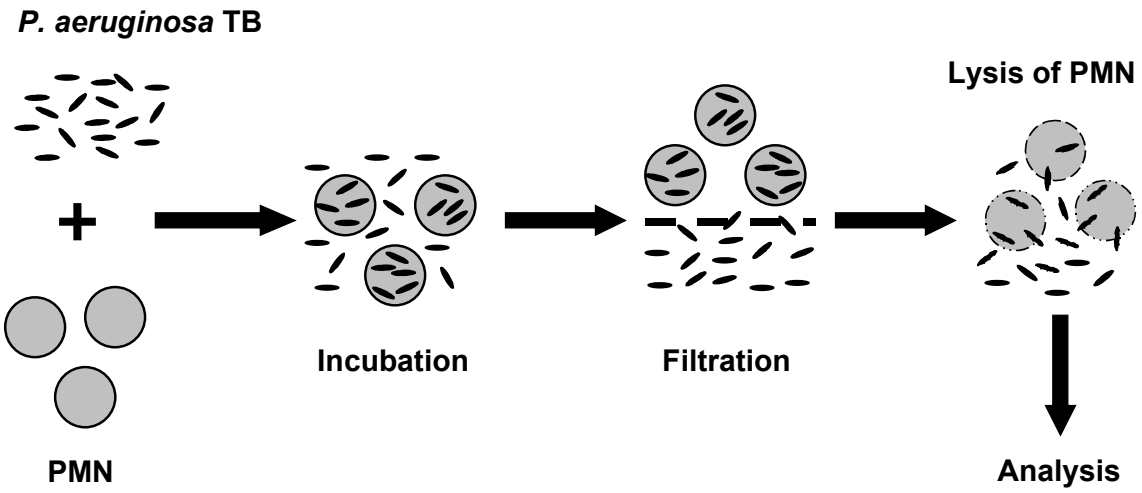


Fig. 2.1. Schematic representation of phagocytosis assay used during screening of the STM library for intracellular survival in PMNs.

The input and output pools were incubated for 120 min (200 rpm, 37°C). After the incubation period, the assay mixture was kept on ice for 10 min. Then the tubes were filled with ice cold RPMI1640 medium until the top and centrifuged (400 x *g*, 10 min). The supernatant was discarded and the granulocyte pellet with the internalized bacteria was resuspended in 200 µl RPMI1640 medium. This suspension was applied on the nitrocellulose filter paper (pore size 5 µM) and washed with 5 ml PBS to remove the extracellular adhered bacteria. Then this filter with the adhered granulocytes was transferred to a fresh tube containing 3 ml distilled water and vortexed for 5 min. This resulted in the separation of intracellular bacteria from granulocytes by lysis. The bacterial suspension was transferred in a new tube and centrifuged (4000 x g, 10 min). The supernatant was discarded and the pelleted bacteria were resuspended in 100 µl PBS buffer and plated on LB agar. 50−100 µl aliquot from the output pool was also plated on LB agar. Bacteria were recovered on

the next day from plates, resuspended in the 10 mM MgSO4 solution. Then this assay was repeated by using the recovered bacteria for input and output pools separately. According to this protocol, the bacteria were grown on LB- agar overnight after the second selection assay The schematic representation of phagocytosis assay is shown in figure 2.1.

While doing this experiment, following precaution was taken.

- Experiment was completed within the 6 hours after withdrawing blood to the filtration of granulocytes. Because granulocytes can stop functioning after 6 hours due to the bacterial selection pressure applied during this procedure.
- Bacteria were not incubated with granulocytes for more than two and half hours, since apoptosis starts and intracellular bacteria can become free.

Genomic DNA was isolated from the survived mutants after second selection and a PCR was performed on this genomic DNA to amplify the signature tags. Here, 8 PCR reactions (each of 25 µl) from one pool were used to get the amount of DNA sufficient for digestion. The PCR product was digested for 16 h with *Hin*dIII and the specific 40 bp sequences were separated from the unspecific 20 bp sequences and unused primers by gel filtration and the DNA was precipitated with ethanol. After resuspending DNA in water, the 40 bp sequences were labeled at 3' end with Dig-ddUTP using a terminal transferase as described by manufacture. These labeled signal sequences from input pool and output pool were hybridized separately at 65 ^{o}C on dot-blots which were previously prepared from all 48 pTnModOGm SigTag-donor plasmids. Then these hybridized dot blots were detected with CDP-Star and exposed to X-ray films. The developed film was scanned and the optical density (od/mm^2) of the individual dot was quantified by using PCBAS program, version 2.09f (raytest Isotopenmeßgeräte, GmbH). The signal strength of each dot (input pool) was compared to the corresponding signal of a probe prepared from pooled bacteria grown on LB agar without selection (output pool). This evaluation was performed in MS Excel and the dot blots were washed and could be used 3–4 times.

Signals out of the 95 % confidence interval of the mean were interpreted to be significantly different from the average signal. These mutants were retested 5 times

and those mutants with consistently strong differences in their ability to survive were selected for further examinations.

2.3.5. Cloning, sequencing and analysis of mutated *P. aeruginosa* DNA

The plasmid rescue for the virulence attenuated mutants was performed. Then the DNA sequences obtained were compared with the *P. aeruginosa* PAO1 genome at http://www.pseudomonas.com. These sequences were also analyzed with the BLAST N program for nucleotide-nucleotide homology sequences and BLAST X program for nucleotide-protein homology sequences on the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) database.

2.4. Screening of STM library for mutants defective in protease secretion

P. aeruginosa maintains a large genetic capacity (approx. 5500 genes; Stover *et al*., 2000), amongst which are encoded a battery of virulence factors localized on the cell surface or released into the external environment. The expression of many virulence factors is controlled by quorum sensing (QS), the use of small, diffusible signaling molecules (acylated homoserine lactones, AHLs) by a bacterial population to monitor cell density (Fuqua *et al*., 2001; Whitehead *et al*., 2001; Erickson *et al*., 2002). These virulence factors consist a major fraction of proteases which are secreted in the extracellular environment. Therefore the whole STM library was screened for protease secretion on casein agar plates. For this, the frozen mutants from glycerol stocks were inoculated on casein agar plates with the help of multi-channel pipette. Then these plates were incubated at 37 ^{o}C for 24–48 hours. During this period, bacterial growth and the zone of clearance was monitored at different time intervals. The transparent zone was formed around the colony since casein is hydrolyzed by the proteases that are secreted by bacteria. So the mutants that were unable to grow or formed small zone of clearance were considered as attenuated for protease secretion. These mutants were retested 4 times by the similar procedure and those with consistent in attenuation for protease secretion or growth on casein were selected for further investigations.

The attenuated mutants from the above assay were also checked for the protease secretion due to the diffusible products secreted by the wild type. For this, the *P.* aeruginosa TB wild type was inoculated in the form of thick line in the center on casein agar plate. The attenuated mutants were inoculated on the same plate perpendicular to the wild type (figure 3.7) without touching to wild type. This plate was incubated for 24–60 h at 37 ^{o}C and the zone of clearance due to the induction by wild type was monitored. A previous student has also screened the un-organized mutant library for the defect in protease secretion by the similar method during his Ph. D. thesis (Wiehlmann, 2001).

The genomic insertions (plasmid rescue) were determined by the similar procedure as described previously.

2.5. Characterization of STM knockout mutants

2.5.1. In silico analysis

The STM allowed screening of large number of mutants for the intracellular survival in PMNs in a reasonably less number of experiments. After screening, one can receive only the information which genes are essential for the intracellular survival in PMNs. However, the function of most of these genes is still not assigned in the *P. aeruginosa* database. So in silico analysis was performed for each identified gene to get the information about genomic context, structural features and homology. This information would help to describe the mutant phenotypically and to assign the function of the identified gene with respect to the intracellular survival in PMNs. The degree of intracellular survival is also quantified which gives idea about the intracellular survival ability of mutant with respect to the positive control (*P. aeruginosa* 892).

2.5.2. Sensitivity towards Hydrogen peroxide

The attenuated virulence mutants from granulocyte selection assays were tested for sensitivity to hydrogen peroxide (H_2O_2). For this, hydrogen peroxide with a defined final concentration was added in the LB agar when the temperature was around 40 ^{o}C and plated in the big quadrate plates. After solidification, wild type and mutants were inoculated in the form of separate dots on agar plates and incubated at 37 ^{o}C

for 24–48 h. The growth pattern was compared at different concentrations of hydrogen peroxide.

2.5.3. Motility assay

The swarming and swimming motility was checked for the mutants that have transposon insertion in the type IV pili and flagella biogenesis genes. The procedure described by Köhler *et al.* (2000) was used to check swimming and swarming motility.

Swarming: Swarm agar was based on M9 salts (Sambrook *et al.*, 1989) without NH_4Cl (termed here M8 medium, for convenience), supplemented with 0.2% glucose, 2 mM $MgSO_4$, and solidified with 0.5 % (w/v) agar. Aspartate was added as a sole nitrogen source at a final concentration of 0.05 % (w/v). After solidification, plates were briefly dried and then 2 µl of an overnight culture of *P. aeruginosa* were spotted and observed up to 12–24 h at 37 ^{o}C.

Swimming: 2 µl of an overnight grown culture of *P. aeruginosa* were inoculated beneath the surface of a 0.2 % (w/v) LB agar plate and observed up to 12–24 h at 37 ^{o}C.

2.5.4. Cytotoxicity towards macrophage J774 cells

Cytotoxicity assays were performed using the murine macrophage cell line J774.A1 (ATCC TIB-67) grown in DMEM medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum. For infection experiments, the macrophages were seeded in culture plates for 20 h before addition of the bacteria. Each *P. aeruginosa* strain was grown in 3 ml of LB medium for overnight and adjusted to an optical density (OD_{600}) of 1.0-1.5 by dilution in LB medium. Bacteria were then centrifuged, resuspended in eukaryotic medium, and added to cells at a multiplicity of infection (MOI) of 5. Cytotoxicity was assayed by measuring the lactate dehydrogenase (LDH) released into culture supernatants as previously described in Dacheux *et al.*, 2001, using the cytotoxicity detection kit (Roche Diagnostics). The 100 % value represented the LDH released from cells lysed by 0.1 % (v/v) TritonX-100.

2.6. Transcriptional analysis of *P. aeruginosa*

During the screening for intracellular survival in PMNs, we identified subset of genes having regulatory function. The other subset of identified genes were not present in the sequenced genetic reference PAO1 strain. In order to determine the function these strain specific genes, we decided to use the *P. aeruginosa* PAO1 microarrays (GeneChips). The global superoxide and oxidative stress response of the sequenced reference strain PAO1, a burn wound isolate from the 1950s, was also compared with that of the two clonal variants TB and 892 isolated from cystic fibrosis lungs that differ substantially in their resistance to PMNs (Tümmler, 1987).

2.6.1. RNA handling and storage

RNases are found everywhere, including laboratory workers hands and in airborne micro-organisms. Therefore special precautions were taken when working with RNA. All reagents and equipments were specially treated to inactivate RNases prior to use.

- The devices were autoclaved at 121 ^{o}C for 60 min. Heat unstable devices were wiped with sterile double distilled water and then with 70 % (v/v) ethanol. Metal devices such as scalpel and forcebs were dipped in the 70% (v/v) ethanol.
- Glass wares were baked at 250 ^{o}C for 5 h.
- All the solutions were prepared in the double distilled Diethylpyrocarbonate (DEPC) treated water. For this, the DEPC (0.05 %, v/v) added water was incubated for overnight at 37 ^{o}C and then autoclaved to hydrolyze any unreacted DEPC. Solutions containing Tris buffer were prepared in the double distilled DEPC autoclaved water.
- RNase free gel chambers were prepared by treating them with 3 % (v/v) H_2O_2 for 10 min and then rinsed with 70 % (v/v) ethanol.
- Fresh stock of plastic wares were used every time.
- When working with RNA, all the samples were placed on ice and RNase inhibitors were also added whenever needed.
- RNA dissolved in RNase-free buffer or water was stored at 70−80 ^{o}C.

2.6.2. *P. aeruginosa* PAO1 microarray

The *P. aeruginosa* microarray (GeneChip) from Affymetrix was designed by the initiative of the Cystic Fibrosis Foundation Therapeutics Inc. (CFFTI, USA) which has

also financed for the *P. aeruginosa* genome sequencing project (Stover *et al.*, 2000). The *P. aeruginosa* GeneChip (Affymetrix) contains probes for 5549 open reading frames, four rRNA operons (23S, 16S, 5S rRNA), 18 tRNA genes as well as 199 selected intergenic regions exceeding 600 base pairs from the completely sequenced and annotated genome of *P. aeruginosa* PAO1 (Stover *et al.*, 2000). In addition, probes from 117 open reading frames of other *P. aeruginosa* strains are also present on the GeneChip. Altogether 5986 different probe sets can be analyzed on the GeneChip. This GeneChip also contains 14 genes from *Bacillus subtilis, Saccharomyces cerevisiae* and *Arabidopsis thaliana* as controls. The sequences which are present on the GeneChip are extracted from the sequence data base GENE BANK available at (http://www.ncbi.nlm.nih.gov) and the further information for the synthesis and order of the GeneChip is available on the data base NETAFFX under http://www.affymetrix.com/analysis/index.affx. Sequence on the GeneChip which can be analyzed is represented by a varying number of 25-mer oligonucleotides according to its length (Lipshutz *et al.*, 1999). Each oligonucleotide is synthesized as probe cell with the help of a special photo-lithographic technology in combination with solid phase combinatorial chemistry on a glass surface of 20 µm x 20 µm in situ (Fodor *et al.*, 1991). All pairs of probes, which represent a sequence section on the GeneChip, are called probe set. The probe pairs of the probe set are distributed on the 1.28 cm x 1.28 cm large gene chip. Thus the effect of local irregularities (non-uniform hybridization and staining or mechanical damages, e.g. scratches) is decreased during hybridization.

2.6.3. Bacterial growth conditions for RNA isolation

In order to see the expression of the identified genes, bacterial cultures were grown in following conditions in the 125 ml Capsenberg flasks. The growth in the Capsenberg flasks allowed a RNA preparation with less experimental variation since these flasks has caps leading to the same aeration rate for all the bacterial cultures. The overnight grown preculture in the respective medium was inoculated in the flasks for RNA isolation.

2.6.3.1. Bacterial growth in LB medium

In this condition, all the bacterial cultures (*P. aeruginosa* TB and transposon mutants) were grown separately in 15 ml of Luria broth at 37 ^{o}C and 300 rpm in 125 ml

Capsenberg flasks to an optical density of 1.5 (early exponential phase) or up to 3.5 (late exponential phase) at 600 nm (OD_{600}). Then the bacteria from the appropriate growth phase were harvested by centrifugation (3800 x *g*, 2 min) and subjected for RNA isolation as described below (see 2.6.4).

2.6.3.2. Bacterial growth in presence of superoxide and oxidative stress generating agents

All the bacterial cultures were grown separately in 15 ml Luria broth at 37 ^{o}C and 300 rpm in 125 ml Capsenberg flasks to an optical density of 5.0 (early stationary phase) at 600 nm. The stationary phase grown culture (3 X 10^{10} cells) was resuspended in 5 ml of Luria broth and kept in a dialysis tube (14 kDa cut-off, 25 mm width) with the effective length of 6 cm for the exchange of fluids. Then the dialysis tube was resuspended in a 1l Erlenmeyer flask containing 600 ml of Luria broth without or with 0.5 mM paraquat or 10 mM hydrogen peroxide. The flasks were incubated at 37 ^{o}C and 200 rpm on a rotary shaker for 2 h. Flasks containing Luria broth without the addition of paraquat or hydrogen peroxide were served as controls. After the incubation period, bacteria were immediately recovered from the dialysis tube and subjected to RNA isolation.

2.6.3.3. Bacterial growth in iron depleted LB medium

The LB medium was depleted for iron by using Chelex-100 beads. For this, 1 gm of sodium azide sterilized Chelex-100 beads were added in the 1l Erlenmeyer flask containing 200 ml of LB medium and incubated on shaker at 37 ^{o}C. After the overnight incubation period, the beads were allowed to settle down at the bottom and LB medium was transferred into a other 1l flask. This procedure was repeated thrice by taking fresh beads every time. LB medium obtained after this procedure was considered to be depleted from iron. Then all the bacterial cultures were grown separately in 15 ml of iron depleted Luria broth at 37 ^{o}C and 300 rpm in 125 ml Capsenberg flasks to an optical density of 1.5 (early exponential phase) at 600 nm. The bacteria from this growth phase were harvested by centrifugation (3800 x *g*, 2 min) and subjected to RNA isolation.

2.6.3.4. Bacterial growth in presence of PMNs

Bacteria were grown separately in 15 ml of Luria broth at 37 ^{o}C and 300 rpm in 125 ml Capsenberg flasks to an optical density of 5.0 (early stationary phase) at 600 nm. The stationary phase grown culture (2 X 10^{10} cells) was resuspended in 2 ml of RPMI1640 medium and kept in a dialysis tube (14 kDa cut-off, 25 mm width) with the effective length of 3 cm for the exchange of fluids. The PMNs were isolated (see 2.3.2) from 30 ml blood provided by the healthy donor. These PMNs were divided into two parts. One part of PMNs were lysed and then PMNs from both parts were resuspended in 15 ml of RPMI1640 medium containing 10 % (v/v) serum. Then the dialysis tube containing bacteria was resuspended in the 125 ml Erlenmeyer flasks containing 15 ml of lysed and intact PMNs. The TB wild type was also added in the flask (MOI 10:1) to stimulate the granulocytes and mimic this condition like the phagocytosis assay. The flasks were incubated at 37 ^{o}C and 300 rpm on a rotary shaker for 2 h. During this incubation period, the content outside the dialysis bag travels and come in physical contact with the bacteria so that the bacteria can respond to the host defense factors. After the incubation period, bacteria were immediately recovered from the dialysis tube and subjected to RNA isolation.

2.6.3.5. Bacterial growth in minimal medium

For quorum sensing experiments, bacterial cultures were grown in the ABC-minimal medium. So the same medium was used to grow bacteria for RNA isolation. The TB wild type and D8A6 transposon mutant (defect in quorum sensing) were grown separately in 15 ml of ABC-minimal medium at 30 ^{o}C and 250 rpm in 125 ml flask to an optical density of 1.0 at 600 nm. Then the bacteria from this growth phase were harvested by centrifugation (3800 x *g*, 2 min) and subjected for RNA isolation.

2.6.4. RNA isolation

The bacterial cells grown under various conditions were harvested by centrifugation at 3800 *g* for 2 min at 4 ^{o}C. Total RNA from approximately 3 x 10^{10} cells was extracted with a modified hot phenol method (Tao *et al.*, 1999). Bacteria were quickly resuspended in 0.5 ml distilled water and lysed in 7.5 ml of preheated (65 ^{o}C) phenol/lysis-buffer mix (pH 5.5) with vigorous shaking for 10 min. The cell lysate was centrifuged (3800 x *g*, 20 min) and the supernatant was extracted with 3 ml of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and then subsequently with 3 ml of

chloroform:isoamyl alcohol (24:1, v/v). To pellet the nucleic acids, 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol were added, incubated at −20 ^{o}C overnight and centrifuged (3800 x *g*, 30 min). The pellet was washed with 5 ml 70% ethanol and resuspended in 175 µl of diethyl pyrocarbonate-treated (DEPC) water. DNA was digested by the addition of 40 U DNase I and 20 U SUPERaseIn in DNase I buffer (pH 6.5) for 30 min at 37 ^{o}C in a total volume of 200 µl. Then the RNA was purified by using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions and the yield of total cellular RNA was quantified by measuring the light absorption at 260 nm. RNA with a size below 200 bp (e.g. tRNAs, 5 S rRNA) is below the cut-off of the column and therefore could not be recovered. All the steps were carried out at 4 ^{o}C or RNA was also incubated on ice intermittently during the RNA isolation procedure.

2.6.5. Separation of RNA by formaldehyde agarose gel electrophoresis

Only completely denatured RNA can be separated as a function of its length by electrophoresis (Ausubel *et al.*, 1988). In order to check the RNA preparations, formaldehyde agarose gel electrophoresis was performed in mini gel chambers (5 x 7 cm, gel volume 40 ml). If the RNA should be transferred afterwards to a nylon membrane for hybridization, large blot gel chamber was used which accommodated 65 µl sample volume (8 x 11 cm with 15 slots, gel volume 220 ml). Every time the chambers were cleaned with 3 % (v/v) H_2O_2 and 70% (v/v) ethanol separately. RNase free agarose (SeaKemGTG, Biozym) at a concentration of 1.2 % (w/v) and 2 % (w/v) was used for mini gels and blot gels, respectively. The agarose was

Table 2.1. Sample preparation for formaldehyde gel electrophoresis.

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

autoclaved (121 ^{o}C, 30 min) in 1x MOPS buffer and after cooling to approximately 50 ^{o}C, 2.4 ml or 13.3 ml formaldehyde (37 %, w/v) was added for mini gel or blot gel, respectively to the other components mentioned in table 2.1. The RNA samples were denatured afterwards at 65 ^{o}C for 10 min, cooled down on ice and centrifuged (14.000 x *g*, 30 s, 4 ^{o}C). 5 µg of RNA molecular weight standard (0,16−1.77 Kb RNA ladder) was loaded on the blot gel and 1x MOPS buffer was used as running buffer. The samples were loaded on the gel after the pre-electrophoresis run (5 min, 60 V). The constant applied field strength for mini gels and blot gels was 5 V/cm (running time approx. 1 h) and 3 V/cm respectively (running time approx. 5 h). After the run, the agarose mini gels were stained with ethidium bromide (10 µg/ml) for 15 min and destained 2 X 15 min in distilled water and photographed under UV transilluminator. The electrophoresis of the blot gels was stopped when the bromophenol blue dye has migrated two-thirds of the gel length. The blot gel with a molecular weight standard was photographed under UV transilluminator (figure 2.2) and the purity and integrity of RNA preparation was checked using the 16S and 23S ribosomal bands as reference.

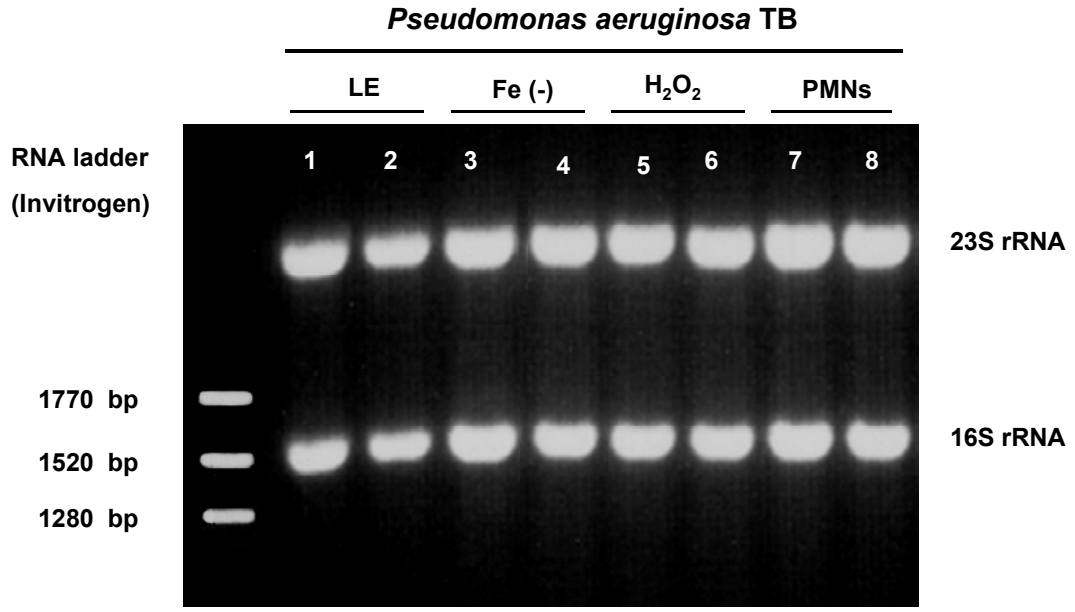


Fig. 2.2. Formaldehyde agarose gel electrophoresis of isolated RNA under different growth conditions [LE, Fe (-), H_2O_2 & PMNs]. The molecular weights of 16S and 23S rRNA are 2985 & 1536 bp respectively (Stover *et al.*, 2000). RNA molecular weight standard is shown on the left side of gel. This RNA was subsequently transferred to a positively charged nylon membrane for hybridization with different probes. (LE: late exponential phase; Fe(-): iron limiting; H_2O_2: in presence of 10 mM H_2O_2; PMNs: in presence of PMNs).

2.6.5.1. Northern transfer

The completely denatured RNA was separated by formaldehyde agarose gel electrophoresis and transferred (Ausubel *et al.*, 1988) on a positively charged nylon membrane (Hybond N^+) by the following procedure.

1. The blot gel was kept in double distilled water for 20 min before making the assembly of the blot equipment and then in 20x SSC buffer for 20 min.
2. For the Blotting by a sandwich method, the previously mentioned arrangement (see 2.2.9.1) was performed except that the 20x SSC buffer was used as a tank buffer. The glass trays and metal stands which are necessary for this arrangement were heat sterilized at 250 ^oC for 5 hours.
3. Transfer process was performed at room temperature for 20–24 h.
4. Then the membrane was removed and washed with 2x SSC buffer for 1 min.
5. Membrane was dried, cross linked and directly used or stored at –20 ^oC until use.

2.6.5.2. RNA-DNA (Northern) hybridization

The Northern hybridization was performed by using following procedure.

1. The required volume of prehybridization buffer was preheated to 42 ^oC and the membrane/blot was kept in a large glass tubes (Biometra) or in 50 ml plastic Falcon tubes. The membrane was kept in the tube with the RNA adhered side inward in the tube. The prehybridization buffer (10 ml/100 cm^2) was added in the tube and prehybridized at 42 ^oC for at least 2–3 hours with constant shaking.
2. The Dig labeled probe was added in 10 ml total volume of prehybridization buffer and denatured by boiling for 10 min then added in the tube containing blot.
3. The blot was hybridized for 16–24 hours at 42 ^oC with gentle shaking in the hybridization oven.
4. After hybridization, the hybridization buffer was poured in a sterile tube and the probe was saved for future use at –20 ^oC.
5. The blots were detected by a procedure described below.

2.6.5.3. Detection of the Northern blot

Non-specifically bound probe to the membrane was removed by several washing steps:

5 min with 6 x SSC + 3 % (w/v) SDS at room temperature

20 min with 2 x SSC + 3 % (w/v) SDS at 42 ^oC and then

20 min with 0.2 x SSC + 3 % (w/v) SDS at 42 ^{o}C.

After these washes, the membrane was removed from the tube and further procedure was carried out as described previously (see 2.2.9.4) except that all the detection solutions used belong to RNA work. The developed membrane was exposed to X-ray film at different time intervals.

2.6.6. cDNA generation

cDNA synthesis, fragmentation and labeling were performed as described by Affymetrix (Santa Clara, California) whereby the conditions for the fragmentation of the synthesized cDNA by DNase I was optimized according to the recommendations of the protocol. Briefly, 10 µg of total RNA were mixed with 750 ng random primers and 8.67 pM (final concentration) control in-vitro transcripts from 10 non-pseudomonal gene sequences (which was kindly provided by S. Lory and co-workers, Harvard) in a total reaction mixture of 30 µl. The control in-vitro transcripts were used as an internal quality control for the yield of labeling. This mixture was incubated for 10 min at 70 ^{o}C followed by 10 min at 25 ^{o}C and then chilled to 4 ^{o}C. 30 µl of cDNA reaction mixture were added to the reaction mixture containing RNA. cDNA synthesis was initiated by incubating the reaction mixture at 25 ^{o}C for 10 min, at 37 ^{o}C for 60 min, at 42 ^{o}C for 60 min and then the enzyme was inactivated at 70 ^{o}C for 10 min.

RNA was hydrolyzed by the addition of 20 µl 1N NaOH and incubation at 65 ^{o}C for 30 min. The reaction mixture was neutralized by the addition of 20 µl 1 N HCl. The cDNA was purified using the QIAquick column (Qiagen, Hilden, Germany) and quantified by light absorbance at 260 nm (see 2.2.4.3).

2.6.7. Fragmentation of cDNA

3 to 5 µg of cDNA were fragmented in One Phor-All Buffer with 0.5 U DNase I per µg cDNA in a 50 µl reaction volume for 10 min at 37 °C. The DNase I enzyme was subsequently inactivated at 98 ^{o}C for 10 min. To check if the majority of cDNA fragments were in a 50–200 bp range, 5 µl fragmented product was loaded on a 2 % (w/v) agarose gel and then stained for 40–60 min by keeping the gel in TBE buffer bath containing 0.0001 % (v/v) SYBRGreen (figure 2.3).

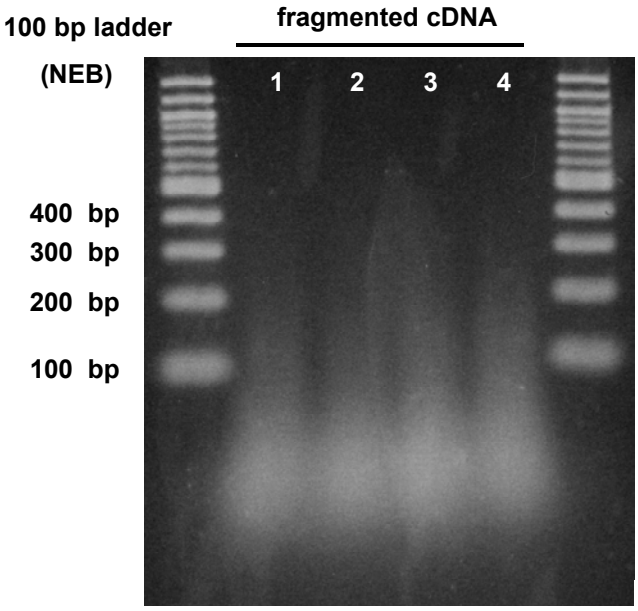


Fig. 2.3. Representative SYBR-Green stained agarose gel of fragmented cDNA. After fragmentation, the majority of cDNA fragments has a size of approximately 50−200 bp.

2.6.8. Labeling of fragmented cDNA with biotin

The fragmented cDNA was labeled by using the Enzo BioArray^{Tm} Terminal Labeling Kit with Biotin-ddUTP. 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 ^{o}C for 60 min. The reaction was stopped by adding 2 µl of 0.5 M EDTA and the target was ready to hybridize onto probe arrays.

2.6.9. *P. aeruginosa* PAO1 GeneChip hybridization and washing

Prior to the cDNA hybridization, the probe arrays were equilibrated at room temperature. The hybridization mixture contained 51 µl of fragmented and labeled cDNA in a MES hybridization buffer, 50 pM B2 control oligonucleotide, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated bovine serum albumin (BSA), 7 % v/v DMSO in a total reaction volume of 130 µl. This hybridization mix was loaded onto a GeneChip and incubated at 50 ^{o}C for 16 h at 60 rpm in a Affymetrix hybridization oven. After hybridization, the mixture was removed and the GeneChips were put into the Affymetrix Fluidics station for washing. The GeneChips were washed 20 times with nonstringent buffer (pH 7.7) at 25 ^{o}C and then 60 times with stringent buffer at 50 ^{o}C.

Following washing, the GeneChips were treated with a streptavidin solution mix for 10 min at 25 °C and washed 40 times with nonstringent buffer at 30 °C. Then each GeneChip was subjected to a secondary stain at 25 °C for 10 min and then to a tertiary stain at 25 °C for 10 min. Finally excess label was removed by 60 washings with nonstringent buffer at 30 °C.

The schematic representation of all the individual steps performed during GeneChip experiments are shown in figure 2.4.

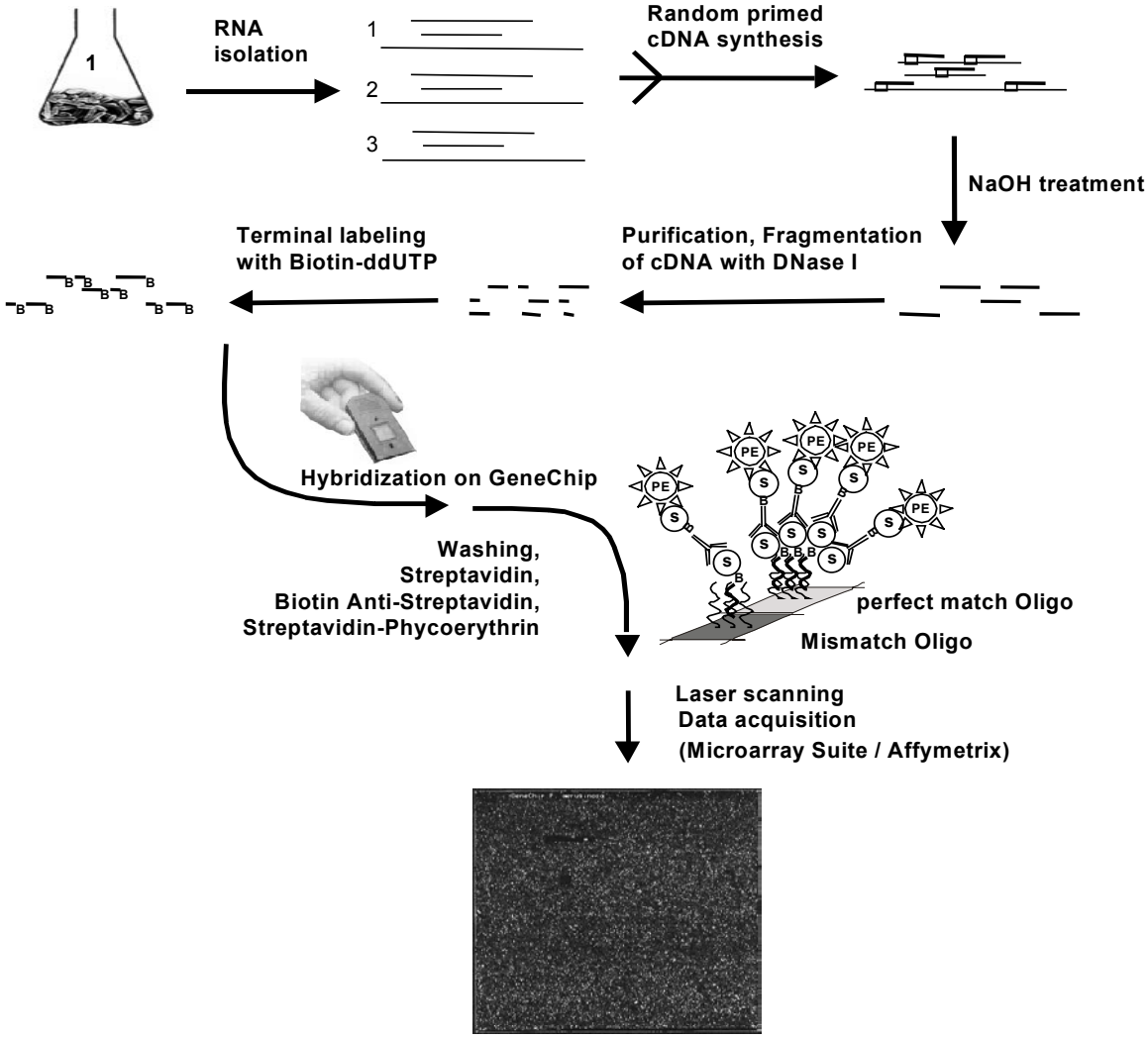


Fig. 2.4. Schematic representation of the individual steps performed during a GeneChip experiment (Courtesy by Goetz, 2003).

2.6.10. GeneChip analyses

Data analysis was performed using the Affymetrix Microarray Suite Software 5.0 with Affymetrix default parameters. A total of 2 GeneChips per strain and per growth condition were scanned at 570 nm with 3 µm resolution in an Affymetrix GeneChip scanner. The signals were calibrated by a scaling factor of 150. Two GeneChips for the test were paired with the control of the same strain resulting in 4 total pairs. The data was imported into a Microsoft Access databank to be capable to search for genes which significantly changed their signal intensities by Wilcoxon rank test and at least 2 fold change (MicroArray Suite 5.0 version) in all the 4 comparisons, described as four-comparison survival method (Chen *et al.*, 2000; Bakay *et al.*, 2002). The arithmetic average and the standard deviation (SD) of the 4 comparisons were calculated. As an independent criterion for significantly changed signal intensities, a Bonferroni correction of the signal ratios obtained from the MicroArray Suite 5.0 Programme was applied to account for the number of tests, i.e. in this case the total number of 5900 ORFs on the chip. First, the ratio of calibrated and corrected hybridization signals per gene S_i obtained of cultures grown under identical condition was verified to follow a Gaussian distribution and the variance σ was calculated. mRNA transcript levels of a gene i were considered to be significantly differentially expressed, if the ratio $S(i)_{test} / S(i)_{control}$ or $S(i)_{control} / S(i)_{test}$ exceeds the threshold $(1 + u\ \sigma)$, whereby the factor u defines that upper boundary of the normalized Gaussian integral $\Phi(u)$ where $\Phi(u) = x^n$ matches the Bonferroni-corrected 95 % confidence interval:

$$(1-\alpha) = x^n \qquad (\text{here: } n = 5900,\ \alpha = 0.025,\ 0.975 << x < 1.0).$$

The annotation of ORFs was extracted from the website of the *P. aeruginosa* PAO1 sequence project provided at http://www.pseudomonas.com. Differentially transcribed genes were classified into different functional groups according to metabolic categories except for orphan genes and conserved hypotheticals.

The bendability/curvature propensity plot was calculated with the bend.it server (http://www2.icgeb.trieste.it), using the DNase I based bendability parameters of Brukner *et al.* (1995) and the consensus bendability scale (Gabrielian *et al.*, 1996).

2.7. Examination of *P. aeruginosa* pathogenicity in murine infection model

During this work, we found some mutants which have impaired survival in PMNs and some mutants that even can survive better as compared to the *P. aeruginosa* TB wild type. In order to asses the roles of these attenuated genes on virulence, we used mouse infection model which was already established in Dr. Niels Hoiby's laboratory (Copenhagen). Dr. Hoiby is a member of European Graduate Program "Pseudomonas: Pathogenicity and Biotechnology". For this, the bacteria were entrapped into seaweed alginate, a substance which is chemically similar to the alginate produced in vivo by *P. aeruginosa* (Pedersen *et al.*, 1990). This model has been used in their laboratory for more than 10 years for different purposes (Johansen *et al.*, 1993; Johansen *et al.*, 1995; Song *et al.*, 1996; Song *et al.*, 1997; Moser *et al.*, 1997).

2.7.1. Mice

Female C3H/HeN mice were purchased from M & B Laboratory Animals (Ry, Denmark) at 10 to 11 weeks of age. The mice were of equal size and were maintained on standard mouse chow and water *ad libitum* for one week before challenge. All experiments were conducted in accordance to the existing permissions from appropriate ethical committees.

2.7.2. Bacterial inoculation and challenge procedure

2.7.2.1. Free bacteria

The infection inoculum was generated by growing *P.* aeruginosa TB in 250 ml flask containing 100 ml of LB broth at 37 ^{o}C for 18 h on a shaker. The cells were centrifuged (12,000 x *g*, 10 min, 4 ^{o}C) and the pellet was resuspended in LB broth, and adjusted to the appropriate challenge dosage in sterile, isotonic NaCl solution. The 0.04 ml of bacterial suspension having different cfu/ml was used for intratracheal challenge.

2.7.2.2. Immobilization of bacteria in alginate beads

The bacteria that were chosen depending on the requirement of experiment, were embedded into seaweed alginate beads as described previously (Johansen and Hoiby, 1999; Pedersen *et al.*, 1990). In brief, one bacterial colony was inoculated in 100 ml of LB broth and grown for 18 h at 37 ^{o}C (250 rpm). The culture was centrifuged (12.000 x *g*,10 min, 4 ^{o}C) and the pellet was resuspended in 5 ml of LB medium, then 1 ml suspension was serially diluted in the LB broth. This diluted 1 ml culture (depending on requirement of cfu) was mixed with 9 ml of autoclave sterilized seaweed alginate solution with 60 % guluronic acid content. The mixture was transferred to a sterile plastic cylinder equipped with an 18 G needle at the bottom. By applying a vertical pressure (0.3 hPa) to the top of the cylinder and simultaneously with a horizontal pressure (1.0 hPa) through the needle, the solution was forced to split into droplets of embedded bacteria in alginate beads. To stabilize the alginate beads, the droplets were collected in 200 ml of sterile Tris-buffer (pH 7.0) containing calcium chloride while stirring. Beads were continuously stirred for 1 h and then collected by centrifugation at 600 to 800 rpm for 10 min, washed twice in sterile isotonic NaCl solution. The final bead pellet was resuspended in 10 ml of sterile isotonic NaCl solution and the content of embedded bacteria in terms of colony forming units was estimated by serial dilutions. The beads were stored at 4 ^{o}C until further use. The challenge solution was composed by diluting the embedded bacteria in sterile isotonic NaCl to the desired final concentration (10^4 to 10^9 cfu/ml) according to the necessity of experiment. This concentration was verified by counting colony forming units from the serially diluted challenge solution.

2.7.3. Infection of mice with *P. aeruginosa* TB

The detailed procedure for bacterial inoculation was described by Dr. Johansen and Hoiby (1999). In brief, before surgical procedure, mice were anaesthetized by subcutaneous injection of 0.2 ml of Hypnorm and Midazolam mixture in sterile water. Anesthetized (sedated) mice were fixed. The trachea was exposed (figure 2.5 left) and then alginate beads were intratracheally instilled with an 18 G bead-tipped needle at a dose of 0.04 ml. The inoculum was installed in the left lung approximately 11 mm from the tracheal penetration site (figure 2.5 right). The incision was sutured with silk and healed without any complications.

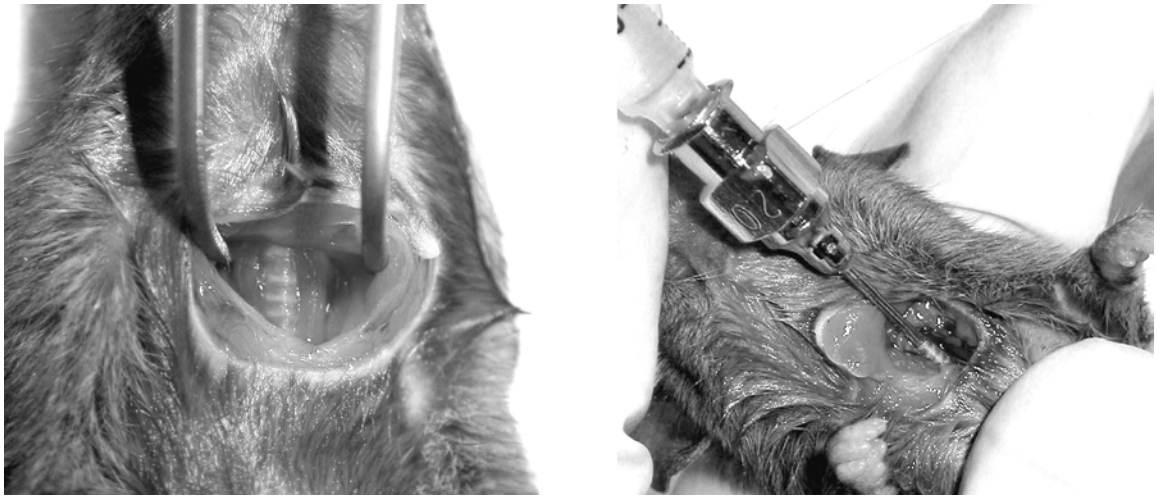


Fig. 2.5. Exposed trachea of an anaesthetized C3H/HeN mouse (left) and Intratracheal installation of the inoculum by a curved bead tipped needle in the left lung of same mouse (right).

2.7.4. Evaluation of different parameters

The mice were sacrified by intraperitoneal injection of 0.05 ml of 20 % pentobarbital at different time-points according to the requirement of the experiment. Then the thoracic cavity was exposed without damaging the lungs. These mice were used for the evaluation of different parameters.

2.7.4.1. Collection of peripheral blood

Blood was collected from the heart by cardiac puncture (figure 2.6) in a 1 ml syringe containing 20 µl heparin (5000 IE/ml) and immediately put on ice until further use.

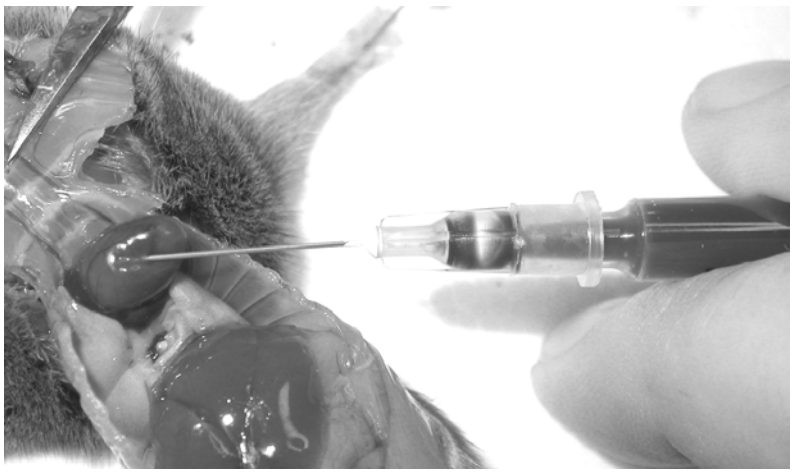


Fig. 2.6. Collection of peripheral blood from the heart

2.7.4.2. Collection of sera

Blood was obtained by cardiac puncture and collected in a 1 ml syringe and stored on ice for 1 h. Then the serum was separated by using centrifugation (14,000 x *g*, 10 min, 4 ^{o}C) and stored at −20 ^{o}C until further use.

2.7.5. Detection of lung bacteriology

The lungs removed from animals were immediately put into sterile containers and stored at 4 ^{o}C (less than 2 hours) until homogenization. The lung samples in 3−5 ml of cold autoclaved PBS buffer were homogenized with a blender (Heidolph, Struers, Denmark) at 4 ^{o}C. The series of diluted samples plated on blue agar-plates (States Serum Institute, Copenhagen, Denmark) containing selective culture medium for Gram-negative bacilli and the colony forming units were determined after 20−24 h of incubation at 37 ^{o}C.

2.7.6. Collection of bronchoalveolar lavage

The trachea was exposed and canulated with a size 22G catheter (OPTIVA* 2, Johnson & Johnson Medical, Brussels, Belgium). Lavage was performed by repeated flushing of lung with 1.5 ml of phosphate buffered saline (5 ^{o}C, without Ca^{2+} and Mg^{2+}) (PBS) for 6 times (figure 2.7). The bronchoalveolar lavage (BAL) fluid was stored on ice until examination of leukocytes.

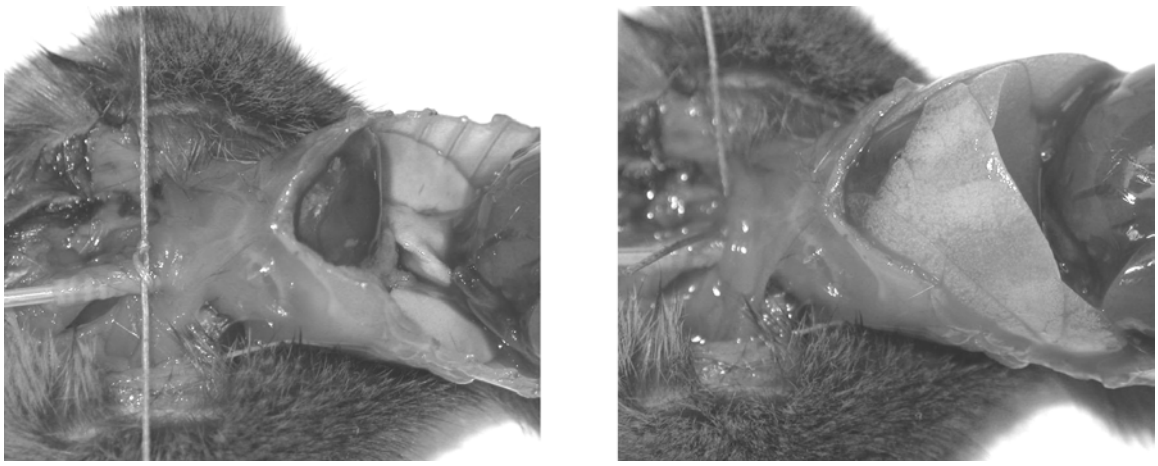


Fig. 2.7. Bronchoalveolar lavage (BAL) fluid collection. The collapsed lungs before flushing (left) and the expanded lungs before retrieving BAL-fluid (right).

2.7.7. Measurement of leukocytes from blood and BAL-fluid

The concentration of leukocytes in the peripheral blood and BAL-fluids was determined by estimating the relation between the number of leukocytes in a fixed volume of sample and detectable beads in a known amount. For this, 50 µl of blood or 200 µl of BAL-fluid was added to a TruCount Tube (Becton Dickinson). Cells were fixed, erythrocytes were lysed, and the DNA of the leukocytes was stained for discrimination by adding 450 µl of 10 % (v/v) FACS Lysing Solution (Becton Dickinson) and 100 µg/ml propidium iodide in MiliQ water. The samples were kept on ice in the dark for at least 10 minutes before analysis by flow cytometry. In the blood, mouse PMNs were discriminated by the morphology dependent light scatter.

2.7.8. Estimation of leukocyte phenotypes

Immunofluorescent staining of the phenotype was used for discrimination of the leukocyte subpopulations and determination of the expression of activation markers For this, 75 µl of peripheral blood or 100 µl BAL-fluid was added to 4 ml cold lysing-buffer in Falcon tubes. The cell pellet obtained after centrifugation was washed in PBS before adding the antibodies. The immuno-fluorescent staining was performed by adding monoclonal antibodies (as shown in table 2.2). All antibodies used were monoclonal rat antibodies.

Table 2.2. Monoclonal rat anti-mouse antibodies.

Antibody	Target	Conjugated Fluorochrome	Amount (µl)	Clone	Isotype	Company
Ly-6G	PMNs	PE	1	RB6-8C5	IgG_{2b}, κ	Pharmingen
F4/80	monocytes	FITC	5	CI:A3-1	IgG_{2b}, κ	Serotec
CD11b	Mac-1	APC	5	M1/70	IgG_{2b}, κ	Pharmingen
CD45	leukocytes	PerCP	5	30-F11	IgG_{2b}, κ	Pharmingen

FITC: Fluorescein isothiocyanate; PerCP: Peridinin chlorophyll A protein; PE: Phycoerythrin; APC: Allophycocyanin, Pharmingen, San Diego, CA, US; Serotec: Oslo, Norway.

The cells were incubated on ice in the dark for 30 min. Excess antibodies were removed by adding 4 ml of cold PBS followed by centrifugation (10 min, 350 x *g*) and

decanting. The stained cells were fixed by adding 150 µl 2 % (v/v) paraformaldehyde in PBS and used for flow cytometry.

2.7.9. Measurement of respiratory burst

The respiratory burst was estimated using the BURSTEST kit (Orpegen Pharma). This assay measures the leukocyte oxidative burst by the fluorescence obtained from intracellular rhodamine 123 after the oxidization of dihydrorhodamine 123 by intracellular H_2O_2. The kit was used in accordance to the modification of the manufacturer's prescription.

In brief, 50 µl of peripheral blood or 200 µl of BAL-fluid was added to a Falcon tube and kept on ice for 10 min. To measure the induced burst, 10 µl of opsonized *E. coli* and 10 µl dihydrorhodamine 123 solution were added to the blood samples and 40 µl opsonized *E. coli* and 40 µl dihydrorhodamine 123 to the BAL-fluid samples. To estimate the spontaneous burst, 10 µl dihydrorhodamine 123 solution were added to the blood samples and 40 µl dihydrorhodamine 123 solution were added to the BAL-fluid samples. A corresponding volume of PBS was added to samples for measuring the baseline. The samples were incubated at 37 ^{o}C for 10 min in a water bath. The incubation time was decided in a pilot experiment where the maximum intensity of the specific signal was reached after 40 min in blood samples from 3 mice.

The reaction was terminated by placing the samples on ice and the samples were fixed in 1 ml FACS Lysing Solution (Becton Dickinson) diluted 10 times in MilliQ water for 20 min. The samples were centrifuged (7 min, 350 x *g*, 4 ^{o}C) and the supernatant was discarded. 3 ml PBS were added and the samples were centrifuged (7 min, 350 x *g*, 4 ^{o}C) and the supernatant was discarded**.** 200 µl of DNA staining solution were added and the samples were incubated on ice for at least 10 min before analysis by flow cytometry.

2.7.10. Measurement of cytokine production

The concentration of cytokines was measured in samples derived from lung. Lung homogenizate was centrifuged (5,000 x *g*, 10 min, 4 ^{o}C) and the supernatant obtained was filtered through 0.2 µM filters and stored at −20 ^{o}C until further use.

The concentrations of cytokines in the lung supernatant were determined by sandwich ELISA kits commercially available from two manufactures. Quantikine (R&D Systems, Abingdon, UK) kits were used for detection of mouse MIP-2, KC, IL-12, and GM-CSF. OptEIA (Pharmingen) kits were applied for measuring the mouse interferon-γ (IFN-γ) and TNF-α. Cytokine measurements were performed according to the manufacturer's description.

a) Measurement of cytokines by Quantikine kit

50 µl of samples and standards in assay-diluent were added in duplicates to the microtiter plates coated with capture antibody and incubated for 2 h. The microtiter plates were washed 5 times in 200 µl washing solution [PBS with 0.05 % (v/v) Tween 20]. 50 µl of detection antibody in conjugate-diluent were added for 2 h. Excess detection antibody was removed by washing 5 times in 200 µl washing solution and colour reagents were added. After 30 min incubation, coloring reaction was terminated by adding stop-solution and the microtiter plates were analyzed on a ELISA reader (Bio-Rad, Tokyo, Japan).

b) Measurement of cytokines by OptEIA kit

The microtiter plates were coated with capture antibody overnight. Excess capture antibody was removed by washing 5 times in 200 µl washing solution. To reduce unspecific binding, 200 µl of 10 % (v/v) FCS in PBS were added for 30 min and the microtiter plates were washed 5 times. 50 µl of samples and standards in assay-diluent were added in duplicates and incubated for 2 h. The microtiter plates were washed 5 times with 200 µl washing solution. 100 µl of biotin conjugated detection antibody and avidin conjugated horse radish peroxidase (HRP) were added and incubated for 1 h. Excess detection antibody and HRP were removed by washing 5 times with 200 µl washing solution. 100 µl TMBPlus ready to use substrate (Kementec, Copenhagen, Denmark) was added in the plate. When appropriate color intensity was achieved, 50 µl of 2N H_2SO_4 were added and the microtiter plates were analyzed on ELISA reader.

The optical density (OD) of stained microtiter plates was immediately recorded at 492 nm by an ELISA reader (Bio-Rad, Tokyo, Japan). The mean OD of the duplicates was calculated and the cytokine concentration was estimated by projection of the

standard curve. The analytical variation was calculated from the standard curves using the formula for the coefficient of variation (CV): CV = SD/m where SD is the standard deviation and m is the mean. The analytical variation did not exceed more than 20 %.

2.7.11. Flow cytometric measurements

The samples were analyzed using a FACSort (Becton Dickinson) equipped with a 15 mW argon-ion laser tuned at 488 nm for excitation and a red diode laser for excitation at 635 nm. Light scatter and logarithmically amplified fluorescence parameters from at least 10,000 events, when possible, were recorded in list mode after gating on light scatter to avoid debris, cell aggregates, and bacteria. The instrument was calibrated using Calibrite (Becton Dickinson). Leukocytes were identified according to their morphology, by their expression of CD45, and their content of DNA. The specific fluorescence from an immunochemically stained sample was calculated by subtracting the fluorescence intensity from samples stained with irrelevant isotypic control antibodies. In samples stained for respiratory burst, the baseline was determined from samples not incubated with 123-dihydrorhodamine.

2.7.12. Statistical analysis

Data were analyzed by Mann-Whitney U test, Kruskal-Walis test, Chi-squared test, t-test, Wilcoxon-signed pair-differences rank test, and Spearman Rank Correlation test using Staview 4.51 (Abacus Concepts, Inc., Berkeley, CA, US) software for Macintosh.

3. RESULTS AND DISCUSSION

A highly virulent *P. aeruginosa* TB (TBCF10839) strain was isolated from the lung of a cystic fibrosis patient (Miethke, 1985). This isolate belongs to a clone which was responsible for nosocomial infections in the burn unit and the cystic fibrosis unit at the Medizinische Hochschule Hannover (Tümmler, 1987). The severe problems in eliminating *P. aeruginosa* TB infection after heavy antibiotic therapy indicated that the bacterium either exhibits high resistance or an atypical pathogenicity mechanism. After further investigation in the supervisor's laboratory, it turned out that *P. aeruginosa* TB (TBCF10839) survives and multiplies both in the phagolysosomal compartment and the cytosol of polymorphonuclear granulocytes. When *P. aeruginosa* isolates (PAO1 or SG17M or C) were incubated with the freshly isolated granulocytes, all the cells were lysed within a 30 min incubation period (figure 1.4A). However, this CF isolate survived in the granulocytes for the same incubation period (figure 1.4B), and it showed the cell division intracellularly in granulocytes when incubated for 1 h (figure 1.5).

The dominant-negative selection by signature-tagged mutagenesis (STM) (Hensel *et al.*, 1995) has been applied to unravel the etiology of this uncommon virulence trait. As the first step, the available STM protocol had to be adapted and optimized to the needs of the GC-rich *P. aeruginosa* (Wiehlmann *et al.*, 2002). STM allows for the simultaneous examination of a large number of isogenic transposon mutants that are differentiated by unique DNA marker sequences. Genes that are essential for survival in the habitat of interest are identified by dominant-negative selection. Persistence of bacteria in professional phagocytes is an optimal bioassay to perform genomewide scans by signature tagged mutagenesis. *P. aeruginosa* TB transposon mutants that do not survive the negative selection in PMNs are defective in the expression of a gene or operon that is essential for the bacterium's intracellular survival and hence are interesting for further examination. As a first step, we have screened the remaining pool of mutants for key genes that are essential for intracellular growth of *P. aeruginosa* TB.

3.1. Screening of STM library for intracellular survival in PMN

We have screened the STM library in the phagocytosis (selection) assays for the identification of genes that are necessary for intracellular survival in this CF isolate. *P. aeruginosa* 892 was also added in each selection experiment to maintain the constant selection pressure on the individual mutant and the granulocytes. Thus the results obtained were comparable. The phagocytosis assays were performed according to the previously described procedure (Wiehlmann, 2001). Each selection assay comprised of 46 *P. aeruginosa* TB transposon mutants and *P. aeruginosa* 892 (A1) and *P. aeruginosa* PAO1 (A2) that contain the transposon episomally. Before beginning the experiment, bacteria were grown separately in LB medium until stationary phase by overnight incubation. This growth up to stationary phase was highly important before incubating the *P. aeruginosa* TB transposon mutants with granulocytes, because *P. aeruginosa* produces a high number of cytotoxic factors particularly during the growth in logarithmic phase which damage the bacterial phagocytosis by granulocytes. In contrast, production of these cytotoxic factors by *P. aeruginosa* was strongly reduced during the growth in stationary phase (Dacheux *et al.*, 2000). The objectives of the present investigation were to find out the genes which are necessary for intracellular survival in PMNs. So if the phagocytosis of *P. aeruginosa* TB in granulocytes is damaged, the selection pressure on the bacteria can decrease making the identification more difficult.

A pool of separately grown 48 mutants was examined for intracellular survival in PMNs in one experiment. The pooled mutants were mixed with the granulocytes and incubated on a shaker. During the initial period, the mutants were phagocyted and exposed to different environmental conditions in the PMNs. Two hours of incubation period was kept for all the selection experiments. A longer incubation was not possible since the whole experiment should finish within 6 hours after withdrawal of blood.

After the selection, the amplified signal sequences were hybridized on the dot blots which were previously prepared by the PCR amplification of signal sequences from

donor plasmids. The signal intensities from the output pool were compared with the signal intensities from the input pool.

3.1.1. Evaluation of selection

The evaluation of the selection results was performed by determining the optical density of the radiographic (X-ray) film after hybridization. The membranes developed by CDP star were exposed to X-ray films for a short time as to identify not only loss-of-function mutants (arrow 2 in figure 3.1) but also gain-of-function mutants (arrow 3 in figure 3.1). These X-ray films were scanned and densities of the respective dots was determined with the PCBAS program, version 2.09f. The example of the scanned blot for selection is shown in the figure 3.1. The signal intensities from selection (input pool) and control (output pool) were normalized separately with the intensities of respective control strains at the positions A1 and A2. The ratio (quotient) from the normalized signal intensities of selection and control was determined. Then the survival of each *P. aeruginosa* TB transposon mutant was compared with the control strains (A1 and A2). The control strains *P. aeruginosa* 892 and *P. aeruginosa* PAO1 were used due to their different intracellular survival ability.

***P. aeruginosa* 892 (position A1):** This bacterium is a clonal variant of *P. aeruginosa* TB and survived in the granulocytes nearly as good as TB. So this was considered as the positive control. *P. aeruginosa* TB transposon mutant in which a gene essential for intracellular survival is mutated, should exhibit a lower survival rate than *P. aeruginosa* 892.

***P. aeruginosa* PAO1 (position A2):** This bacterium can not survive intracellularly in PMNs. *P. aeruginosa* TB transposon mutant in which a gene essential for intracellular survival is mutated, should exhibit a survival rate comparable to *P. aeruginosa* PAO1. The positions of A1 and A2 are shown in the figure 3.1.

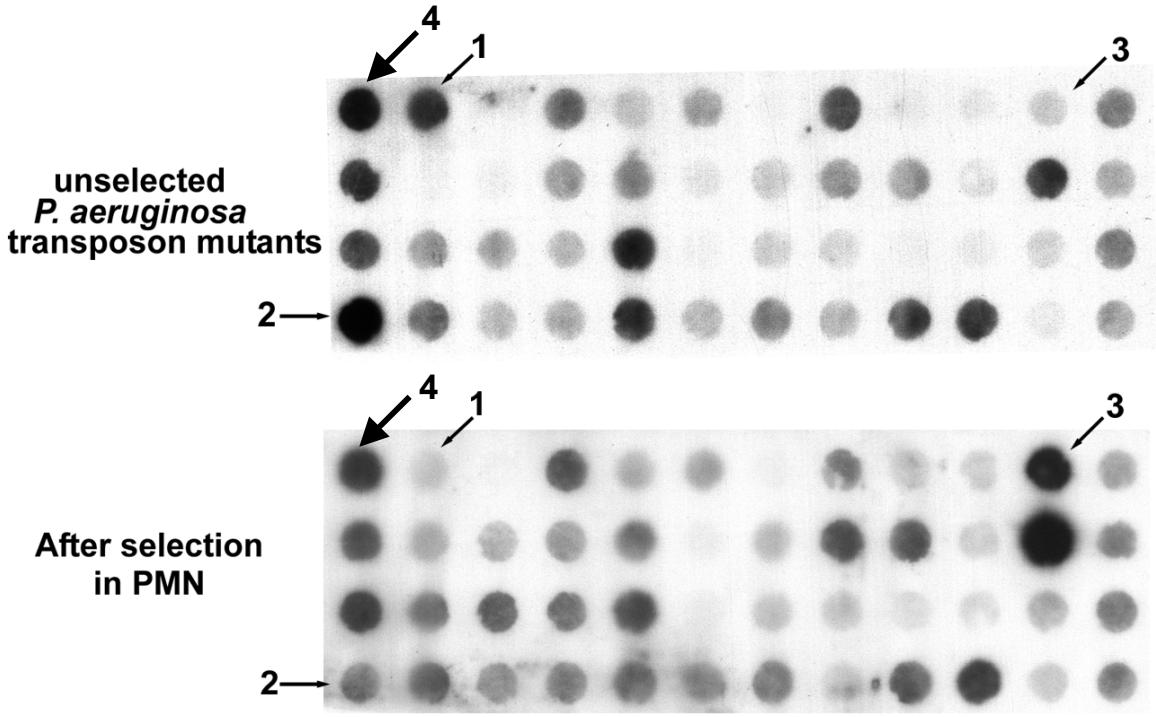


Fig. 3.1. Detection of the signature tags to determine the intracellular survival rate of *P. aeruginosa* TBCF10839 transposon mutants. 48 transposon mutants were checked for their intracellular survival. DIG-labeled, *Hin*dIII- digested signal sequences of the bacteria not subjected to selection ("unselected *P. aeruginosa* transposon mutants", upper panel) and of bacteria recovered from the PMN phagocytosis assay ("after selection in PMN", lower panel) were hybridized onto dot blots of the signal sequences of the pTnModOGm SigTag. The membranes were developed with CDP- Star for a short time as to identify not only loss- of- function (arrow 2) but also gain of function mutants (arrow 3). Mutants indicated by significantly different signal strength on the two blots were subjected to further analysis. A co- selected transposon mutant of *P. aeruginosa* PAO1 that is not able to survive intracellularly in PMN served as negative control (A2 position indicated by arrow 1) and transposon mutant of *P. aeruginosa* 892 that is able to survive intracellularly in PMN served as positive control (A1 position indicated by arrow 4).

After determining the signal intensity, those mutants were selected which showed the signal intensity maximum of 20 % to the positive control or the average value for loss of function mutant and double or more for gain of function mutant. From each pool, 5–10 mutants were identified as attenuated in the infection model. At some positions in the input pool, signals were absent or very weak, indicating that the particular mutant does not survive in the PMNs or the signals observed were very strong as compared to the signals from the output pool indicating that the corresponding mutant survives better than the wild type *P. aeruginosa* TB. About 200 transposon

mutants with significant differences in signal strength compared to the average were identified in the first round of selection. These mutants were re-tested in the additional selection assays. In this way, we screened the remaining pool of non-screened library consisting of 1344 transposon mutants for their intracellular survival ability in human PMNs and 960 mutants were screened by the previous student during his Ph. D. studies (Wiehlmann, 2001).

Out of these 1344 mutants, we found 29 mutants with consistently high differences in their survival rates in comparison to the *P. aeruginosa* TB wild type. Out of these 29 mutants, 22 mutants which has impaired survival in PMNs (loss of function mutants), while 7 mutants which survived even better (gain of function mutants) as compare to the *P. aeruginosa* TB wild type. The results of the STM library screening for intracellular survival in PMNs are presented below in short.

Loss-of-function mutants (with reduced survival):

9B9	10B11	10B12	11D1	11D7	25A12
25D12	30B5	30B7	30B12	30D5	31B11
32B12	32C1	32D9	36C8	36D6	36D10
40B9	42D8	45A7	45A10		

Gain-of-function mutants (with increased survival):

25C8	25D6	26C2	29D2	35D1	38A11
40D12					

The name of transposon mutant refers to their position in the library of *P. aeruginosa* TB. One selection level consist of 48 mutants, which were arranged in four rows (A-D) with 12 positions in each row (1−12). For example, a transposon mutant that was arranged in the selection level 25 and in the row C at position 8 gets the name 25C8.

The disrupted genes of these 29 mutants with consistently high differences in their survival rates in comparison to the wild type were sequenced. After comparing the gene sequences with the PAO1 genome, it was observed that most of the genes for the intracellular survival in PMN are already known from PAO1 genome. 27 of these genes exhibited about 99.5 % sequence identity to known *P. aeruginosa* PAO1

genes. Two other sequences are strain specific, one with no homologies to any known sequence, the other with some homology to an alcohol dehydrogenase.

3.2. Characterization of STM knockout mutants

STM technology was applied for the identification of genes which are essential for the survival in a particular habitat. After screening the STM library, one can receive the information which genes are essential for survival under the particular selection conditions. However, the function of these genes with respect to intracellular survival is not known. For this, each identified mutant should be characterized further to yield more functional information.

3.2.1. In silico characterization of *P. aeruginosa* TB mutants

In silico analysis was performed for each mutant to get more information about the influence on the neighbouring genes due to the transposon insertion. A survival rate with respect to the positive control (*P. aeruginosa* 892) was also calculated for each mutant. It indicates the degree of intracellular survival ability in PMNs. This information is tabulated in the following table (Table 3.1).

Table 3.1. *P. aeruginosa* TBCF10839 genes involved in virulence.

Mutant	PAO No.[1]	Degree of intracellular survival (%)[2]	Possible polarity effect[3]
Loss-of-function mutants (with reduced survival):			
9B9	PA4613, *katB*	31	No, transcription termination behind gene
10B11	PA1510	37	Yes, unknown ORFs PA1509–PA1508
10B12	PA2239	29	Yes, unknown ORFs PA2240–PA2242, probable polysaccharide biosynthesis
11D1	PA1954	15	No, transcription termination behind gene
11D7	PA1104, *fliI*	25	Yes, *fliJ*
25A12	TB02	26	Yes, unknown ORFs (see 3.4.1)
25D12	PA4489	42	Yes, unknown ORFs PA4487–PA4488
30B5	PA1174, *napA*	22	Yes, *napB*, *napC*
30B7	PA2822	19	No, terminal gene of operon
30B12	PA3933	15	No
30D5	PA1920	16	No
31B11	PA4065	32	Yes, unknown ORF PA4066

Mutant	PAO No.[1]	Degree of intracellular survival (%)[2]	Possible polarity effect[3]
32B12	PA0694, *exbD2*	21	Yes, unknown ORF PA0695
32C1	PA5177	35	No
32D9	PA0880	26	Yes, unknown ORFs PA0881–PA0883
36C8	PA5220	29	No, last gene of operon
36D6	PA4034, *aqpZ*	37	No
36D10	PA5248	22	Yes, unknown ORF PA5247
40B9	PA5373, *betB*	28	Yes, *betI*
42D8	PA2242	15	Yes, unknown ORFs PA2243-PA2245
45A7	TB01	26	Yes, unknown ORFs (see 3.4.1)
45A10	PA1452, *flhA*	18	Yes, *flhF, flhN, fliA*
Gain-of-function mutants (with increased survival):			
25C8	PA4554, *pilY1*	540	Yes, *pilY2, pilE*
25D6	PA3270	625	No, transcription termination behind gene
26C2	PA4186	1170	No, transcription termination behind gene
29D2	PA4228, *pchD*	850	Yes, *pchA*, *pchB*, *pchC*
35D1	PA3704, *wspE*	720	Yes, *wspA*, *wspB*, *wspC*, *wspD*
38A11	PA0845	710	No
40D12	PA1677/ 1678	1480	Yes, intergenic region of unknown ORFs PA1677 or PA1678

[1]: Gene names are given in italics.

[2]: Degree of intracellular survival was calculated from five independent experiments, survival of *P. aeruginosa* 892 (positive control) was considered as 100%.

[3]: Polarity, the downstream genes are considered from *P. aeruginosa* PAO1 database (http://pseudomonas.com).

A previous student has also identified 15 mutants during the screening for intracellular survival in PMNs (Wiehlmann 2001). Altogether, we have found 44 mutants, out of which 35 mutants have impaired survival ability (loss of function), while the other 9 mutants survived even better than the TB wild type. The disrupted genes of these 44 mutants with consistently high differences in their survival rates in comparison to the wild type were sequenced. Forty of these genes exhibit about 99.5 % sequence identity to already known *P. aeruginosa* PAO1 genes. Three other sequences are unknown, two with no homologies to any known sequence, the other with some homology to an alcohol dehydrogenase. The identified genes were categorized according to their known or estimated function (Table 3.2). From this data, the identified genes with known functions are related to oxidative stress

response, flagella and Type IV pili biosynthesis, membrane proteins and strain specific or are involved in a regulatory function.

Table 3.2. Screening of *P. aeruginosa* TBCF10839 STM mutants in PMN phagocytosis assay: localization of the Tn*5* insertion in gain- of- function and loss- of-function mutants.

Mutant[a]	PAO1 No.	Identity (%)	Annotation[e]
Category I: loss of function (reduced survival in granulocytes)			
Oxidative stress response			
3D1[a]	PA1288	99.6	outer membrane protein precursor fadL, putative
14C5[a]	PA3344	99.4	ATP-dependent DNA helicase, *recQ*
9B9	PA4613	99.6	promoter region of catalase, *katB*
2D5[a]	PA4621	99.9	oxidoreductase, putative
41D3[a]	PA5349	99.8	rubredoxin reductase, putative
40B9	PA5373	99.5	betaine aldehyde dehydrogenase, *betB*
Oxidative stress response, putative			
32D9	PA0880	99.6	ring cleaving dioxygenase, putative
30B5	PA1174	100	periplasmic nitrate reductase, *napA*
Flagella biogenesis			
11D7	PA1104	98.6	flagellum-specific ATP synthase, *fliI*
22D11[a]	PA1441	99.6	hypothetical protein, 47% similar to fliK of *Salmonella typhimurium*
45A10	PA1452	99.7	flagellar biosynthesis protein, *flhA*
Membrane protein and receptors			
32B12	PA0694	99.6	transport protein, *exbD*
30B12	PA3933	99.3	choline transporter betT3, putative
36D6	PA4034	99.3	aquaporin Z, *aqpZ*
31B11	PA4065	99.9	hypothetical protein; membrane protein
36D10	PA5248	98.7	hypothetical protein; membrane protein
15A3[a]	PA5252	99.8	ATP-binding component of ABC transporter, putative
Regulation			
23B9[a]	PA2468	99.8	sigma-70 factor, putative
24A12[a]	PA3192	99.6	two-component response regulator, *gltR*
Unknown and hypothetical			
10B11	PA1510	99.5	hypothetical protein
19C2 [a]	PA1572	99.7	conserved hypothetical protein
30D5	PA1920	99.6	conserved hypothetical protein
11D1	PA1954	99.4	hypothetical protein
10B12	PA2239	99.5	transferase, putative

Mutant[a]	PAO1 No.	Identity (%)	Annotation[e]
18A12[a]	PA2613	99.7	conserved hypothetical protein
30B7	PA2822	99.5	conserved hypothetical protein
20A6[a]	PA3953	99.9	conserved hypothetical protein
25D12	PA4489	99.6	hypothetical protein
32C1	PA5177	99.4	conserved hypothetical protein
42D8	PA2242	99.5	hypothetical protein
36C8	PA5220	99.7	hypothetical protein
24C5[a]	PA5551	99.6	hypothetical protein
Not present in PAO1			
45A7	TB01 [b]	71%	dehydrogenase, putative
25A12	TB02 [c]	0%	no homologies
47D7[a]	TB03 [d]	0%	no homologies
Category II: enhanced survival in granulocytes			
Type IV pili biogenesis			
35D1	PA3704	100	chemotaxis sensor/effector fusion protein, *wspE*
25C8	PA4554	99.3	type 4 fimbrial biogenesis protein, *pilY1*
15B10[a]	PA5040	99.4	type 4 fimbrial biogenesis protein, *pilQ*
Other genes			
14B12[a]	PA1992	100	two-component sensor, putative
29D2	PA4228	99.4	pyochelin biosynthesis protein, *pchD*
Unknown and hypothetical			
38A11	PA0845	99.8	conserved hypothetical protein
40D12	PA1677/PA1678	99.8	intergenic region between hypothetical ORF and DNA methylase
25D6	PA3270	100	hypothetical protein
26C2	PA4186	99.3	hypothetical protein

[a]: These genes were identified in the previous screening for intracellular survival in PMNs by a previous student (Wiehlmann Ph.D. thesis, 2001).

[b]: Strong homology with dehydrogenases

nucleotide level:

gi|887430|emb|X73835.1|ECFDH *E.coli* plasmid DNA for formaldehyde-dehydrogenase,
Expectation value = 1e-92

gi|15829254|ref|NC_002695.1| *E. coli* O157:H7, complete genome,
Expectation value = 2e-69

amino acid level:

gi|887431|emb|CAA52057.1| (X73835) formaldehyde dehydrogenase (glutathione) *E. coli*;
Expectation value = e-180

gi|15800087|ref|NP_286099.1| (NC_002655) alcohol dehydrogenase class III; formaldehyde dehydrogenase, glutathione-dependent *E. coli* O157:H7, Expectation value = e-173

[c]: No significant homologies (all expectation values > 0.3) at nucleotide and protein level to any gene deposited in the NCBI database, only present in clonal variants of *P. aeruginosa* TBCF10839.

[d]: Present in other clonal variants of *P. aeruginosa* TBCF10839 and clone C; no significant homologies (all expectation values > 0.1) at nucleotide level; low homology at amino acid level to (gi|16505895|emb|CAD09774.1| (AL513383) hypothetical protein *Salmonella enterica* subsp. enterica serovar Typhi, Expectation value = 3e-08

[e]: Gene names are indicated in italics.

A transposon insertion in 35 of the identified 44 ORFs resulted in a decreased intracellular survival rate, while with an insertion in 9 genes, the bacterium was able to survive even better in PMNs than the TB wild type. The function of the majority of ORFs has yet to be elucidated. The search in the databases did not provide a clue about the encoded function for 22 out of the 44 identified genes. Two of these 22 genes have no known homolog in the databases yet. A remarkable feature of 95 % of the sequenced genes is that they are already known sequences from the PAO1 genome and exhibit an average of 99.7 % nucleotide homology (Table 3.2).

Taking into account that the transposon mutants of the constructed library were selected not to be auxotrophic mutants (Wiehlmann, Ph. D. thesis, 2001) and that the redundancy of the library is less than 10 %, the 2300 asservated mutants should represent mutations in a majority of all phenotypically relevant genes or operons. The high amount of known PAO1 genes indicate that strain PAO1 is equipped with almost all genes necessary for intracellular survival in PMNs. The change from an opportunistic pathogen to a highly virulent pathogen apparently requires only a few minor genetic changes. Amongst the 22 clearly annotated genes (Table 3.2), the two largest subsets of genes are either associated with oxidative stress response or the flagellar export and synthesis machinery. The respective mutants were subjected to functional analysis.

3.2.2. Oxidative stress response in *P. aeruginosa* TB

Analysis of the resistance of the bacteria to hydrogen peroxide allowed for the examination of the oxidative stress response of the mutants. As hydrogen peroxide is an unstable compound, the bacteria are only initially subjected to this particular stress factor. After only a few hours, the hydrogen peroxide concentration in the

medium has decreased sufficiently to allow for the surviving *P. aeruginosa* to grow. The conditions of this experiment correspond to those found in PMN, where the bacteria are also initially subjected to considerable oxidative stress. Afterwards, the severe damage to the PMN results in cell death. The outcome of the STM- screening demonstrates that tolerance to initial oxidative stress is instrumental to persist in the hostile environment of the PMN.

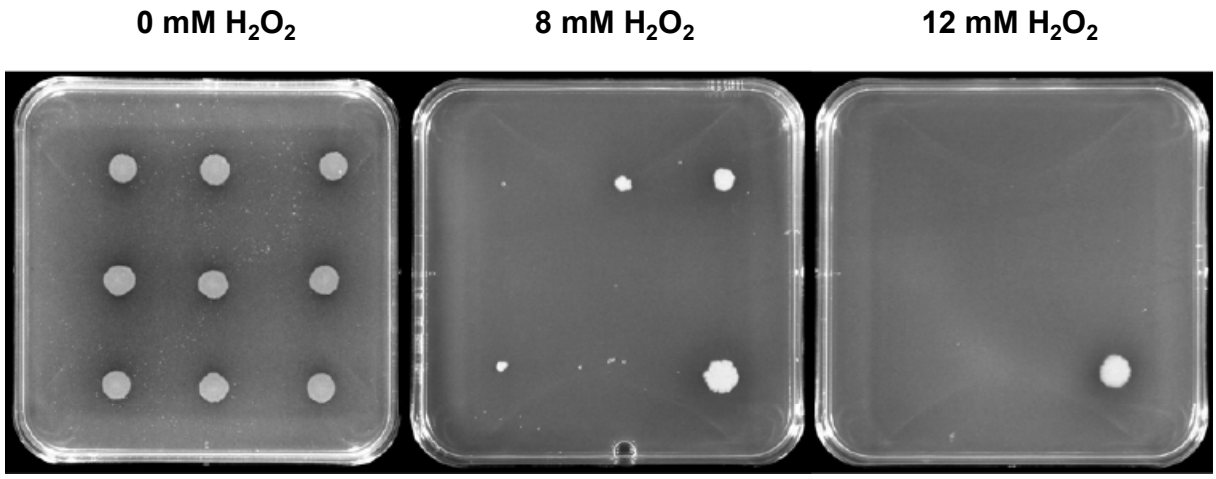


Fig. 3.2. Sensitivity of *P. aeruginosa* TBCF10839 transposon mutants with impaired survival in PMNs towards hydrogen peroxide. TBCF10839 wild type and transposon mutants were incubated overnight at 37°C on LB agar containing initially 8 or 12 mM hydrogen peroxide in order to assay the sensitivity towards oxidative stress. LB agar without hydrogen peroxide was used as control (left plate) and significant growth was observed for all strains. Only the TBCF10839 wild type strain exhibited comparable growth on hydrogen peroxide-containing LB medium (right plate). The two transposon mutants in the choline transporter betT3 and aquaporin Z were also impaired in growth. The other six mutants whereby each carried the transposon in a gene assigned to the category 'oxidative stress response' (Table 3.2) did not grow at all. (From left to right: upper row (mutants with defects in osmoregulation): PA5373 (*betB*), PA4034 (*aqpZ*), PA3933 (*betT3*), middle row: PA5349 (*rubR*), PA4621, PA4613 (*katB*), lower row: PA3344 (*recQ*), PA1288 (*fadL*), *P. aeruginosa* TBCF10839 (wt)

Eight out of the 44 mutants did not grow in the presence of 12 mM hydrogen peroxide (figure 3.2). Besides obvious candidates such as catalase (PA4613) and helicase (PA3344) which are involved in the inactivation of peroxide or in the repair of oxidative DNA- damage, the relevance of other elements such as the rubredoxin reductase (PA5349) for the resistance against oxidative stress is less obvious. Rubredoxin reductases have been identified in many bacteria, can substitute for superoxide dismutase (Lumppio *et al.*, 2001; Pianzzola *et al.*, 1996) and are involved in the oxidation of aliphatic hydrocarbon compounds (Eggink *et al.*, 1990). The

homologs of PA1288 (FadL) transport long-chain aliphatic hydrocarbons in numerous Gram-negative bacteria (DiRusso and Black, 1999) and it is reasonable to assume that in *P. aeruginosa* FadL also provides the substrate for rubredoxin reductase. Hence we would like to conclude that the capture of oxygen radicals by long-chain hydrocarbons is an essential pseudomonal weapon against PMNs.

3.2.3. Flagellar export and synthesis machinery

Three mutants with decreased survival in PMNs carry the transposon in the *fliI* (PA1104), *fliK* (PA1441) and *flhA* (PA1452) genes of the flagellar biogenesis pathway. Motility was assessed in terms of swimming and swarming on agar plates. Swarming which requires pili and flagellum (Köhler *et al.*, 2000) was found not to be impaired in the three mutants (data not shown). The *flhA* and *fliK* mutants were non-motile, whereas motility of the *fliI* mutant was somewhat lower than that of the wild-type TBCF10839 strain (figure 3.3).

The cellular morphotypes and the structure of the flagellum were characterized by electron microsocopy in co-operation with G. Brandes (Medizinische Hochschule Hannover). The *flhA* Tn*5* mutant turned out to be non-flagellated (figure 3.3). The *fliK* transposon mutant showed an elongated hook structure and misassembled filaments (figure 3.3 & 3.4) as it has been observed in severely affected *fliK* mutants of *Salmonella enterica* serovar Typhimurium (Williams *et al.*, 1996; Minamino *et al.*, 1999). The flagellin FliC was demonstrated to be incorporated into these aberrant hooks by both immunogold labeling (figure 3.4) and immunoblot with anti-FliC (type b) antibody (data not shown). The hook-type mutant flagella of the mutant Tn*5*::fliK strain are decorated with anti flagellin- gold particles along the whole surface which shows up by the negatively stained oblique stripes. The density of the antibody - protein A - complexes is higher on Tn*5*::fliK mutant flagella than on those of wild type strain TBCF10839. Apparently the flagellin monomers of the Tn*5*::fliK mutant are assembled to a flagellar structure with a hook- like relief that exposes more epitopes on its surface than the wild- type flagella. The *fliI* Tn*5* mutant exhibited a variable number of one to six polar flagella (figure 3.3). The bacterial suspension contained many isolated flagella with no basal body. Preparations of isolated flagella were a mixture of about 10 % normal flagella and about 90 % of flagella without the MS and C rings of the basal body suggesting that the physical interaction between the

external rings and the flagellar protein export apparatus (Minamino and Macnab, 1999) is very labile. In other words, FliI seems to be necessary for the tight association of the external ring with the export apparatus, rod and hook (figure 3.5).

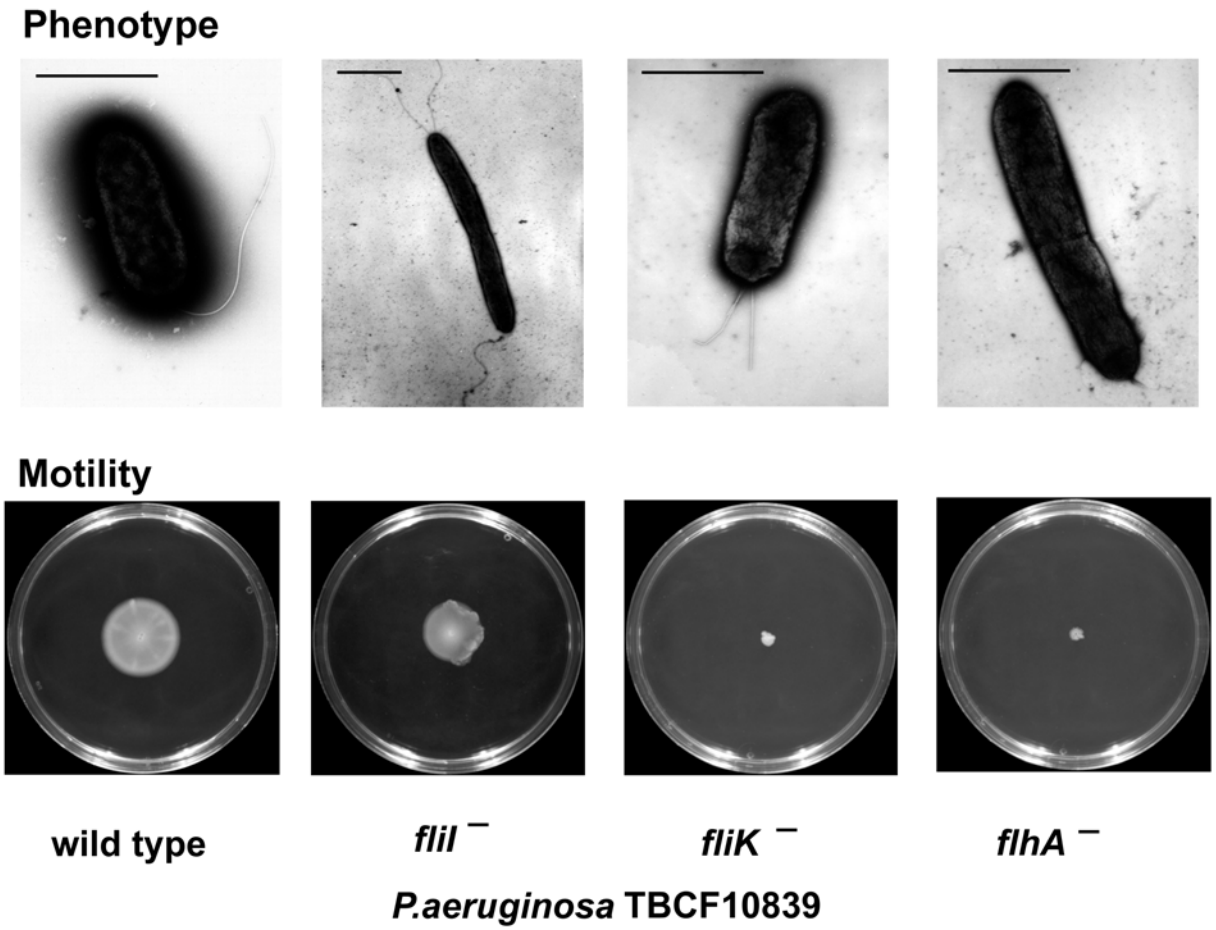


Fig. 3.3. Morphotype (upper panel) and swimming phenotype (lower panel) of the *P. aeruginosa* TBCF10839 strain and its isogenic transposon mutants in *fliI, fliK* and *flhA*. The bar indicates the length of 1 µm. (Courtesy by G. Brandes for EM of flagella mutants).

So far only few elements of the flagellar export and synthesis machinery have been characterized in *P. aeruginosa* (Simpson *et al.*, 1995; Arora *et al.*, 1996; Fleiszig *et al.*, 2001). The primary sequence of the individual genes and the overall genetic organization (Stover *et al.*, 2000) is highly homologous to that in *S. enterica* serovar Typhimurium. This homology provides the rationale to interpret findings on the *P. aeruginosa* flagellum in analogy to the extensively studied *Salmonella* flagellum (Macnab, 1996). In *Salmonella* FliI, FlhA and FliK are components of the flagellar protein export apparatus (Minamino and Macnab, 1999). If we assume that the highly homologous proteins in *P. aeruginosa* exert analogous functions and have a similar

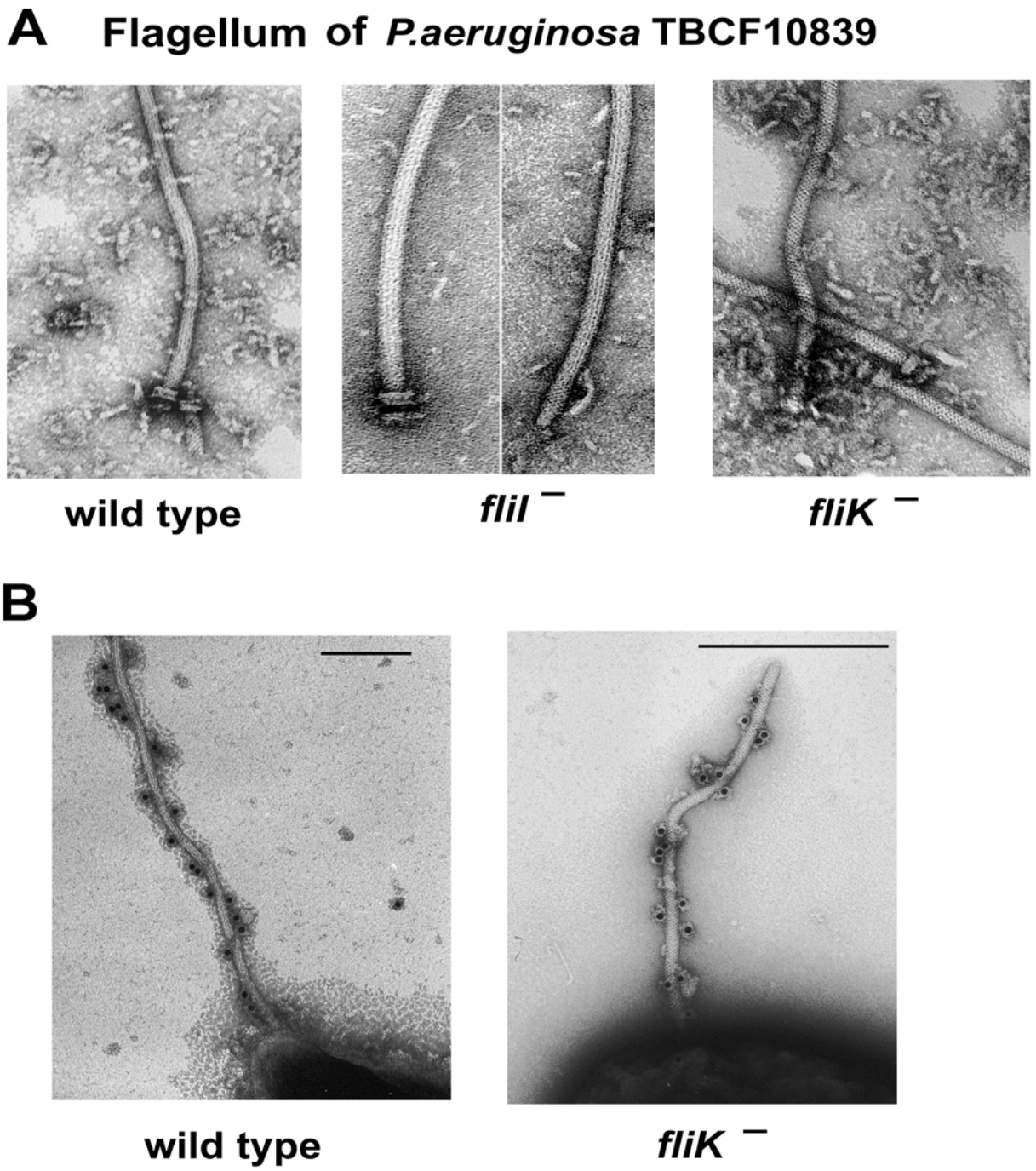


Fig. 3.4. Electron microscopy of flagella preparations. (A) Negative stain of flagella of the *P. aeruginosa* TBCF10839 strain and of its isogenic transposon mutants in *fliI* and *fliK.* A normal flagellum is characterized by basal body, hook and extended filament. The preparations of the *fliI* transposon mutants consisted of a minority of apparently normal flagella (left) and a majority of filaments with a convex tip at one end probably representing parts of the flagellar export apparatus (see figure 3.5). The *fliK* transposon mutant produced basal bodies and elongated hook-like structures. (B) Immunogold labeling of the flagella of the wild-type strain and its *fliK* transposon mutant with anti-flagellin FliC (type b) polyclonal goat antibody and secondary gold-labeled anti-goat antibody. The bar indicates the length of 0.5 µm (Courtesy by G. Brandes).

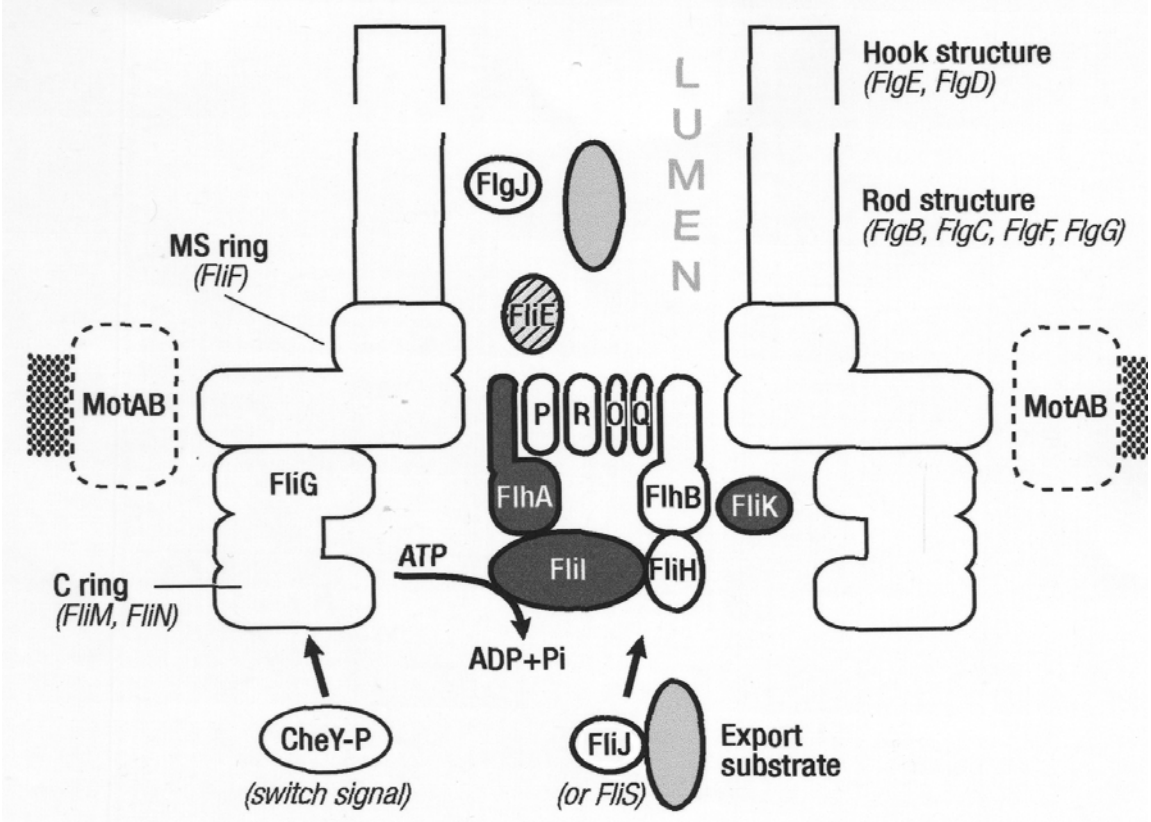


Fig. 3.5. Hypothetical model for the flagellar protein export apparatus (adapted from Minamino and Macnab, 1999).

spatial organization, they are not exposed to the exterior. Consequently, the interaction with the PMNs in our STM selection assays was not executed by neither of the three proteins but rather by a yet unknown factor secreted by the flagellar export apparatus. This conclusion is consistent with the uniform phenotype of the three mutants in the phagocytosis assays, albeit their impact on flagella synthesis and structure was different. The *fliI* mutant lacks sufficient stability of the basal body, in the *fliK* mutant with its elongated hooks the hook cap protein FlgD is not properly replaced by the hook-filament junction protein FlgK (Makishima *et al.*, 2001), and the *flhA* mutant does not produce any flagella. FliK (own unpublished data) and FhlA (Fleiszig *et al.*, 2001) the latter of which is homologous to the invasion protein InvA in *Salmonella* have been shown in *P. aeruginosa* to be essential for motility and internalization by epithelial cells underlining the double role of these proteins for secretion and flagella biogenesis. In *Yersinia enterocolitica*, for example, the FhlA-homologue is essential for the secretion of a virulence associated phospholipase (Young *et al.*, 1999).

3.2.4. Cytotoxicity towards macrophage J774 cells

The flagella mutants and wild type were subjected to cytotoxicity test on murine macrophage J774 cells. The results showed that *fliK*, *flhA*, *CHAexsA* mutants were non-cytotoxic in addition to the *P. aeruginosa* 892 wild type (figure 3.6) and *P. aeruginosa* TB was equally cytotoxic like another clinical isolate CHA. However, the *fliI* mutant was also equally cytotoxic like the TB wild type which could be due to a cell specific or unknown mechanism. *P. aeruginosa* CHA is a highly cytotoxic strain and serves as a positive control for the assay, while the *CHAexsA* mutant exhibits no cytotoxicity due to the defect in the type III secretion system regulator and serves as a negative control for this assay. Recently, It has been reported that the CF clinical isolate, *P. aeruginosa* CHA, is able to induce rapid oncosis of PMNs and macrophages. The cytotoxicity is type III secretion system dependent but is independent of the toxin ExoU (Dacheux *et al.*, 1999; Dacheux *et al.*, 2000). So these results also support the hypothesis that the flagellar export apparatus may be involved in the secretion of virulence factors. Further research is required to get insights of flagella mediated virulence factor secretion in this CF isolate.

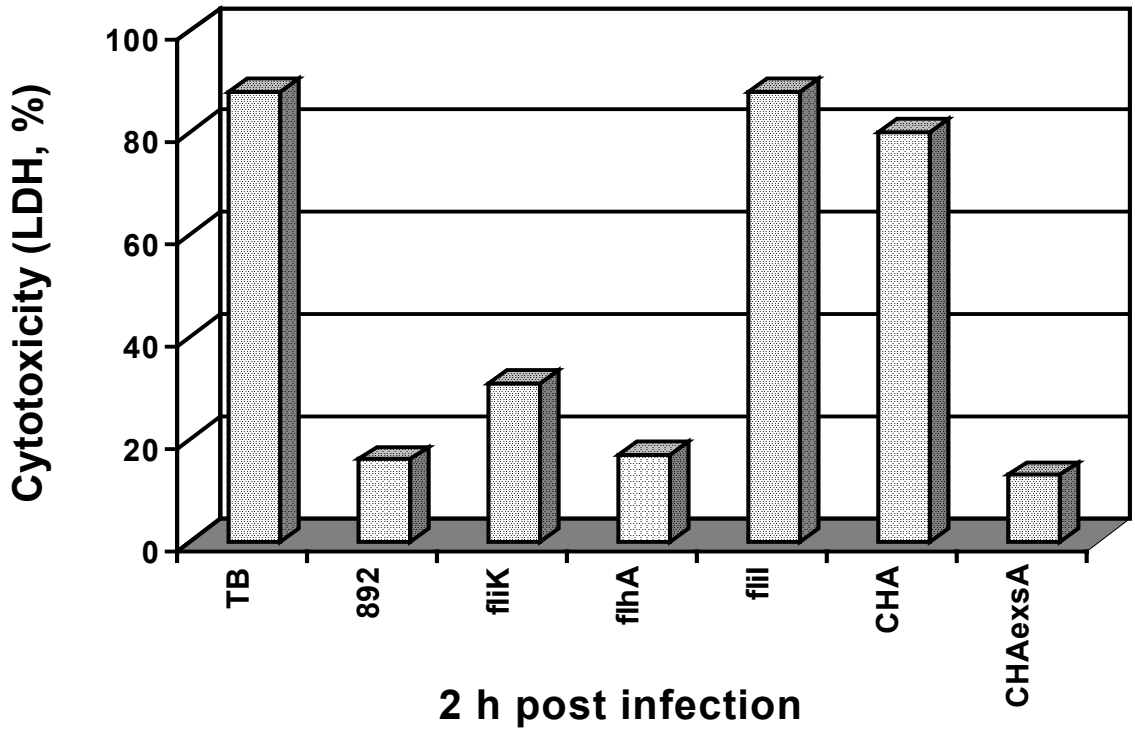


Fig. 3.6. Cytoxicity of *P. aeruginosa* strains toward J774 macrophage cells. The macrophages were infected with *P. aeruginosa* and CHA strains (MOI, 20) and the cytotoxicity percentage was calculated from the release of LDH activity.

3.3. Screening of STM library for mutants defective in protease secretion

In *P. aeruginosa*, quorum sensing has been proposed as a global mechanism for controlling virulence factor expression and the development of biofilms (Davies *et al*., 1998). Several genes are regulated by quorum sensing, including *toxA* (exotoxin A; Gambello *et al*., 1993), *lasB* (elastase; Brint & Ohman, 1995; Latifi *et al*., 1995; Pearson *et al*., 1997) and *lasA* (LasA protease; Toder *et al*., 1991), *aprA* (alkaline metalloproteinase; Gambello *et al.*, 1993; Latifi *et al*., 1995), *rhlAB*, encoding proteins involved in the synthesis of rhamnolipid (Ochsner *et al*., 1994; Pearson *et al*., 1997), *katA* and *sodA* (catalase and superoxide dismutase; Hassett *et al*., 1999), *lecA* (lectin; Winzer *et al*., 2000) and genes involved in pyocyanin synthesis (Brint & Ohman, 1995; Latifi *et al*., 1995). The influence of the *las* and *rhl* quorum sensing systems on the extracellular protein profile in *P. aeruginosa* was also examined by Nouwens *et al.* (2003). All mutant strains showed a significant reduction in the total concentration of secreted proteins, suggesting that the lack of any *las* or *rhl* gene severely disrupts protein secretion, and/or expression of previously abundant extracellular constituents (Nouwens *et al.*, 2003).

In order to screen the transposon library for mutants defective in quorum sensing, a casein plate agar assay was developed. This is a fast assay where most of the *lasI-rhlI* dependent proteases form a zone of hydrolysis on casein agar plates. The test assay was developed by a previous student in our laboratory who detected 8 protease deficient mutants in a preliminary screen (Wiehlmann, 2001). We have systematically screened the STM library for the defect in quorum sensing by this protease secretion assay. Altogether, 2306 transposon mutants were screened on casein agar where casein was supplied as sole carbon source. The transposon mutant with a defect in quorum sensing did not grow or formed a weak zone of clearance on the M9 medium containing casein. On the other hand, other mutants formed a zone of clearance like the wild type due to the hydrolyzed proteins which surrounded the colony. In this way, we have found about 200 mutants in the first round of screening which did not grow or have a defect in protease secretion on casein agar. These mutants were subsequently tested in the same assay for four times and the comparative quantification of proteolytic activity/zone of clearance was

monitored for 60 h. Induction of proteolytic activity was also monitored in further experiments where the mutants were inoculated in a close proximity in the form of the thick line perpendicular to the wild type TB (see figure 3.7). Since the homoserine lactone molecules produced by wild type can diffuse to the mutant through the casein agar, the quorum sensing in mutant can be induced resulting in the production of proteases which form weak zone of clearance on the side of TB wild type. After these assays, those mutants which had a defect either in the formation of zone of clearance or formed a weak zone of clearance or formed a zone of clearance after stimulation by the wild type or did not grow completely on casein agar were selected.

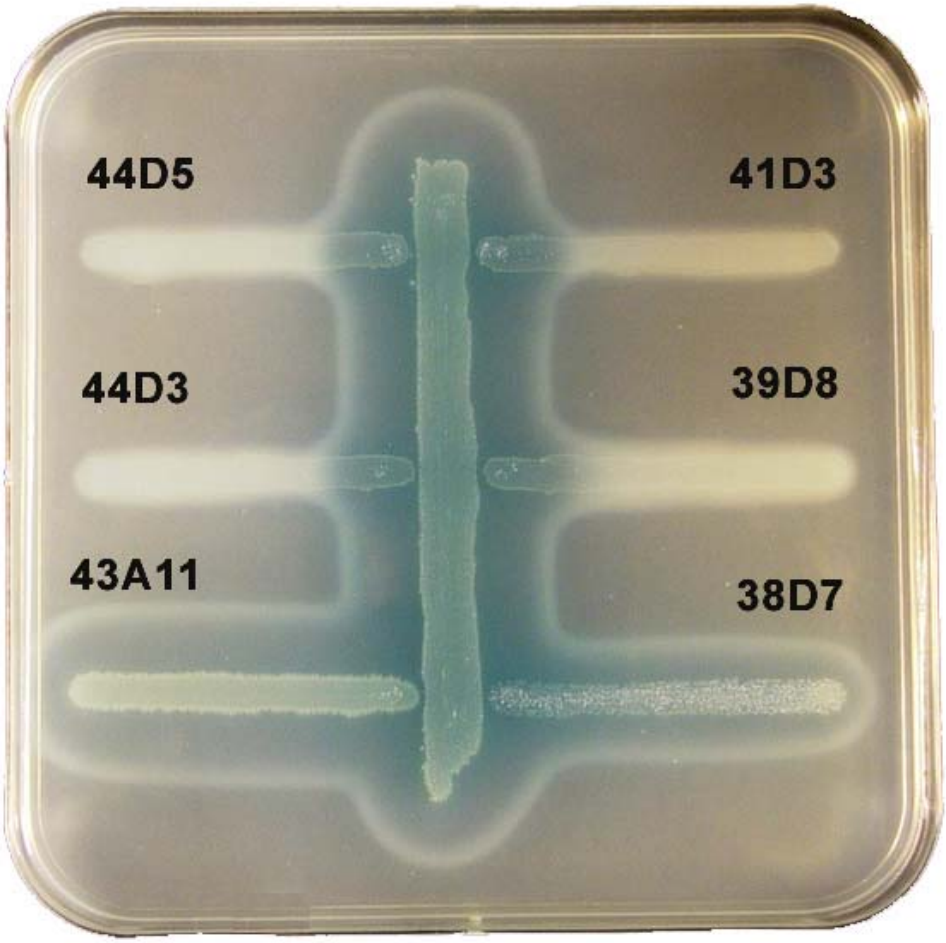


Fig. 3.7. Examination of inducible proteolytic activity of *P. aeruginosa* TB transposon mutants on casein agar. The *P. aeruginosa* TB wild type was inoculated in the center while the transposon mutants were inoculated perpendicular to wild type in a close proximity on casein agar plates and incubated for 60 h. 44D5, 44D3, 41D3 and 39D8 showed proteolytic activity due to the diffusible products secreted by wild type while 43A11 showed weak zone of hydrolysis from beginning and 38D7 showed zone of hydrolysis after 48 h.

Table 3.3. Characteristics of the mutants that are defective in proteolytic activity on the casein plate agar assay.

Mutant	No zone of clearance (up to 48 h)	Weak zone of clearance (from beginning)	Weak zone of clearance (after 48 h)	Stimulation by wild type	Defect in QS assay[1]
1C4		+			nt
2B6	+		+		nt
2D1	+			+	+
3B6	+		+		nt
4B6	+		+		nt
5B6	+		+		nt
6B6	+		+		nt
15D9		+			nt
16C12	+			+	nt
18A8		+			+
18D3		+			nt
20D1	+		+		nt
23B11		+			nt
37D7	+		+		+
38D7	+		+		nt
39D8	+			+	+
43A11		+			nt
44D3	+			+	+
44D5	+			+	+
48A8		+			nt
48D12	+		+		nt

+: Indicates that the mutant is positive for a mentioned characteristic.

[1]: These mutants were confirmed in the quorum sensing assays (see 2.4.1) and some mutants were not tested (nt) in the same assays.

We have found 21 mutants, out of which 14 mutants were unable to produce a zone of clearance, while the other 7 mutants which produced a weak zone of clearance as compared to the TB wild type. For example, the mutant 38A11 showed a zone of clearance after 48 h, the other mutant 43A11 showed a zone of clearance from the beginning, while the other mutants 39D8, 41D3, 44D3 and 44D5 did not show a zone of clearance but showed induction in protease secretion due to the wild type. The characteristics of the individual mutants are given in the following table (Table 3.3).

The subset of these attenuated mutants was confirmed by testing in a further assay for quorum sensing in Prof. Dr. Leo Eberl's lab. Then these mutants were subjected to plasmid rescue to identify the disrupted genes. Out of the 21 mutants, we obtained transformants from 14 mutants. Plasmid rescue failed for 7 mutants even after successive efforts. The disrupted genes of these 14 mutants with consistently high differences in their protease secretion characteristics in comparison to the TB wild type were sequenced. Out of these 14 mutants, 5 mutants (2B6, 3B6, 4B6, 5B6 and 6B6) have turned out to have a transposon insertion in the same gene. All of the genes from other 9 mutants exhibit about 99.5 % sequence identity to already known *P. aeruginosa* PAO1 genes (Table 3.4).

Table 3.4. Screening of *P. aeruginosa* TB STM mutants for defect in protease secretion: localization of the Tn*5* insertion in the attenuated mutants.

Mutant	PAO1 No.	Identity (%)	Annotation[1]
1C4	PA4468	99.6	hypothetical protein
6B6	PA3095	99.1	general secretion pathway protein M, *xcpZ*
18A8	PA3477	99.2	transcriptional regulator, *rhlR*
18D3	PA0928	98.9	sensor/response regulator hybrid
20D1	PA3102	100	general secretion pathway protein F, *xcpS*
39D8	PA1436	99.3	probable RND efflux transporter
44D3	PA2751/ 2752	99.6	intergenic region between hypothetical proteins on either side
44D5	PA0447/ 0448	99.8	intergenic region between glutaryl-CoA dehydrogenase, *gcdH* and probable transcriptional regulator
48D12	PA3105	100	general secretion pathway protein D, *xcpQ*

[1]: Gene names are indicated in italics.

The identified genes from the protease secretion assay belong to the general secretion pathway such as *xcpZ* (PA3095), *xcpS* (PA3102) and *xcpQ* (PA3105), unknown function (PA2751/PA2752, PA4468), RND efflux transporter (PA1436) and some have regulatory functions (PA0928, PA3477) (Table 3.4). It has been reported that loss of quorum sensing results in reduced expression of several secreted proteins, including elastase (*lasB*) (Passador *et al*., 1993; Pearson *et al*., 1997). Out

of the 9 mutants, we found a mutant that have transposon insertion in the already known quorum sensing dependent gene (*rhlR*) and in three *xcp* genes (*xcpZ*, *xcpS* & *xcpQ*). The *xcp* genes are not under the direct control of quorum sensing but are positively regulated by *vfr* and *gacA* (Beatson *et al.*, 2002). The *rhlR* is a positive regulator involved in the second quorum sensing system *rhlI-rhlR* (Davis *et al.*, 1998; de Kievit and Iglewski, 2000). The other mutant 44D5 that has the insertion in the intergenic region which can affect the transcription of both genes i.e. *gcdH* and a probable transcriptional regulator because they are transcribed in the opposite direction. The *gcdH* gene was also found to be quorum sensing promoted during the microarray experiment performed by Wagner *et al.* (2003). So we can conclude that this is the fast and easy method to screen genomic libraries and identify genes involved in quorum sensing.

3.4. Transcriptional analysis of *P. aeruginosa*

3.4.1. Global transcriptional analysis of *P. aeruginosa* strains against superoxide and oxidative stress

Background

The polymorphonuclear leukocyte (PMN) is the principle effector cell responsible for clearance of *P. aeruginosa* (Tümmler and Kiewitz, 1999). At the initial stage of infection by *P. aeruginosa*, the pathogen must colonize and proliferate in an hostile environment where it is exposed to several stresses, such as limitation of certain nutrients, thermal stress, osmotic stress and oxidative stress. The latter stress is induced during phagocytosis when a phagosomal NADPH oxidase complex undergoes a burst of oxygen consumption (Babior *et al.*, 1973; Chanock *et al.*, 1994). The electrons are transferred from cytoplasmic NADPH to oxygen on the phagosomal side of the membrane, generating superoxide as a reactive oxygen species. It can form the hydroxyl radicals via the iron-catalysed Haber-Weiss reaction (Cohen *et al.*, 1988) or by reacting with hypochlorous acid (Candeias *et al.*, 1993), and it can also react with nitric oxide to produce peroxynitrite (Zhu *et al.*, 1992). This oxidative burst is essential for killing of number of micro-organisms. Superoxide is a moderately reactive compound capable of acting as an oxidant and reductant in biological systems (Fridovich, 1997). The superoxide produced by PMNs also acts as

a precursor of hydrogen peroxide. A common model compound that continuously produces superoxide is the chemically inert xenobiotic paraquat (Ma *et al.*, 1998).

Bacteria have developed a sequential series of defense strategies to evade the detrimental action of superoxide and the hydroxyl radicals (Kettle and Winterbourn, 1990). Some organisms inhibit the NADPH oxidase dependent oxidative burst and thus reactive oxidant production within the phagosome (Miller and Britigan, 1997). They have also developed highly specific and reactive enzymatic pathways of oxidant inactivation, including those catalyzed by superoxide dismutase (SOD), catalase/peroxidase, and glutathione in combination with glutathione peroxidase and glutathione reductase (Hassett and Cohen, 1989). SOD detoxifies the O_2^- anion by a dismutation reaction that generates H_2O_2 and O_2 , while catalase converts toxic H_2O_2 into H_2O and O_2 (Fridovich, 1978). The production of SOD enzymes is a key defense strategy aimed at the elimination of superoxide.

Prior to the complete sequencing of the *P. aeruginosa* PAO1 genome (Stover *et al.*, 2000), a candidate gene approach has been pursued to uncover the oxidative stress defense in *P. aeruginosa* (Hassett *et al.*, 1992; Brown *et al.*, 1995; Hassett *et al.*, 1995; Hassett *et al.*, 1996; Ochsner *et al.*, 2000b; Ochsner *et al.*, 2001). *P. aeruginosa* possesses numerous enzymes for defense against reactive oxygen intermediates, including two superoxide dismutases (SodB, SodM) (Hassett *et al.*, 1995), three catalases (KatA, KatB, KatC) (Brown *et al.*, 1995) and four alkylhydroperoxide reductases (AhpA, AhpB, AhpCF, Ohr) (Ochsner *et al.*, 2000b; Ochsner *et al.*, 2001). Some of the genes involved in oxidative stress response, including the major catalase *katA* and the ferredoxin-dependent alkyl hydroperoxide reductase a*hpC* are constitutively expressed at high levels during normal aerobic growth and hence even significant oxidative stress causes only a maximal twofold increase in expression (Ochsner *et al.*, 2000b). Key regulators modulating the oxidative stress response in bacteria are *soxR* and *oxyR*, both of which are activated at the post-translational level. The *soxR*- and *oxyR*-regulons have been studied in detail in *E. coli*, but recently an *oxyR* homolog has been identified in *P. aeruginosa* PAO1 (Ochsner *et al.*, 2000b). The *oxyR* gene is encoded in an operon together with *recG*, the latter being involved in DNA repair.

Phagocytosis and killing by PMNs is the major human defense mechanism against *P. aeruginosa.* We were interested to know whether and, if yes, how *P. aeruginosa* can evade the detrimental superoxide and the oxidative stress. The recent sequencing of the *P. aeruginosa* PAO1 genome (Stover *et al.*, 2000) and the subsequent development of genome arrays provide the opportunity to study the global response of *P. aeruginosa* to superoxide oxidative stress without making any a priori hypotheses on candidate genes. In order to minimize the biological variation and to compare the array data with those by the candidate gene approach (Hassett *et al.*, 1992; Brown *et al.*, 1995; Hassett *et al.*, 1995; Hassett *et al.*, 1996; Ochsner *et al.*, 2001; Ochsner *et al.*, 2000b), the model compounds paraquat and hydrogen peroxide were chosen to unravel the response of *P. aeruginosa* to superoxide and oxidative stress respectively. The expression profile of the sequenced reference strain PAO1, a burn wound isolate from the 1950s, was compared with that of the two clonal variants TB and 892 isolated from cystic fibrosis lungs that differ substantially in their resistance to PMNs (Tümmler, 1987).

3.4.1.1. GeneChip analysis

P. aeruginosa GeneChips (microarrays) were used to study the global expression profile of *P. aeruginosa* in response to paraquat and hydrogen peroxide. Cultures grown in LB broth to stationary phase were exposed to 0.5 mM paraquat or to 10 mM of hydrogen peroxide in a dialysis bag for 2 h. 0.5 mM paraquat was measured to be the highest concentration in a two-fold concentration dilution series that did not affect cell growth. The hydrogen peroxide is degraded by the bacteria and it is a volatile compound, which decreases gradually from the medium. So 10 mM of hydrogen peroxide in excess was added to the medium. Dialysis bags were employed so that the diffusion of paraquat and hydrogen peroxide into the dialysis bag was the rate-limiting step for the equilibration between the two compartments. Hence the bacteria were allowed to adapt to asymptotically increasing concentrations of the stressor. We chose these conditions, because first, we wanted to minimize the activation of global stress pathways, and second, we wanted to investigate the steady state response rather than the early adaptation phase. After 2 hours of incubation, about 6 mM of hydrogen peroxide was still present in the medium (data not shown).

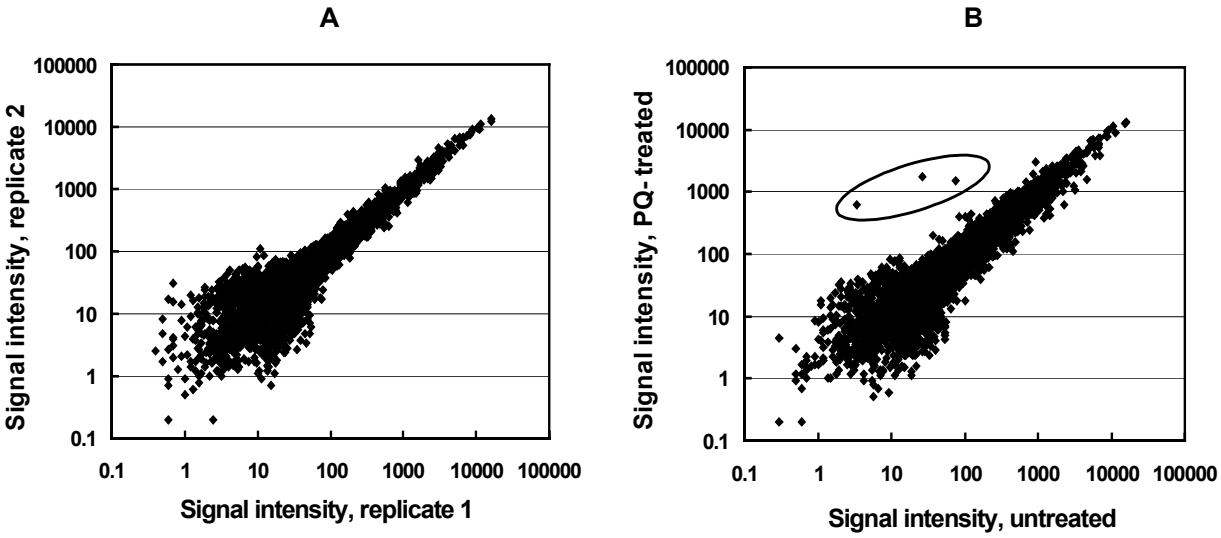


Fig 3.8. Logarithmic scatter plot of normalized signal intensities of all ORFs of the *P. aeruginosa* PAO1 genome represented by 5900 individual array spots.

A: Global expression profiles of two PAO1 cultures grown in LB medium until stationary phase. The normalized signal intensities of one replicate are plotted against the signal intensities of the second replicate.

B: Normalized signal intensities from paraquat-treated PAO1 cells are plotted against the signal intensities of untreated cells. The circle indicates that the expression of the three genes PA0939 - PA0941 was increased by more than one order of magnitude during superoxide stress generated by paraquat.

Total RNA was extracted from treated and untreated cultures and was used to synthesize cDNA which was hybridized on gene arrays. The oligonucleotide-array represents the whole sequenced genome of *P. aeruginosa* PAO1 (5549 CDS, 18 tRNA genes and one rRNA operon, 199 probe sets corresponding to all intergenic regions exceeding 600 base pairs as well as 117 additional genes which are present in other *P. aeruginosa* strains). The signal from each spot in the arrays served as a measure of the expression level of each gene and was used to calculate the expression ratio between the paraquat- treated or hydrogen peroxide- treated and untreated cultures for all the strains in duplicate experiments. Two stringency criteria were applied to each experimental data set. Only those genes were further analyzed that first, exhibited a significant change according to the Wilcoxon rank test, and showed an at least twofold change in expression in all 4 independent comparisons from the same experiment. Third, a Bonferroni correction for multiple comparisons revealed the subset of significantly differentially regulated genes. The genes having

signal intensities below 100 were discarded because of the large experimental variation (figure 3.8).

3.4.1.2. Paraquat mediated superoxide stress response

Figure 3.8 shows the correlation between the treated and untreated culture samples of the averaged expression levels for the 5900 genes and the in vitro control transcripts represented in the arrays for one experiment. The Pearson coefficient of independent experiments was 0.96 and 0.98 for the paraquat- treated and untreated cells respectively. Only 0.5 % of ORFs were significantly modulated in its expression level by paraquat.

Common response to paraquat in *P. aeruginosa*

Table 3.5 and figure 3.9 list the six genes that are up-regulated in all three analyzed *P. aeruginosa* strains. No gene was found to be commonly repressed. Expression was moderately up-regulated for the conserved hypothetical PA2229 and the ferredoxin NADP reductase Fpr.

Table 3.5. Genes up-regulated in all analyzed *P. aeruginosa* strains exposed to paraquat.

PAO	Average fold change[1]			Protein name[2]
No.	TB	892	PAO1	
PA0939	201.65 (58.11)	44.18 (26.27)	51.39 (13.16)	hypothetical protein
PA0940	58.79 (3.42)	26.75 (10.33)	20.01 (5.20)	hypothetical protein
PA0941	106.22 (9.63)	64.49 (26.75)	48.78 (16.30)	hypothetical protein
PA0942	6.85 (0.20)	2.11 (0.39)	3.85 (0.62)	probable transcriptional regulator
PA2229	7.23 (0.56)	2.14 (0.36)	4.32 (0.95)	conserved hypothetical protein
PA3397	2.05 (0.21)	2.08 (0.18)	2.47 (0.30)	ferredoxin NADP+ reductase, *fpr*

[1]: Values are means (SD) of four independent RNA preparations.

[2]: Gene name indicated in italics.

When paraquat is taken up by the bacterial cell, it is reduced by oxidoreductases (Liochev *et al.*, 1994) in a NADPH-dependent reaction and then re- oxidized by O_2 to form superoxide. Thus paraquat exerts its harmful action by superoxide itself and the consumption of NADPH. The up-regulated ferredoxin NADP reductase Fpr rescues some NADPH and could restore the activity of fumarases, aconitases and the other

(4Fe−4S)- containing dehydratases that are inactivated by superoxide molecules (Fridovich, 1997).

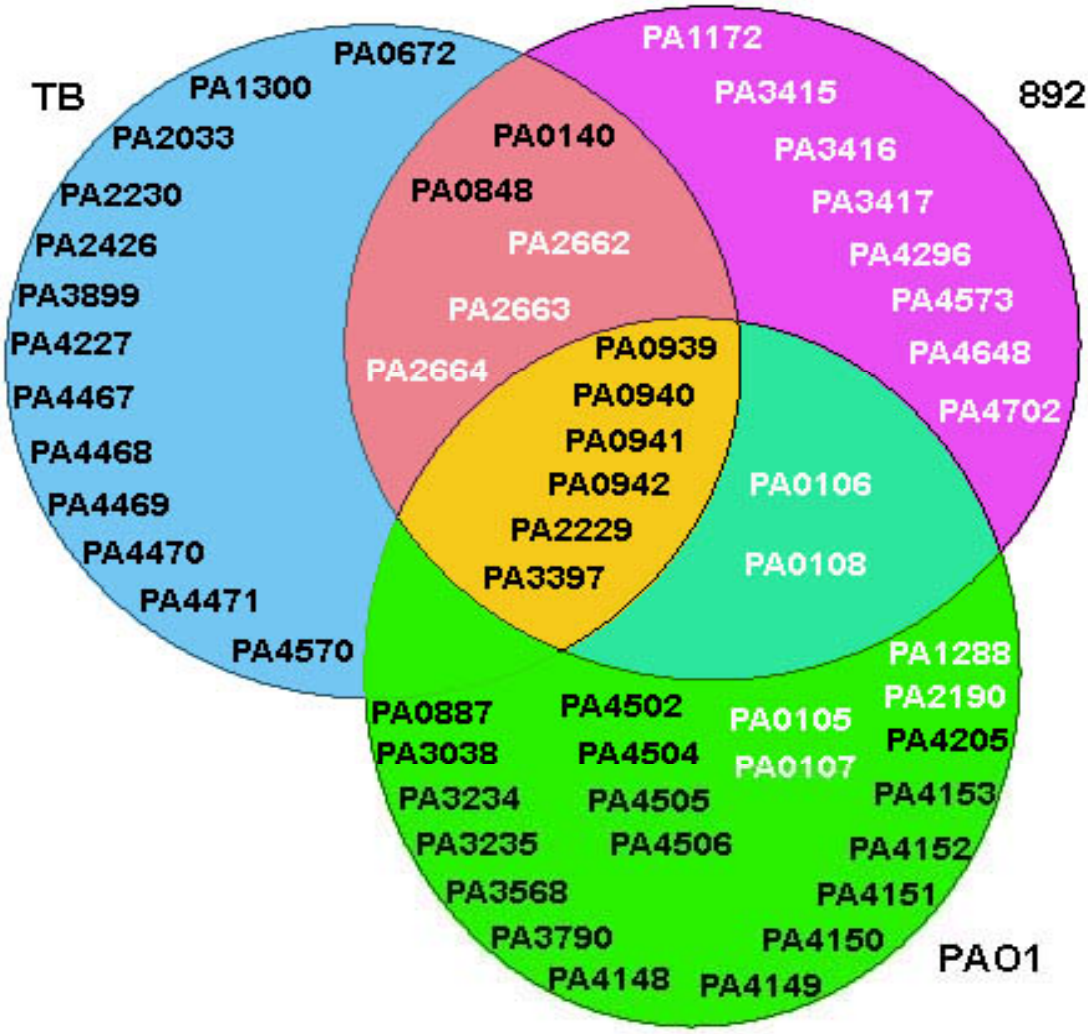


Fig. 3.9. PAO1 genes with significantly changed expression levels in paraquat-treated *P. aeruginosa* TB, 892 and PAO1. Black letters indicate the up-regulated genes while white letters indicate repressed genes. Intersections indicate the genes with changed mRNA transcript levels in two or three strains.

One complete operon (PA0939-PA0942) of yet undescribed function was highly up-regulated in all three strains, whereby the expression levels of putative transcriptional regulator (PA0942) were increased by 2 to 7 fold and those of the structural genes by 20 to 200 fold. The highly virulent strain TB showed the most dramatic increase of gene expression. No homologs are known for the structural genes, but the putative transcriptional regulator exhibits amino acid sequence homology with the regulators EmrR (30 %) (Brooun *et al.*, 1999) and PecS (31 %) (Nasser *et al.*, 1999)

from *Escherichia coli* and *Erwinia chrysanthemi,* respectively. According to the curvature-propensity plot the 94 bp large intergenic region between PA0941 and PA0942 could adopt a curved conformation (figure 3.10). A stretch of bended DNA is a typical target for a transcriptional regulator, and hence we assume that paraquat-derived signals are channeled to the PA0939–PA0941 genes in trans by a DNA binding protein that modulates the bending of the non-transcribed region. This conformational change could first activate the expression of the PecS-homolog PA0942 which then induces the transcription of the structural genes. PA0942 is probably not under the control of OxyR because its consensus binding sequence (Ochsner *et al.*, 2000b) is missing in the promoter region upstream of PA0942. The up-regulation of the operon PA0939–PA0942 is the only prominent and consistent response of all tested *P. aeruginosa* strains to paraquat at the mRNA expression level. Hence we conclude that the operon has the function to confer resistance to paraquat in addition to the constitutively expressed genes (see following sections).

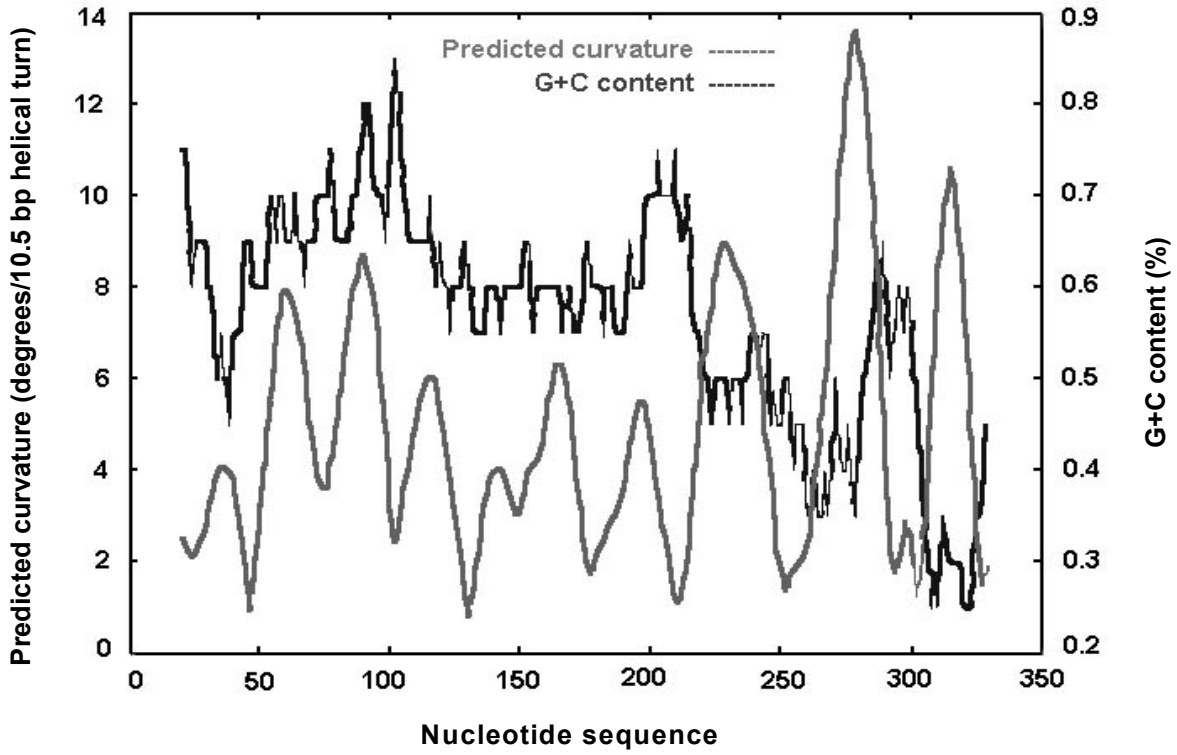


Fig 3.10. Curvature propensity plot of PAO1 gene PA0941 (position 20–235) and its upstream sequence (position 236–330) until the start of PA0942 that is transcribed in the opposite direction. The profile exhibits two peaks of stronger bending with maxima of 13.8 and 10.2 at nucleotide positions 280 and 320.

Strain-specific responses to paraquat

Table 3.6. Differential transcription profile of *P. aeruginosa* TBCF10838 exposed to paraquat.

	PAO1 No.	Fold change[1]	Protein name[2,3]
A. Paraquat induced genes			
Oxidative stress response			
	PA0140	2.11 (0.12)	alkyl hydroperoxide reductase subunit F, *ahpF*
	PA0848	2.04 (0.16)	probable alkyl hydroperoxide reductase
Iron responsive genes			
	PA0672	3.62 (1.03)	heme oxygenase, *hemO*
	PA1300	4.42 (1.87)	probable sigma70 factor (28 %, *fecI*, *E. coli*)
	PA2033	2.89 (0.79)	hypothetical protein (27 %, vibriobactin utilization protein *viuB*, *Vibrio cholerae*)
	PA2426	7.08 (2.88)	sigma factor, *pvdS*
	PA3899	2.73 (1.09)	probable sigma70 factor (75 %, *PupI*, *P. putida*)
	PA4227	2.52 (0.21)	transcriptional regulator, *pchR*
	PA4467	7.99 (4.34)	hypothetical protein
	PA4468	4.14 (1.08)	superoxide dismutase, *sodM*
	PA4469	5.43 (1.32)	hypothetical protein
	PA4470	6.69 (1.61)	fumarate hydratase, *fumC1*
	PA4471	16.89 (4.49)	hypothetical protein
Other genes			
	PA2230	2.68 (0.47)	hypothetical protein
	PA4570	5.46 (1.42)	hypothetical protein
B. Paraquat repressed genes			
Other genes			
	PA2662	4.08 (0.32)	conserved hypothetical protein
	PA2663	4.56 (0.59)	conserved hypothetical protein
	PA2664	6.87 (0.61)	flavohemoprotein, *fhp*

[1]: Values are means (SD) of four independent RNA preparations.

[2]: Brackets indicate % homology with genes from other bacterial species.

[3]: Gene names are indicated in italics.

Tables 3.6, 3.7, 3.8 and figure 3.9 list the genes whose expression was significantly induced or repressed by paraquat in either one or two of the three tested *P. aeruginosa* strains TB, 892 and PAO1. Paraquat stimulates the production of superoxide and hydrogen peroxide (Greenberg and Demple, 1989; Hassan and Fridovich, 1979) which can be degraded by alkyl hydroperoxide reductases (AhpF

and PA0848) and superoxide dismutase (Hassett *et al.*, 1992; Brown *et al.*, 1995). AhpF and PA0848 were up-regulated in strains 892 and TB whereas the superoxide dismutase operon (PA4467–PA4471) was only up-regulated in strain TB. The paraquat-induced stress reduced the expression of the cytochrome c oxidase *cox* operon in strains PAO1 and 892 and that of the flavohemoprotein *fhp* operon in strains TB and 892. Some steps of aerobic (*cox*) and anaerobic electron transport (*fhp*) were apparently down-regulated by the bacteria to escape the deleterious interference by superoxide.

Table 3.7. Differential transcription profile of *P. aeruginosa* 892 exposed to paraquat.

	PAO1 No.	Fold change[1]	Protein name[2]
A. Paraquat induced genes			
Oxidative stress response			
	PA0140	2.54 (0.62)	alkyl hydroperoxide reductase subunit F, *ahpF*
	PA0848	2.00 (0.11)	probable alkyl hydroperoxide reductase
B. Paraquat repressed genes			
Energy metabolism			
	PA0106	3.21 (1.10)	cytochrome c oxidase, subunit I, *coxA*
	PA0108	3.62 (1.82)	cytochrome c oxidase, subunit III, *coIII*
	PA1172	3.03 (1.08)	cytochrome c type protein, *napC*
	PA3415	5.01 (2.96)	probable dihydrolipoamide acetyltransferase
	PA3416	4.77 (2.45)	probable pyruvate dehydrogenase E1 component, beta chain
	PA3417	4.17 (2.08)	probable pyruvate dehydrogenase E1 component, alpha subunit
Other genes			
	PA2662	2.24 (0.51)	conserved hypothetical protein
	PA2663	2.24 (0.32)	hypothetical protein
	PA2664	2.50 (0.65)	flavohemoprotein, *fhp*
	PA4296	3.40 (1.46)	probable two component response regulator
	PA4573	3.51 (1.65)	hypothetical protein
	PA4648	4.34 (2.26)	hypothetical protein
	PA4702	3.60 (1.79)	hypothetical protein

[1]: Values are means (SD) of four independent RNA preparations.

[2]: Gene names are indicated in italics.

Table 3.8. Differential transcription profile of *P. aeruginosa* PAO1 exposed to paraquat.

	PAO1 No.	Fold change[1]	Protein name[2,3]
A. Paraquat induced genes			
TCA cycle and acetoin metabolism			
	PA0887	3.51 (1.30)	acetyl-coenzyme A synthetase, *acsA*
	PA3568	2.50 (0.61)	probable acetyl-CoA synthetase
	PA4148	2.55 (0.48)	probable short-chain dehydrogenase
	PA4149	3.13 (1.15)	conserved hypothetical protein (41 %, acetoin catabolism protein X, *Ralstonia eutropha*)
	PA4150	3.11 (0.73)	probable dehydrogenase (57 %, acetoin: DCIP oxidoreductase alpha subunit, *Ralstonia eutropha*)
	PA4151	4.32 (1.68)	acetoin catabolism protein, *acoB*
	PA4152	2.28 (0.34)	probable hydrolase (67 %, acetoin dehydrogenase component E2, *P. putida*)
	PA4153	2.59 (0.45)	2,3-butanediol dehydrogenase
Transport			
	PA3234	3.69 (1.56)	probable sodium:solute symporter
	PA4502	2.01 (0.22)	probable binding protein component of ABC transporter
	PA4504	2.04 (0.12)	probable permease of ABC transporter
	PA4505	2.01 (0.22)	probable ATP-binding component of ABC transporter
	PA4506	2.92 (0.73)	probable RND efflux membrane protein precursor
Other genes			
	PA3038	4.16 (1.34)	probable porin
	PA3235	3.31 (0.90)	conserved hypothetical protein
	PA3790	2.92 (0.67)	putative copper transport outer membrane porin, *oprC*
B. Paraquat repressed genes			
Energy metabolism			
	PA0105	2.85 (0.28)	cytochrome c oxidase subunit II, *coxB*
	PA0106	3.15 (0.86)	cytochrome c oxidase subunit I, *coxA*
	PA0107	3.78 (1.33)	conserved hypothetical protein (55 %, cytochrome c assembly protein, *Paracoccus denitrificans*)
	PA0108	3.20 (1.33)	cytochrome c oxidase subunit III, *coIII*
Other genes			
	PA1888	2.53 (0.72)	hypothetical protein
	PA2190	2.47 (0.67)	conserved hypothetical protein

[1]: Values are means (SD) of four independent RNA preparations.

[2]: Brackets indicate % homology with genes from other bacterial species.

[3]: Gene names are indicated in italics.

P. aeruginosa superoxide dismutase Mn-SOD is only expressed under iron limitation conditions (Hassett *et al.*, 1995; Hassett *et al.*, 1996). Exposure of strain TB to paraquat presumably affected iron homeostasis because besides Mn-SOD, the heme oxygenase HemO (PA0672), siderophore-associated sigma factors (PA1300, PA2426, PA3899, PA4227) and a homolog of the vibriobactin utilization protein (PA2033) were up-regulated (Table 3.6). This data most likely reflects the perturbation of the intracellular redox status by hydrogen peroxide and superoxide. More ionized iron is oxidized and correspondingly the demand of *P. aeruginosa* TB for iron increases.

Strain PAO1 changed its central carbon metabolism in response to paraquat. The synthesis of acetyl-CoA (PA0887, PA3568) and the NAD(H)-dependent conversion of acetoin (operon PA4148-PA4153) was stimulated. The relative yield of diacetyl and butane-2,3-diol from acetoin depends on the intracellular redox potential. *P. aeruginosa* PAO1 can exploit acetoin and acetyl-CoA for the use as a carbon source and for the production of ATP by substrate phosphorylation independent of the electrochemical potential. In other words, the PAO1 cells probably activated alternative pathways for carbon and energy metabolism in order to escape the influence of superoxide on the cellular redox status and aerobic ATP production. The data that the expression of the cytochrome oxidase c operon was repressed (see above) is consistent with this interpretation.

Oxidative stress defense genes sensu strictu

Paraquat reduction within *E. coli* is catalyzed by three paraquat:NADPH oxidoreductases (Liochev and fridovich, 1994). *P. aeruginosa* possesses both NADPH and NADH-dependent paraquat oxidoreductases; there are two of the latter and at least five of the former (Ma *et al.*, 1998). With the exception of Fpr (Tables 3.5, 3.6, 3.7 and 3.8) none of these enzymes was up-regulated at the transcriptional level in response to paraquat in our experiments. Unexpectedly the expression of the *zwf* gene was also not induced, although its gene product glucose-6-phosphate dehydrogenase is known to confer some resistance to paraquat in *P. aeruginosa* (Ma *et al.*, 1998).

Table 3.9. Expression of mRNA transcripts involved in oxidative stress response: GeneChip normalized signal intensities in the absence (-) and presence (+) of 0.5 mM paraquat.

Gene	PAO1 No.	strain TB		strain 892		strain PAO1	
		-	+	-	+	-	+
ahpB	PA0847	10	4	22	5	106	69
ahpC	PA0139	2274	2738	2017	2278	2158	2802
ahpF	PA 0140	62	166	54	120	81	182
ohr	PA 2850	45	66	55	51	31	26
katA	PA 4236	1659	1657	1650	1176	3770	3623
katB	PA 4613	22	37	13	30	21	22
katE	PA 2147	6	2	5	8	22	5
katN	PA 2185	11	12	12	10	2	22
sodB	PA 4366	1517	1619	1426	1427	1218	1106
sodM	PA 4468	34	192	26	26	62	36
pqrC	PA 0939	6	1634	1	120	3	371
pqrB	PA 0940	62	3949	38	756	77	2398
pqrA	PA 0941	37	5375	32	1239	26	2559
pqrR	PA 0942	114	682	97	144	101	435

Sensitivity to paraquat is discussed in the literature (Ma *et al.*, 1998) to be dependent on the presence or absence of multiple cellular factors, some of which include alkyl hydroperoxide reductase AhpF, SodM, catalase, methionine sulfoxide reductase, DNA repair systems, glutathione reductase and various regulatory proteins (e.g., OxyR, SoxRS). It is interesting to note that only the expression of *ahpF* and *sodM*, but none of the other genes was induced under our carefully controlled experimental conditions. The up-regulation of *katB*, *ahpB* and *ahpC* mRNA transcript levels upon exposure of paraquat to strain PAO1 reported by others (Ochsner *et al.*, 2000b) was not observed in our GeneChip analyses (Table 3.9). We would like to attribute these discrepant findings to the different exposure kinetics. In Ochsner‘s (2000b) experimental design, the paraquat concentration changed within the few seconds mixing time from zero to final concentration whereas our set-up to exchange the outer medium allowed for a diffusion-controlled equilibration across the dialysis

membrane between the outer and the bacteria-containing inner compartments. In our opinion the two-compartment model is more appropriate to mimic the situation in vivo than the one-step addition of paraquat.

Under our experimental conditions the superoxide dismutase *sodB*, the catalase A *katA* and the alkyl hydroperoxide reductase *ahpC* were constitutively expressed at high levels in the absence and presence of paraquat. Table 3.9 displays the normalized signal intensities of operon PA0939–PA0942 and of the major genes known from the literature to be involved in the handling of reactive oxygen intermediates. *sodB* is expressed above average. *katA* and *ahpC* belong to the 100 most strongly expressed genes of the transcriptome. In other words, the two latter genes are already expressed at such a high level that any further up-regulation upon exposure to oxidative stress would not be biologically meaningful for the cell.

Conclusion

Paraquat-mediated stress affected only few mRNAs of the *P. aeruginosa* transcriptome. Besides some fine tuning to degrade superoxide and hydrogen peroxide and to neutralize the effect of these toxic oxygen species on iron homeostasis and aerobic respiration, the major response was exerted by one yet uncharacterized operon. Although the detoxifying activity of its gene products awaits experimental proof, the unique strong up-regulation of mRNA transcript levels is sufficient evidence to designate the PA0939-PA0942 genes as the *pqrCBAR* operon (paraquat response). This previously unrecognized operon PA0939-PA0942 and the constitutively highly expressed SodB, KatA and AhpC seem to be the key players to combat the paraquat-induced oxidative stress.

3.4.1.4. Hydrogen peroxide mediated oxidative stress response

We performed the global gene expression analysis against hydrogen peroxide induced oxidative stress response in three strains of *P. aeruginosa* (TB, 892 and PAO1) by using DNA microarrays. Cultures grown in LB broth to stationary phase were exposed to 10 mM hydrogen peroxide in a dialysis bag for 2 h. Total RNA was extracted from H_2O_2 treated and untreated cultures and was used to synthesize cDNA which was hybridized onto gene arrays. Thus the expression ratio for each gene was measured in duplicate for treated and untreated cultures. Upon exposure

to hydrogen peroxide, 23.5, 17.5, 15 % of ORFs were significantly differentially regulated in *P. aeruginosa* TB, 892 and PAO1 respectively. The total number of genes expressed in the chosen condition were about 75 % from all the three strains. This indicates that most of the genes were essential for the cell metabolism in response to hydrogen peroxide. It was observed that a greater number of genes were significantly modulated against the H_2O_2 response in TB as compared to 892 and PAO1 (Table 3.16). The modulated genes were sorted and divided into 20 different categories depending upon the known function. The table 3.10 shows a summary of functional categories which were observed in all the three strains against H_2O_2 response.

Table 3.10. Number of up- or down-regulated genes in different functional categories from the three strains of *P. aeruginosa* TB, 892 and PAO1.

Functional categories	**Up-regulation**			**Down-regulation**		
	TB	**892**	**PAO1**	**TB**	**892**	**PAO1**
Energy metabolism	64	39	27	67	65	42
Amino acid biosynthesis & metabolism	54	37	27	15	12	18
Biosynthesis of cofactors, prosthetic groups & carriers	23	18	3	1	1	3
Quorum sensing	0	0	0	26	18	10
Flagella biogenesis & chemotaxis	0	0	0	49	58	30
Sulfur metabolism	11	9	4	0	0	0
Iron metabolism	16	15	18	0	0	0
Cell rescue/defense	23	20	17	7	5	6
Cell processes (including adaptation, protection)	16	7	6	2	2	0
Carbon compound catabolism	10	4	1	9	7	2
Nucleotide biosynthesis and metabolism	32	13	12	7	5	5
DNA replication & recombination	11	5	6	0	0	0
Transcription, RNA processing & degradation	10	1	1	0	0	0
Translation, post-translational modification	52	15	38	7	5	7
Protein secretion/export	7	4	9	6	5	1
Cellular organization	28	12	8	11	9	9
Putative regulatory proteins	15	9	5	24	21	18
Putative transport & binding proteins	36	20	7	17	15	26
Other known	33	21	22	76	69	76
Unknown/unclassified	272	179	116	343	295	293
Total	713	428	327	667	592	546

From the abovementioned data (Table 3.10), it was observed that a significantly greater number of genes were up-regulated in TB from the functional groups such as energy metabolism; amino acid biosynthesis and metabolism; carbon compound catabolism; DNA replication and recombination; transcription, RNA processing & degradation; translation & post-translational modification and cellular organization as compared to 892 and PAO1 when the cells were treated with hydrogen peroxide. In the functional groups, such as biosynthesis of cofactors, prosthetic groups & carriers; sulfate metabolism; iron metabolism; cell rescue; cell processes; nucleotide biosynthesis & metabolism; DNA replication & recombination; translation, post-translational modification, the number of up-regulated genes surpassed those of down-regulated genes significantly, suggesting that these groups of genes were generally induced by hydrogen peroxide. The genes from other categories such as quorum sensing and flagella biogenesis and chemotaxis were down-regulated upon exposure to hydrogen peroxide. The number of up-regulated genes was much higher than down-regulated genes in many functional categories.

The genes from major functional categories are listed in the following table 3.11. The data from other categories are given in a appendix III.

Table 3.11. Differential transcription profile of *P. aeruginosa* TB, 892 and PAO1 exposed to hydrogen peroxide.

PAO No.	Average fold change[a]			Protein name[b]
	TB	**892**	**PAO1**	
I) Hydrogen peroxide induced genes				
A) Cell rescue/defense				
Oxidative stress response				
PA0139			3.81 (0.34)	alkyl hydroperoxide reductase subunit C, *ahpC*
PA0140	23.87 (1.61)	36.19 (7.89)	36.89 (3.74)	alkyl hydroperoxide reductase subunit F, *ahpF*
PA0848	15.96 (3.09)	25.10 (7.56)	32.26 (8.27)	probable alkyl hydroperoxide reductase,
PA2532		2.85 (0.73)		thiol peroxidase, *tpx*
PA2850	3.45 (0.88)	4.67 (1.97)	8.22 (2.70)	organic hydroperoxide resistance protein, *ohr*
PA3397	2.23 (0.20)			ferredoxin-NADP+ reductase, *fpr*
PA3450	6.70 (1.25)	5.89 (0.70)		probable antioxidant protein
PA4613	56.98 (6.59)	118.41 (17.81)	185.57 (71.85)	catalase, *katB*
General stress response				
PA0961	3.94 (0.26)	3.48 (1.02)	3.32 (0.30)	probable cold-shock protein
PA1596		2.89 (0.30)		heat shock protein, *htpG*

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
I) Hydrogen peroxide induced genes				
A) Cell rescue/defense				
General stress response				
PA3126		3.49 (0.28)		heat-shock protein, *ibpA*
PA3266	3.56 (0.41)	2.73 (0.57)		cold acclimation protein B, *capB*
PA3625			2.69 (0.24)	survival protein, *surE*
PA3810	3.16 (0.46)		2.36 (0.37)	heat shock protein, *hscA*
PA3811	2.67 (0.46)		2.52 (0.26)	heat shock protein, *hscB*
PA4428	2.42 (0.16)			stringent starvation protein A, *sspA*
PA4557	3.49 (0.28)	2.89 (0.33)	2.94 (0.35)	LytB protein, *lytB*
PA5193	4.12 (0.75)	3.34 (0.94)	2.47 (0.20)	heat shock protein HSP33, *yrfI*
DNA repair				
PA0382	3.74 (0.30)	3.12 (0.88)		DNA mismatch repair protein, *micA*
PA0750	2.57 (0.37)	2.75 (0.40)		uracil-DNA glycosylase, *ung*
PA1534	4.52 (1.68)	3.46 (0.62)	5.45 (2.22)	recombination protein, *recR*
PA2545	2.98 (0.10)	3.06 (0.20)		exodeoxyribonuclease III, *xthA*
PA3007	4.07 (0.14)	5.47 (0.39)	5.37 (0.19)	repressor protein, *lexA*
PA3617	3.25 (0.00)	4.09 (0.43)	4.52 (0.13)	RecA protein, *recA*
PA3620	2.56 (0.23)	2.44 (0.37)		DNA mismatch repair protein, *mutS*
PA3725	2.26 (0.19)			single-stranded-DNA-specific exonuclease, *recJ*
PA4042	2.63 (0.47)			exodeoxyribonuclease VII small subunit, *xseB*
PA4234			2.23 (0.27)	excinuclease ABC subunit A, *uvrA*
PA4763	4.94 (0.39)	7.23 (0.65)	5.20 (0.35)	DNA repair protein, *recN*
PA5443			2.30 (0.18)	DNA helicase II, *uvrD*
II) Hydrogen peroxide repressed genes				
B) Microaerophilic and anaerobic growth				
Denitrification				
PA0024	3.61 (0.24)	3.28 (0.84)		coproporphyrinogen III oxidase, *hemF*
PA0509	61.86 (2.47)	41.99 (11.54)	2.26 (0.15)	probable c-type cytochrome, *nirN*
PA0510	357.51 (86)	217.37 (182)	2.93 (0.)	probable uroporphyrin-III c-methyltransferase
PA0511	68.67 (15.1)	37.84 (12.95)	2.99 (0.25)	heme d1 biosynthesis protein, *nirJ*
PA0512	123.29 (45)	62.58 (40.42)	2.56 (0.23)	conserved hypothetical protein
PA0513	164.49 (42)	85.02 (66.07)	2.60 (0.22)	probable transcriptional regulator
PA0514	148.96 (54)	73.18 (48.90)	2.88 (0.90)	heme d1 biosynthesis protein, *nirL*
PA0515	528.02 (98)	170.58 (167)	2.75 (0.40)	probable transcriptional regulator
PA0516	95.72 (9.84)	60.13 (40.17)	3.10 (0.32)	heme d1 biosynthesis protein, *nirF*
PA0517	223.37 (94)	161.33 (169)	2.74 (0.24)	probable c-type cytochrome precursor, *nirC*
PA0518	97.24 (7.7)	84.45 (85.36)	2.31 (0.29)	cytochrome c-551 precursor, *nirM*
PA0519	32.08 (2.5)	45.58 (45.37)		nitrite reductase precursor, *nirS*
PA0520	19.01 (4.0)	11.28 (5.59)		regulatory protein, *nirQ*
PA0521	16.98 (4.3)	14.31 (4.87)		probable cytochrome c oxidase subunit
PA0522	2.85 (0.36)			hypothetical protein
PA0523	104.12 (38)	54.95 (53.83)	4.49 (2.37)	nitric-oxide reductase subunit C, *norC*

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
II) Hydrogen peroxide repressed genes				
B) Microaerophilic and anaerobic growth				
Denitrification				
PA0524	243.58 (103)	124.73 (114)	4.56 (2.52)	nitric-oxide reductase subunit B, *norB*
PA0525	65.31 (15.1)	86.19 (52.29)	4.37 (2.21)	probable dinitrification protein
PA1172	34.83 (7.04)	22.04 (5.70)	18.71 (0.66)	cytochrome c-type protein, *napC*
PA1173	40.03 (8.53)	27.64 (7.36)	15.31 (2.16)	cytochrome c-type protein, *napB*
PA1174	35.94 (6.31)	22.93 (6.68)	17.19 (1.37)	periplasmic nitrate reductase protein, *napA*
PA1175	16.85 (3.61)	19.05 (4.16)	14.51 (1.87)	NapD protein of periplasmic nitrate reductase, *napD*
PA1176	26.53 (6.16)	28.70 (8.10)	16.59 (6.13)	ferredoxin protein, *napF*
PA1177	38.93 (4.70)	26.74 (7.49)	16.90 (3.81)	periplasmic nitrate reductase protein, *napE*
PA1546	2.71 (0.42)	4.81 (2.54)		oxygen-independent coproporphyrinogen III oxidase, *hemN*
PA1555	12.75 (4.51)			probable cytochrome c
PA1556	7.13 (1.79)			probable cytochrome c oxidase subunit
PA1557	2.27 (0.27)			probable cytochrome oxidase subunit
PA1861	3.28 (0.47)	2.43 (0.21)		molybdenum transport protein, *modC*
PA1862	2.27 (0.27)	4.86 (0.50)		molybdenum transport protein, *modB*
PA1863	3.13 (0.66)	2.49 (0.41)	2.90 (0.40)	molybdate-binding periplasmic protein, *modA*
PA2664	11.52 (0.4)			flavohemoprotein, *fhp*
PA3032	2.74 (0.68)	2.59 (0.52)		cytochrome c Snr1, *snr*1
PA3391	61.78 (39)	85.11 (91.69)	3.95 (0.41)	regulatory protein, *nosR*
PA3392	86.70 (13)	94.18 (79.88)	2.23 (0.20)	nitrous-oxide reductase precursor, *nosZ*
PA3393	19.72 (1.1)	23.87 (1.61)	2.52 (0.29)	NosD protein, *nosD*
PA3394	26.49 (1.7)	25.84 (9.07)	2.35 (0.27)	NosF protein, *nosF*
PA3395	36.27 (3.7)	17.10 (4.90)		NosY protein, *nosY*
PA3396	37.38 (21)	29.93 (7.01)		NosL protein, *nosL*
PA3870	24.29 (8.9)	26.67 (3.82)	17.50 (5.56)	molybdopterin biosynthetic protein A1, m*oaA*1
PA3871	34.99 (17)	28.22 (18.74)	22.99 (6.89)	probable peptidyl-prolyl cis-trans isomerase
PA3872	36.91 (12)	50.36 (4.50)	17.98 (6.35)	respiratory nitrate reductase gamma chain, *narI*
PA3873	55.17 (22)	76.70 (11.05)	22.10 (10.80)	respiratory nitrate reductase delta chain, *narJ*
PA3874	79.11 (25)	305.34 (2.51)	24.83 (12.93)	respiratory nitrate reductase beta chain, *narH*
PA3875	272.60 (110)	415.01 (421)	20.79 (13.82)	respiratory nitrate reductase alpha chain, *narG*
PA3876	62.39 (16.2)	70.76 (44.30)	12.23 (8.15)	nitrite extrusion protein 2, *narK*2
PA3877	20.10 (6.36)	64.18 (55.25)	10.58 (6.38)	nitrite extrusion protein 1, *narK*1
PA3879	2.70 (0.28)	4.60 (2.47)		two-component response regulator, *narL*
PA3914	45.03 (8.10)	130.25 (79.2)		molybdenum cofactor biosynthetic protein A1, *moeA*1
PA3915	13.86 (2.49)	44.62 (31.54)	5.07 (3.58)	molybdopterin biosynthetic protein B1, *moaB*1
PA3916		4.87 (0.59)		molybdopterin converting factor, *moaE*
PA3917	2.34 (0.15)	6.09 (0.69)		molybdopterin converting factor, *moaD*
PA3918		3.90 (0.64)		molybdopterin biosynthetic protein C, *moaC*

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
II) Hydrogen peroxide repressed genes				
B) Microaerophilic and anaerobic growth				
Denitrification				
PA4196	2.31 (0.29)			probable two-component response regulator
PA4810	3.83 (0.60)			nitrate-inducible formate dehydrogenase, *fdnI*
PA4811	3.94 (0.26)	3.42 (1.56)		nitrate-inducible formate dehydrogenase, *fdnH*
PA4812	2.73 (0.11)			formate dehydrogenase-O, *fdnG*
Arginine fermentation				
PA5051	2.42 (0.16)			arginyl-tRNA synthetase, *argS*
PA5170	2.60 (0.17)	21.76 (20.59)		arginine/ornithine antiporter, *arcD*
PA5171	15.85 (2.27)	34.78 (28.79)	2.30 (0.18)	arginine deiminase, *arcA*
PA5172	52.48 (8.35)	41.11 (32.16)	2.39 (0.21)	ornithine carbamoyltransferase, *arcB*
PA5173	63.24 (7.64)	48.99 (25.57)	2.56 (0.23)	carbamate kinase, *arcC*
Cytochrome biogenesis				
PA0105	13.65 (5.74)	13.12 (3.50)	15.63 (9.18)	cytochrome c oxidase, subunit II, *coxB*
PA0106	12.05 (2.14)	12.03 (3.20)	50.20 (16.05)	cytochrome c oxidase, subunit I, *coxA*
PA0108	12.08 (2.33)	6.51 (2.39)	11.54 (3.50)	cytochrome c oxidase, subunit III, *coIII*
PA0113	3.11 (0.43)		3.92 (1.13)	probable cytochrome c oxidase assembly factor
PA0918	7.39 (1.06)	5.27 (1.87)	3.57 (0.67)	cytochrome b561
PA2475	3.70 (0.61)	3.43 (0.23)		probable cytochrome P450
PA3331		3.69 (1.22)		cytochrome P450
PA4571	3.44 (0.42)	5.96 (3.39)		probable cytochrome c
PA5328			6.94 (1.99)	probable cytochrome c(mono-heme type)

[a]: Values are means (SD) of four independent RNA preparations.

[b]: Gene names are indicated in Italics.

Up-regulated genes in *P. aeruginosa* strains

The following categories of genes (figure 3.11) were up-regulated in the *P. aeruginosa* strains after exposure to hydrogen peroxide. Most of the genes show a common expression pattern in all the three strains of *P. aeruginosa* after exposure to hydrogen peroxide except that few of the genes were not up-regulated in 892 and PAO1. The most obvious category of genes i.e. cell rescue/defense is discussed further in detail.

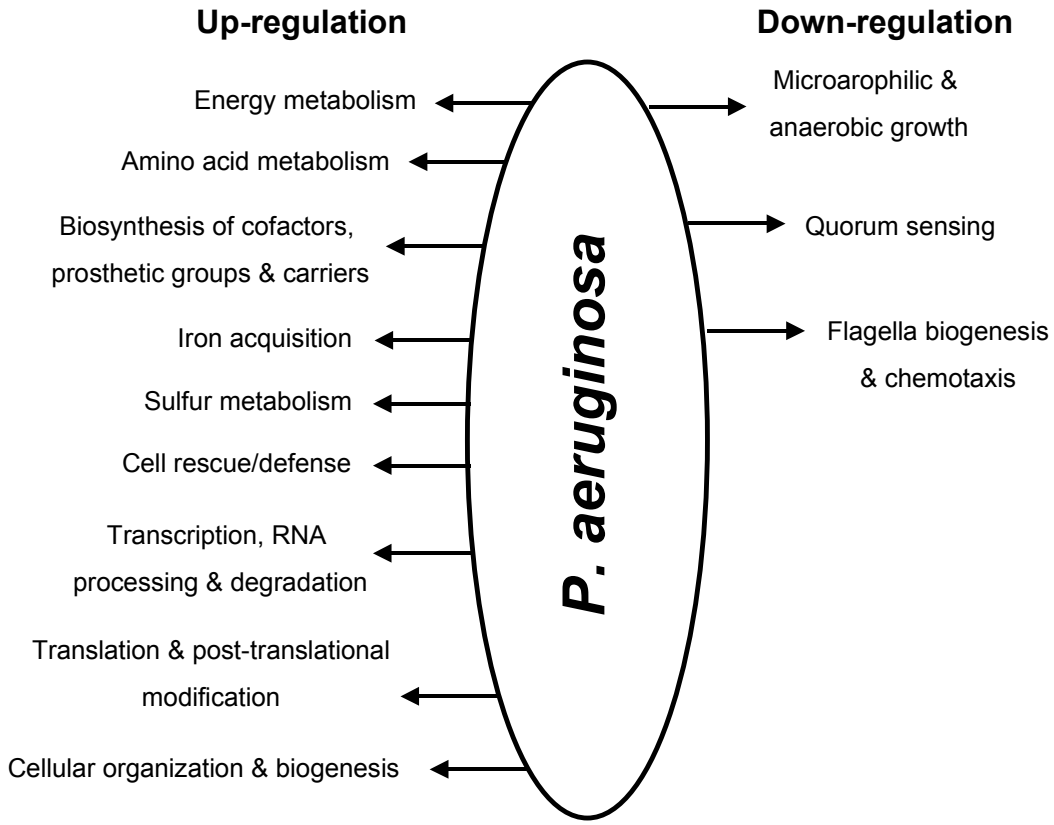


Fig. 3.11 . Various categories of differentially regulated genes in *P. aeruginosa* TB, 892 and PAO1 after exposure to hydrogen peroxide.

Cell rescue/defense

A hallmark of the *P. aeruginosa* response to hydrogen peroxide is the rapid and strong induction of a set of OxyR-regulated genes, including *ahpC*, *ahpF*, PA0848, *ohr* and *katB*. We observed >10-fold induction of all the genes except *ahpC* in all the strains, provided an internal validation of the microarray experiment (Table 3.11). It was observed that all the above mentioned genes (except *ahpC*) showed 2−3 times higher up-regulation in PAO1 as compared to TB. Each of these genes is important in combating hydrogen peroxide mediated stress. Aerobic respiration in *P. aeruginosa* leads to the production of toxic metabolic products including the superoxide anion, H_2O_2, hydroxyl radicals and organic hydroperoxides. Sublethal level of superoxide and H_2O_2 are detoxified by two superoxide dismutases and two catalases respectively. In our experiments, bacterial cells were always encountered with the exogeneously added hydrogen peroxide which generated oxidative stress. In this stress response, both superoxide dismutases (*sodB* & *sodM*) and catalases (*katA* & *ahpB*) were not up-regulated in any of the strains used. Some of the genes involved in oxidative stress defense, including *katA*, *sodB* are expressed at high

levels during aerobic and microaerophilic growth (Table 3.12). These results suggest that the further up-regulation of superoxide dismutase (*sodB*) and catalase (*katA*) would not be meaningful to the cell. The differential regulation of Oxy-R dependent genes: *katE*, *katN*, *ahpB* and *sodM* was not observed in all the three *P. aeruginosa* strains upon exposure to hydrogen peroxide during our carefully monitored growth conditions (Table 3.12).

Table 3.12. Expression of mRNA transcripts involved in oxidative stress response: GeneChip normalized signal intensities in the absence (-) and presence (+) of 10 mM hydrogen peroxide.

Gene	PAO No.	strain TB		strain 892		strain PAO1	
		-	+	-	+	-	+
ahpB	PA0847	13	4	17	5	112	19
katA	PA 4236	1770	2374	1510	2363	3712	5742
katE	PA 2147	4	4	3	3	18	5
katN	PA 2185	11	12	11	5	12	7
sodB	PA 4366	1490	2053	1434	2188	1121	2014
sodM	PA 4468	30	75	24	45	52	112

It appears that redundancy of oxidative stress defense system allows *P. aeruginosa* to optimally cope with reactive oxidative intermediates (ROIs) generated by its own aerobic metabolism and respond rapidly to exogenous hydrogen peroxide mediated ROIs. Here, we have found that some of the Oxy-R independent genes were also up-regulated in either one or two or all the three of *P. aeruginosa* strains. These genes encodes cold and heat shock proteins (HSPs). Recently it has been reported that HSPs stabilize and protect intracellular proteins and prevent damage caused by heat and oxidative stress (Kitagawa *et al.*, 2002). All organisms living in aerobic environments are exposed to reactive oxygen by-products that can damage most cellular components, including lipids, proteins and DNA. Active oxygen species are generated during normal aerobic metabolism, as well as by exogeneously added chemicals. Although all organisms have evolved mechanisms of defense against oxidative damage, those have been best characterized in the bacterium *E.coli*. When *E. coli* encounters oxidative stress during aerobic growth, it induces the expression of a set of genes encoding enzymes that degrade the reactive oxygen species and repair the damaged macromolecules, such as DNA repair systems (Farr and Kogoma, 1991). These genes are organized in two independent stimulons that are

turned on by different oxidative stresses, the superoxide stress response (induced by the superoxide radical and regulated by *soxR* and *soxS*) and the peroxide stress response (induced by hydrogen peroxide and organic peroxides and regulated by *oxyR*). These inducible responses are critical for survival of *E.coli* against oxidative stress, as well as for decreased mutagenesis. During our studies, the set of genes involved in DNA repair were up-regulated in either one or two or in all the three *P. aeruginosa* strains. The *recR*, *lexA*, *recA* and *recN* were up-regulated nearly at the same level in all the three *P. aeruginosa* strains after exposure to hydrogen peroxide (Table 3.11). The RecA protein plays a central role in homologous recombination and, in conjunction with the LexA protein, induces the SOS regulon in response to DNA damage (Lusetti and Cox, 2002). It was also observed that other DNA repair proteins encoded by *micA*, *ung*, *xthA*, *mutS*, *recj*, and *xseB* were up-regulated only in TB and 892. DNA glycosylase (encoded by *ung*) excise damaged or unconventional bases in DNA and initiate the base excision repair (BER) pathway to maintain genomic integrity. XthA play an important role in the repair of abasic sites in DNA (Doetsch and Cunningham, 1990). Unless lesions are repaired prior to DNA replication, the non-coding lesions promote misincorporation of nucleotides and mutagenesis. Our findings suggest that the more number of genes involved in DNA repair in TB could give advantage for the efficient DNA repair and more resistant against the hydroxyl radicals.

The other categories of up-regulated genes belongs to energy metabolism, amino acid biosynthesis and metabolism, biosynthesis of cofactors, prosthetic groups & carriers, iron acquisition, sulfur metabolism, transcription RNA processing & degradation, translation & post-translational modification and cellular organization and biogenesis (figure 3.11 and appendix III).

The expression profile of genes involved in cellular processes including glycolysis, TCA cycle, anpleurotic reactions, aerobic electron transport chain components, fatty acid and phospholipid metabolism, NADH dehydrogenase and ATPase was up-regulated in all the three strains after exposure to hydrogen peroxide (appendix III). This suggest that the oxygen was produced in presence of hydrogen peroxide and the bacterium utilizes aerobic respiratory metabolism for energy conservation. Most of the genes involved in the biosynthesis and catabolism of acidic, basic, aromatic

and sulfur amino acids were up-regulated. This global change in amino acid pattern suggests that the amino acids could be utilized for the synthesis of various building blocks and thus aiding adaptation in presence of hydrogen peroxide. We could speculate that the up-regulation of genes those are involved in cobalamin and vitamin biosynthesis, can adapt the physiological and metabolic status of the organism to respond rapidly and sometimes dramatically to the environmental changes. Some of the genes involved in iron acquisition and sulfur metabolism were up-regulated in all the three strains after exposure to hydrogen peroxide. It observed that more number of genes which are involved in iron acquisition were up-regulated in PAO1 and most of the genes showed higher up-regulation in PAO1 as compared to TB. These genes are presumed to be involved in iron homeostasis. Iron and sulfur is also necessary for the reductive reconstitution of oxidatively inactivated (4Fe−4S) containing dehydratases. The up-regulation of genes involved in iron and sulfur metabolism are important for the cycle of oxidative inactivation by hydroxyl radicals, followed by reductive reactivation of iron-sulfur clusters, could act as a sink for hydroxyl radicals. The global response of the cells when grown in presence of hydrogen peroxide in the experiments analyzed here was up-regulation of reasonably high number of transcription and translation apparatus genes. We considered the expression profiles of all the genes that are involved in transcription and translation, including the major subunits of RNA polymerase, ribosomal proteins, and transcription and translation factors. The GeneChip experiment revealed that there is the global expression and co-ordination of the ribosome number and components of the transcription and translation apparatus for faster growing cells. We could observe the up-regulation of genes involved in cellular organization and biogenesis suggesting that there was rapid cell wall turnover and/or repair, presumably in response to hydrogen peroxide-mediated cell wall damage.

Down-regulated genes

The three major categories of genes involved in microaerophilic and anaerobic growth, quorum sensing and flagella biogenesis and chemotaxis were down-regulated in *P. aeruginosa* strains after exposure to hydrogen peroxide (figure 3.11). Out of these, only one category of genes is discussed in detail.

Microaerophilic and anaerobic growth

After exposure to hydrogen peroxide, it was observed that 55, 49 and 33 genes those are involved in denitrification were down-regulated in TB, 892 and PAO1 respectively (Table 3.11). The interesting finding was that most of the genes from operon PA0509–PA0525 were 10–200 times more strongly down-regulated in TB and 892 as compared to the PAO1 strain, while the other operon PA3870–PA3879 was just 2–10 times more strongly down-regulated in TB and 892 as compared to the PAO1. Then we checked the absolute signal intensities of genes from above mentioned operons (Table 3.13). We found that most of the genes has almost same signal intensity in absence of hydrogen peroxide in all the three *P. aeruginosa* strains. However, the signal intensities for PAO1 strain are significantly higher as compared to the intensities of TB and 892 strains in presence of hydrogen peroxide. The observed difference in the expression of these genes for PAO1 is due to the different expression pattern in the presence of hydrogen peroxide. The *P. aeruginosa* TB and 892 strains belongs to the same clone. This similar down-regulation pattern of denitrification genes could be due to the clonal similarity. This data suggests that TB and 892 do not prefer denitrification for the energy generation in presence of hydrogen peroxide. The other genes involved in heme biosynthesis, cytochrome biogenesis and molybdopterin biosynthesis showed random pattern of down-regulation. Many species of Gram-negative as well as Gram-positive bacteria can use inorganic N oxides as alternative electron acceptors for respiratory growth under oxygen-limiting conditions by a process referred to as denitrification. We incubated the bacteria in dialysis bags in presence of hydrogen peroxide (test sample) and in absence of hydrogen peroxide (control sample), for 2 h before RNA isolation. This can lead to fully aerobic conditions in presence of hydrogen peroxide and oxygen-limiting conditions in absence of hydrogen peroxide. Oxygen-limiting conditions are usually necessary for denitrification. The expression of *nir* and *nor* gene clusters are regulated by two FNR-like regulators, *anr* and *dnr*, in *P. aeruginosa* (Arai *et al.*, 1995; Arai *et al.*, 1997). FNR regulate the expression of many genes that are required for anaerobic growth when oxygen is depleted. But in our experiment, we could not see the differential regulation of both regulators *anr* & *dnr* required for denitrification. One possibility is that, they may be expressed below the cut-off value. This down-regulation of whole number of denitrification genes indicates that fully aerobic condition was created in presence of hydrogen peroxide.

Table 3.13. Expression of mRNA transcripts involved in microaerophilic and anaerobic growth: GeneChip normalized signal intensities in the absence (-) and presence (+) of 10 mM hydrogen peroxide.

Gene	PAO No.	strain TB		strain 892		strain PAO1	
		-	+	-	+	-	+
nirN	PA0509	1537	28	1627	46	1578	637
	PA0510	1545	5	1369	14	1202	410
nirJ	PA0511	1813	32	1680	52	2459	830
	PA0512	1656	12	1373	25	962	371
	PA0513	1503	8	1403	27	1023	361
nirL	PA0514	1504	6	1393	27	2144	726
	PA0515	2297	3	2198	49	2702	960
nirF	PA0516	2319	18	2070	48	2014	676
nirC	PA0517	3080	16	3045	100	3878	1391
norC	PA0523	3366	32	3045	130	1983	363
norB	PA0524	2666	8	2348	35	959	218
	PA0525	1143	13	981	14	1348	315
nosR	PA3391	586	6	554	27	639	170
nosZ	PA3392	2332	22	2555	47	3145	1520
nosD	PA3393	674	31	853	36	562	233
nosF	PA3394	338	10	386	10	412	168
moaA1	PA3870	278	11	482	8	558	31
	PA3871	524	10	697	10	1589	59
narI	PA3872	994	29	1754	23	3306	204
narJ	PA3873	1016	14	1774	25	1880	81
narH	PA3874	1135	20	1984	15	1908	75
narG	PA3875	1008	2	1697	12	1208	50
narK2	PA3876	686	11	1202	25	4925	42
narK1	PA3877	785	63	1309	47	1106	96
arcA	PA5171	4057	200	3885	185	9525	4190
arcB	PA5172	4842	79	4652	178	11199	4754
arcC	PA5173	3465	37	3651	81	5571	2216

We found that the whole operon (PA5170–PA5173) for arginine deiminase pathway was also down-regulated in all the three strains in presence of hydrogen peroxide. It has been reported that *anr* acts on a sequence related to *fnr* box to regulate the

expression of the arginine deiminase pathway under oxygen limiting conditions in *P. aeruginosa*. It was observed that this operon was 10–20 times down-regulated in TB and 892 as compare to PAO1. This could be also due to the clonal similarity of TB and 892. The absolute signal intensities of *arcABC* operon were higher in PAO1 strain as compared to the TB and 892 in presence of hydrogen peroxide (Table 3.13). The *arcABC* operon is strongly up-regulated in PAO1 in presence and absence of hydrogen peroxide. This suggests that *P. aeruginosa* PAO1 prefers arginine deiminase pathway for the energy generation in presence of hydrogen peroxide. In other words, the *arcABC* operon is a paradigmatic example for the clone specific regulation of metabolic features. In summary, the genes involved for growth in oxygen limiting conditions were severely down-regulated in *P. aeruginosa* TB and 892 as compared to PAO1 strain after exposure to hydrogen peroxide.

Summary

The growth and survival of bacteria in diverse environments is achieved through homeostatic mechanisms that have evolved to maintain relatively constant cytoplasmic conditions. In parallel, gene expression is modulated so that the overall activity of metabolic pathways remains adequate for growth and survival. Despite these mechanisms, the cell has to cope with significant variations in the cytoplasmic constitution, e.g. solute composition and concentration, and pH, as well as oxidative stress generated during aerobic growth. Hydrogen peroxide-mediated stress response modulated a higher number of genes in TB as compared to 892 and PAO1. Being clonal variants, TB and 892 shared the common differential expression pattern on transcriptome. The global gene response against hydrogen peroxide showed the up-regulation of genes necessary for the energy generation during the aerobic growth by creating the oxygen-rich environment and up-regulation of amino acid metabolism genes, as the preferred nitrogen source. The transcriptome data indicated that bacterial cell needs adequate amount of iron and sulfur for the synthesis of oxidatively damaged iron-sulfur clusters. It was observed that most of the key players to combat hydrogen peroxide mediated oxidative stress response and DNA repair genes were up-regulated in all the three strains. Consistent with a need for increased energy and biosynthetic activity for production of *P. aeruginosa* cell wall components and oxidative damage protection proteins, most of the genes necessary for nucleotide metabolism, transcription and translation were up-regulated

in TB as compared to the PAO1. This result can be explained that the turnover number of TB could be much higher as compared to the PAO1 in response to hydrogen peroxide. These findings indicated that enough oxygen was generated during our growth conditions to down-regulate the genes necessary for arginine fermentation and anaerobic respiration. Down-regulation of quorum sensing system, chemotaxis genes show that it is not necessary for the cell against the hydrogen peroxide mediated oxidative stress response. Several lines of evidence indicate that global changes in *P. aeruginosa* gene expression are essential for survival in presence of hydrogen peroxide. We discovered that in addition to genes involved in oxidative damage and DNA repair, several genes for pathways in energy generation and cell biogenesis likely contribute to the ability of *P. aeruginosa* TB to evade the hydrogen peroxide mediated oxidative stress.

3.4.2. Transcriptional analysis of non-PAO1 ORFs

During our screening for intracellular survival in PMNs, we have found putative transcriptional regulators and strain specific genes called non PAO1 genes which are attenuated for intracellular survival in PMNs. The function of these genes is not characterized so far. So we have used a microarray (GeneChip) approach to obtain functional details about these genes.

3.4.2.1. Gene organization of non-PAO1 sequences

Altogether, we have found three mutants that have insertions in genes which do not show any homology with the sequenced PAO1 strain. Out of these, two mutants were further characterized in this dissertation. The plasmids from these mutants were sequenced further on either side to obtain more sequence information. The G+C content from these sequence was about 60 % which is low as compared to the average G+C content (67 %) of *P. aeruginosa*. This suggests that these sequences could have extrachromosomal origin and integrated through horizontal gene transfer. In order to determine the abundance of these sequences in other *P. aeruginosa* strains, we hybridized these non-PAO1 sequences on Southern blots prepared from the genomic DNA of *P. aeruginosa* clinical isolates CSGB8 and 892 and environmental isolate SG17M in addition to the reference PAO1 strain. The *P. putida* KT2440 was used as a negative control during the Southern hybridization. We could see signals on the genomic DNA of *P. aeruginosa* TB and 892 but there were no

signals obtained on the genomic DNA from other isolates (figure 3.12 and 3.13). The *P. aeruginosa* TB and 892 belongs to the same clone. The results from Southern hybridization suggests that this non-PAO1 sequence is present only in the TB clone.

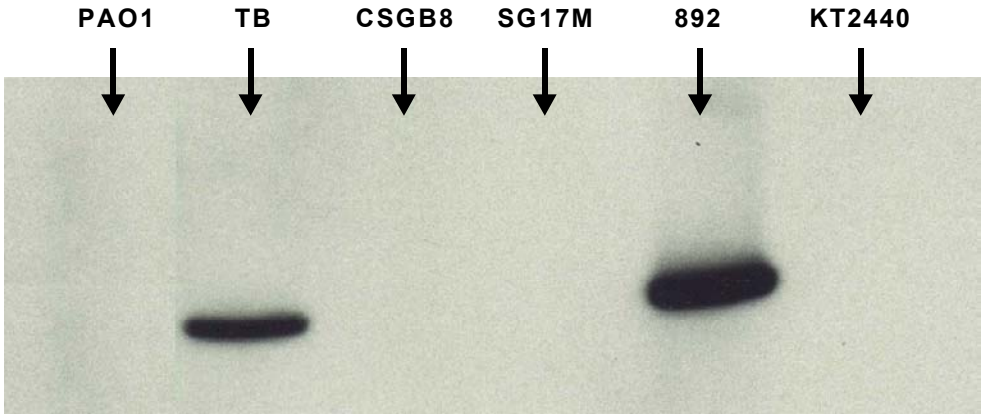


Fig. 3.12. Genomic abundance of the non-PAO1 sequence from 25A12 mutant. The genomic probe from 25A12 mutant was hybridized on the Southern blot prepared from genomic DNA of different *P. aeruginosa* isolates.

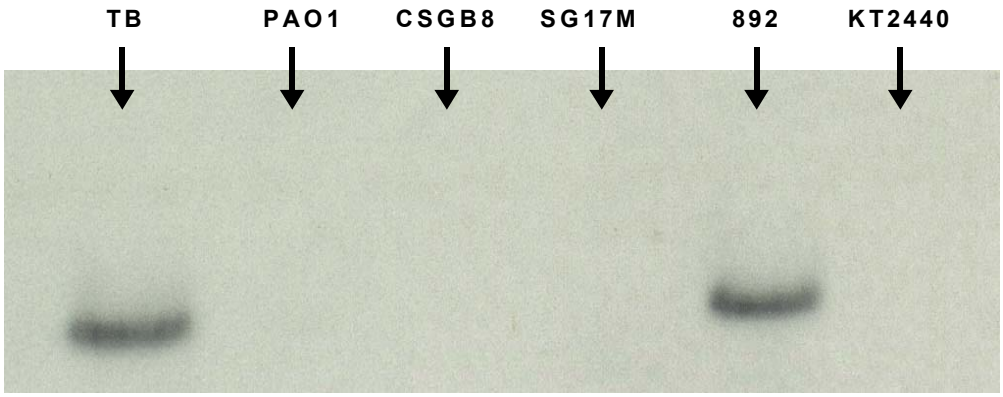


Fig. 3.13 . Genomic abundance of the non-PAO1 sequence from 45A7 mutant. The genomic probe from 45A7 mutant was hybridized on the Southern blot prepared from genomic DNA of different *P. aeruginosa* isolates.

Then the sequences from 25A12 and 45A7 mutants were subjected to the gene finder program to predict the open reading frames (ORFs) and gene organization (see figure 3.14 and 3.15).

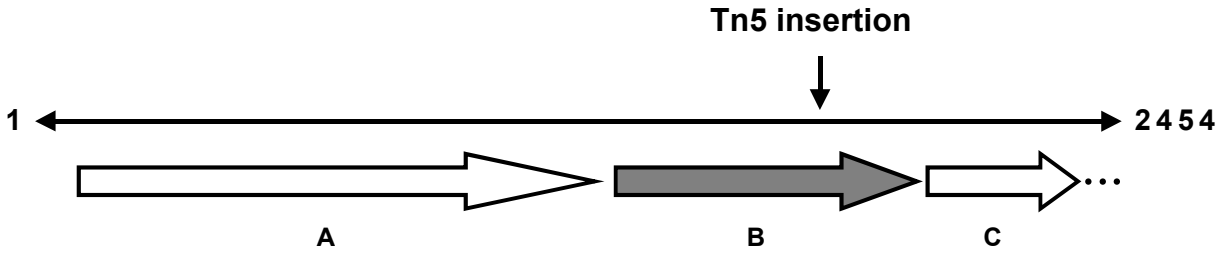


Fig. 3.14. Gene organization of non-PAO1 sequence (2454 bp) from 25A12 mutant.

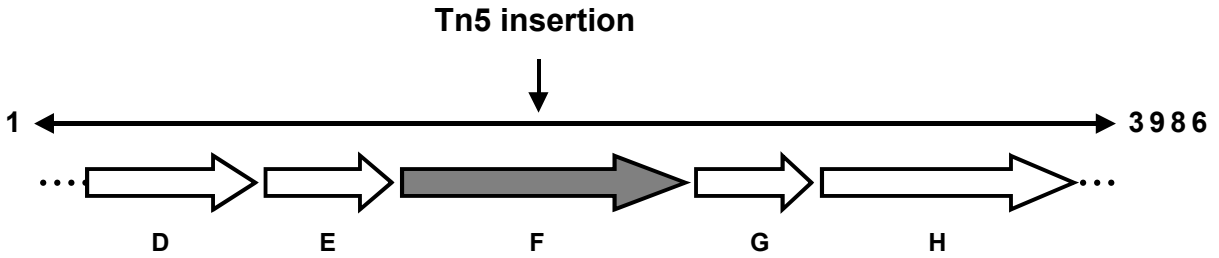


Fig. 3.15. Gene organization of non-PAO1 sequence (3986 bp) from 45A7 mutant.

We predicted three ORFs, one truncated on the right side and two complete on the left side from 25A12 non-PAO1 sequence (Table 3.13). Five ORFs, two truncated on either side and three complete in the middle were predicted from 45A7 non-PA01 sequence. The truncated ORFs on either side in both the mutants were due to the small size of the inserted DNA. We could not detected the anti-termination loops in both non-PAO1 sequences by in silico analysis. These ORFs were blasted for protein homology searches on NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/), including finished and unfinished genome projects. The homologies obtained are listed below (Table 3.14).

It was observed that the ORFs from 25A12 non-PAO1 sequence did not show significant homology with any other gene from the available databases. The ORFs from 45A7 non-PAO1 sequence showed homology on protein level from 55–84 % with nitrilase or α-helix motif or hypothetical proteins. This in silico analysis did not give any functional clue for these ORFs with respect to the intracellular survival in PMNs. Hence we used *P. aeruginosa* PAO1 microarrays to obtain the functional information and how these genes react with the core *P. aeruginosa* genome.

Table 3.14. Predicted ORFs and homology on protein level from non-PAO1 sequence.

ORF	Start	End	Size (bp)	Homology on protein level	Identity (%)
A	113	1606	1494	no	-
B	1628	2215	588	no	-
C	2371	>2454	>84	no	-
D	>1	693	>693	nitrilase (*Xanthomonas campestris pv. campestris*)	68
E	943	1251	309	α-helix chain (*E. coli*)	58
F	1311	2420	1110	alcohol dehydrogenase (*E. coli*)	83
G	2464	2862	399	hypothetical protein (*Proteus vulgaris*)	84
H	2976	>3986	>1011	hypothetical protein (*Mesorhizobium loti*)	55

3.4.2.2. Expression of 25A12 non-PAO1 ORF

Before going to GeneChip analysis, the critical and essential requirement was to see the expression of these unknown ORFs. For this, we used standard conditions like growth in LB medium to early and late exponential phase, exposure to stress conditions like growth in iron limiting LB medium, growth in presence of chemicals generating oxidative stress (paraquat and hydrogen peroxide) and growth in presence of PMNs. For the last two conditions, the bacterial cells were grown in LB medium up to early stationary phase, harvested and were exposed to chemicals generating oxidative stress or to PMNs in a dialysis bag for about 2 h. There was virtually no direct contact of cells with PMNs. Because of difficulties in the separation of eukaryotic and prokaryotic RNA, this system was developed. After the incubation period, the cells were directly recovered from the dialysis bag and RNA was isolated as mentioned previously (see 2.6.4). Then the expression pattern of these non-PAO1 ORFs was compared on Northern blots (figure 3.16 and 3.17).

We could not see the signal of 25A12 non-PAO1 ORF on the Northern blot when the TB wild type was grown under above mentioned conditions. However, a strong signal was obtained when the mutant 25A12 was grown under the same conditions (figure 3.16). These results suggested that this may be a very lowly expressed and essential gene and could modulate the gene expression very precisely at low levels. The signal intensity of the mutant was weak when grown in iron depleted medium as compared to the other growth conditions. The strong signal on the Northern blot for mutant could be due to the feedback mechanism because the mutant tries to express

a truncated and non-functional ORF at high levels. However, the pattern of truncated ORFs vary when the mutant was grown under standard and stress conditions. We could see the high molecular weight truncated ORFs when the mutant was grown in presence of oxidative stress generating agents and PMNs. We did not find the anti-termination loops in the sequence from 25A12 mutant indicating that all the genes from this sequence are expressed simultaneously. These results suggests that it could be polycistronic mRNA and form an operon structure. However, there were not clear cut bands obtained on the Northern blot indicating the faster degradation of mRNA.

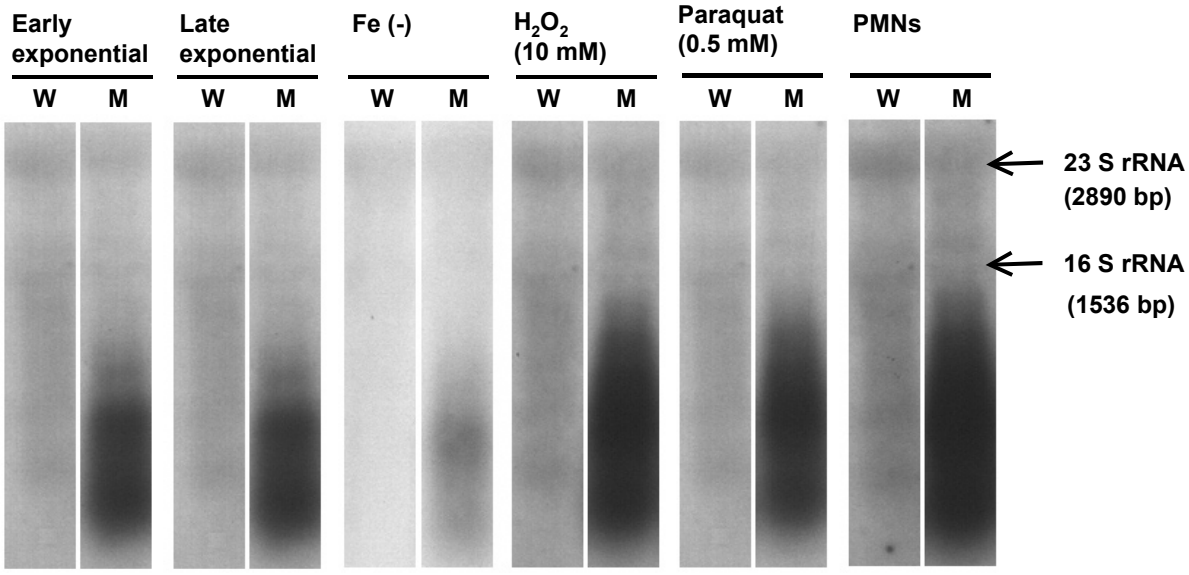


Fig. 3.16. Expression profile of non-PAO1 ORF from 25A12 mutant on Northern blot under various growth conditions. RNA from various growth conditions was hybridized with the probe prepared from 25A12 non-PAO1 sequence.

3.4.2.3. Expression of 45A7 non-PAO1 sequence

This non-PAO1 gene showed three strong signals on the Northern blot when the wild type was grown in the above mentioned conditions, whereas we could see only the low molecular weight signal at the bottom on the mutant which is weak as compared to the TB wild type (figure 3.17). This indicates that this is a highly expressed gene in all the tested conditions. The approximate molecular weight sizes of these signals corresponds to 3.1 Kb, 2.5 Kb and 4.5 Kb. The intergenic region of almost 249 and 113 nucleotides is present between E and F ORFs and G and H ORFs, respectively

(figure 3.15). We could not find the anti-termination loop in the intergenic region and in the whole sequence from 45A7 mutant by in silico analysis. The expression pattern of 45A7 non-PAO1 sequence suggests that it could be a polycistronic mRNA and form an operon structure.

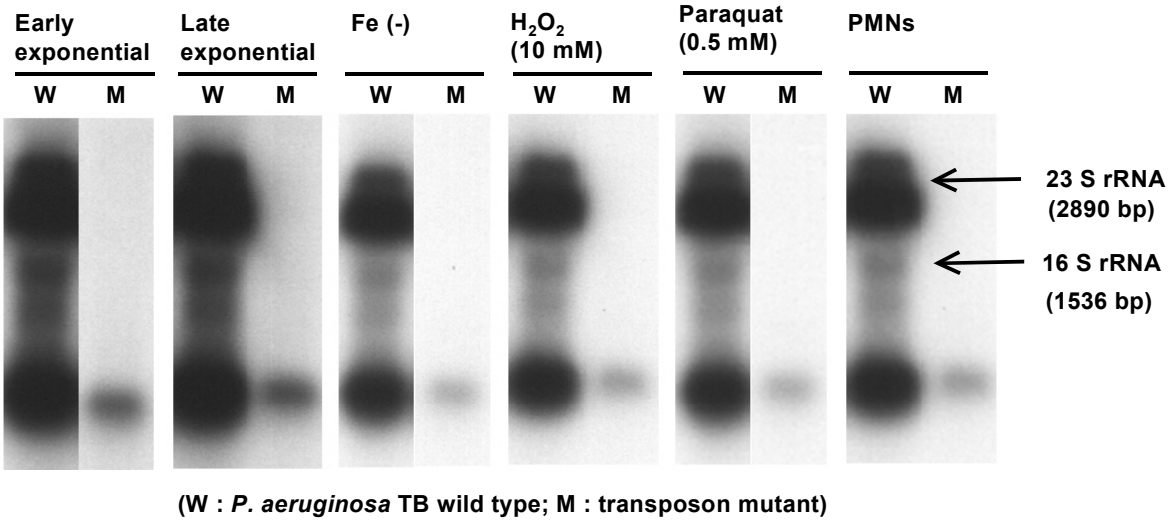


Fig. 3.17. Expression profile of non-PAO1 ORF from 45A7 mutant on Northern blot under various growth conditions. RNA from various growth conditions was hybridized with the probe prepared from 45A7 non-PAO1 sequence.

3.4.2.4. GeneChip analysis of 25A12 and 45A7 non-PAO1 sequences

GeneChip expression analysis was performed for these two mutants that have insertion in the non-PAO1 genes. For this, three expression conditions were chosen: growth of wild type and mutants in LB medium up to late exponential phase, growth in presence of hydrogen peroxide and PMNs. The idea of choosing the standard condition was that it would show the impact of the transposon insertion on the expression of the PAO1 defined core genome and on the metabolism of *P. aeruginosa* TB. The latter two conditions can mimic the situation close to the PMNs because oxidative stress response is the major host defense mechanism exerted by PMNs for microbial killing. RNA from these conditions was isolated and processed further for GeneChip hybridization as previously described (see 2.6.4–2.6.10).

3.4.2.4.1. Down-regulation in the non-PAO1 mutants

Differentially regulated genes from both mutants having known function were classified. The differential expression pattern of both the mutants were compared from all the three conditions. The most striking observation is that both mutants showed similar pattern on the transcriptome in the two conditions i.e. when grown in the presence of hydrogen peroxide and PMNs. For the third condition i.e. growth in LB medium up to late exponential phase, we could see the slight down-regulation of genes involved in arginine metabolism in the 25A12 mutant (see table 7.2 in appendix III). It was observed that 14 genes involved in arginine metabolism were modestly down-regulated in the 25A12 mutant when grown in LB medium up to late exponential phase. The arginine metabolism genes from arginine transport (PA0888–PA0893), arginine succinyltransferase (AST) pathway (PA0895–0899) and arginine deiminase (ADI) pathway (PA5171–PA5173) and the transcriptional regulator *anr* were down-regulated in the mutant 25A12. *P. aeruginosa* can utilize arginine under aerobic conditions as a sole source of carbon, energy, and nitrogen via the arginine succinyltransferase pathway (Haas *et al.*, 1990). Despite the preference for an aerobic environment, *P. aeruginosa* can utilize arginine under anaerobic conditions via the arginine deiminase pathway (Luthi *et al.*, 1990). The ADI pathway in association with *anr* was modestly down-regulated in the 25A12 mutant. This pathway catabolizes L-arginine to L-ornithine, with concomitant formation of ATP from ADP. So these results suggests that this unknown sequence has modest impact on the arginine utilization as a energy source. We could not see the significant down-regulation of arginine utilization genes in 45A7 mutant when grown under the same condition.

There were virtually no differentially regulated genes in the mutant 45A7 when grown in LB medium up to late exponential phase. This GeneChip data uncovered that there is no interference with the core genome of *P. aeruginosa* under standard growth conditions and these two genes could be regulated their own.

Differential regulation of genes involved in denitrification

Table 3.15 shows the genes whose expression was significantly down-regulated by hydrogen peroxide or PMNs in either one or both tested *P. aeruginosa* transposon

Table 3.15. Genes down-regulated in 25A12 and 45A7 mutants compared to the wild type strain when grown in presence of hydrogen peroxide or PMNs[1].

PAO No.	Average fold change				Protein name[2]
	H_2O_2		PMNs		
	25A12	45A7	25A12	45A7	
Denitrification					
PA0024	2.12	2.15			coproporphyrinogen III oxidase, *hemF*
PA0291	2.19	1.89	1.62	1.76	outer membrane porin, *oprE*
PA0518		1.93			cytochrome c-551 precursor, *nirM*
PA0519	6.71	15.85			nitrite reductase precursor, *nirS*
PA0527	22.00	29.92	1.83	3.14	transcriptional regulator, *dnr*
PA0538	2.95	3.26			disulfide bond formation protein, *dsbB*
PA1544	3.05	2.34	1.52	1.62	transcriptional regulator, *anr*
PA1546	10.19	14.12	1.62	1.57	oxygen-independent coproporphyrinogen III oxidase, *hemN*
PA1556	6.44	13.77	1.74	1.61	probable cytochrome c oxidase subunit
PA1557	11.11	15.05	2.96	2.51	cytochrome oxidase subunit (cbb3-type)
PA2664	30.20	22.08			flavohemoprotein, *fhp*
PA3873			1.81	1.58	respiratory nitrate reductase delta chain, *narJ*
PA3874			2.05	1.60	respiratory nitrate reductase beta chain, *narH*
PA3875			2.69	2.29	respiratory nitrate reductase alpha chain, *narG*
PA3876			2.47	2.27	nitrite extrusion protein 2, *narK2*
PA3877	9.43	6.80	2.14	2.17	nitrite extrusion protein 1, *narK1*
PA3878	2.53	2.35			two-component sensor, *narX*
PA3879	2.69	3.52	2.73	2.50	two-component response regulator, *narL*
PA4133	1.60	1.71	1.52	2.81	cytochrome c oxidase subunit (cbb3-type)
PA4464	2.04	2.30			nitrogen regulatory IIA protein, *ptsN*
PA4812	1.72	1.46			formate dehydrogenase-O, *fdnG*

[1]: No down-regulated genes from above mentioned categories were observed when both the mutants were grown in LB medium up to late exponential phase.

[2]: Gene names are indicated in italics.

mutants 25A12 and 45A7. It was observed that the down-regulation gene pattern in both mutants was similar in the presence of hydrogen peroxide and PMNs. But the number of down-regulated denitrification genes in presence of hydrogen peroxide were higher and the absolute values were high on the negative side in both the mutants as compared to the PMNs. This pattern reflects that the signal provided by

direct contact of hydrogen peroxide for gene modulation could be stronger than the indirect contact with PMNs. It was observed that the cells in presence of hydrogen peroxide were more active metabolically. That could be the reason why they need strong gene modulation.

The expression of the *nir* and *nor* genes involved in denitrification is regulated by two FNR-like regulators, ANR and DNR, in *P. aeruginosa* (Arai *et al.*, 1995; Arai *et al.*, 1997). We could see the down-regulation of these regulators in both the mutants when exposed to hydrogen peroxide and PMNs. These FNR-like regulators regulates the expression of many genes that are required for anaerobic growth when oxygen is depleted. During our experiments, we observed the down-regulation of some of the genes from *nir* (*nirM*, *nirS*) and *nar* (*narJ*, *narH*, *narG*, *narK2*, *narK1*, *narX*, *narL*) gene cluster, heme biosynthesis (*hemF*, *hemN*) and anaerobically induced genes (*oprE*, *dsbB*, *ptsN*, *fdnG*) in both the mutants 25A12 and 45A7. *Fhp* was also strongly down-regulated in both the mutants after exposure to hydrogen peroxide. It is able to scavenge oxygen molecules, improve microaerobic cell growth and enhance oxygen-dependent product formation in several organisms (Frey *et al.*, 2000). These results suggest that most of the genes necessary for growth in oxygen limiting condition or in anaerobic respiration were down-regulated in both mutants.

3.4.2.4.2. Up-regulation in the non-PAO1 mutants

Differential regulation of genes involved in type III secretion system

The type III secretion system (TTSS) is widespread in Gram-negative pathogens, including *Yersinia*, *Salmonella* and *Shigella*, and in plant pathogens such as *Pseudomonas syringae* and *Erwinia chrysanthemi* (Galan and Collmer, 1999). This unusual system requires close contact between infecting bacteria and the host cell to secrete and deliver toxic bacterial proteins directly to the cytosol of the host cell. TTSS and its secreted effector proteins are considered the most prominent virulence factor of *P. aeruginosa*.

Table 3.16 lists the genes that were up-regulated in both mutants 25A12 and 45A7 which are involved in building the type III secretion machinery. Out of the 36 TTSS genes, 6 were up-regulated when grown in LB medium up to exponential phase and 12 were up-regulated after exposure to PMNs. Both mutants showed a similar

expression pattern under these two conditions. *P. aeruginosa* secretes at least four effector proteins through its type III secretion system, namely ExoS, ExoT, ExoY and ExoU (Frank, 1997). But we could not see the up-regulation of these effector proteins in both examined *P. aeruginosa* TB mutants. Results from several laboratories strongly suggest that *P. aeruginosa* strains collected from diverse infection environments express different type III system-secreted effectors (or effector combinations), and this therefore results in different phenotypes (Dacheux *et al.*, 2001). In our case, the signal from PMNs may not be enough to stimulate the secretion of the effector proteins since there was not direct contact with the PMNs.

Table 3.16. Genes up-regulated from type III secretion system in 25A12 and 45A7 mutants when grown in LB medium up to late exponential phase and in presence of PMNs[1].

PAO No.	Average fold change			Protein name[2]
	LE	PMNs		
	25A12	25A12	45A7	
PA1692	2.30	2.27	2.94	translocation protein in type III secretion
PA1697		5.50	5.68	ATP synthase in type III secretion system
PA1698		3.26	3.81	type III secretion outer membrane protein, *popN*
PA1703	3.48	4.52	4.61	type III secretary apparatus protein, *pcrD*
PA1705	2.42	3.00	4.33	regulator in type III secretion, *pcrG*
PA1707		2.07	2.14	regulatory protein, *pcrH*
PA1713		1.87	1.87	transcriptional regulator, *exsA*
PA1715		4.21	5.87	type III export apparatus protein, *pscB*
PA1716		2.46	2.73	type III secretion outer membrane protein, *pscC*
PA1721		3.51	3.62	type III export protein, *pscH*
PA1723	2.78	3.26	3.93	type III export protein, *pscJ*
PA1724	2.64			type III export protein, *pscK*
PA1725	2.88	4.51	3.39	type III export protein, *pscL*

[1]: No up-regulated genes from type III secretion system were observed when both the mutants grown in presence of hydrogen peroxide.

[2]: Gene names are indicated in italics.

Differential regulation of genes involved in quorum sensing and iron uptake

Table 3.17 shows the genes involved in quorum sensing and iron uptake which were up-regulated in both mutants after exposure to hydrogen peroxide. *P. aeruginosa* produces a wide spectrum of secreted virulence factors, including LasB elastase,

Table 3.17. Genes up-regulated from quorum sensing system and iron uptake in 25A12 and 45A7 mutants when exposed to hydrogen peroxdie[1].

PAO No.	Average fold change		Protein name[2]
	25A12	45A7	
PA1247	3.73	3.10	alkaline protease secretion protein, *aprE*
PA1249	3.48	3.61	alkaline metalloproteinase precursor, *aprA*
PA1871	3.55	4.33	LasA protease precursor, *lasA*
PA4210	4.00	11.85	probable phenazine biosynthesis protein, *phzA1*
PA4211	4.76	3.52	phenazine biosynthesis protein, *phzB1*
PA1901	59.90	42.96	phenazine biosynthesis protein, *phzC2*
PA1902	27.00	12.21	phenazine biosynthesis protein, *phzD2*
PA1903	5.02	3.49	phenazine biosynthesis protein, *phzE2*
PA1904	47.04	33.24	phenazine biosynthesis protein, *phzF2*
PA1905	8.02	4.19	pyridoxamine 5'-phosphate oxidase, *phzG2*
PA2194	3.37	4.57	hydrogen cyanide synthase, *hcnB*
PA2195	2.85	4.51	hydrogen cyanide synthase, *hcnC*
PA3478	4.86	4.46	rhamnosyltransferase chain B, *rhlB*
PA3479	4.21	3.41	rhamnosyltransferase chain A, *rhlA*
PA3724	12.31	7.06	elastase LasB, *lasB*
PA4224	2.36	2.15	pyochelin biosynthetic protein, *pchG*
PA4225	2.61	2.38	pyochelin synthetase, *pchF*
PA4226	2.04	2.14	dihydroaeruginoic acid synthetase, *pchE*
PA4228	2.35	2.14	pyochelin biosynthesis protein, *pchD*
PA4229		2.22	pyochelin biosynthetic protein, *pchC*
PA4230		2.83	salicylate biosynthesis protein, *pchB*
PA4231	2.78	3.03	salicylate biosynthesis isochorismate synthase, *pchA*

[1]: No up-regulation of genes from the abovementioned categories were observed when both mutants were grown in LB medium and when grown in presence of PMNs.

[2]: Gene names are indicated in italics.

rhamnolipids, pyocyanin, lipase, and hydrogen cyanide (Van Delden and Iglewski, 1998). The *las* and *rhl* quorum-sensing systems regulate the production of these factors in a cell density dependent manner. This regulation relies on the accumulation of two autoinducer (AI) molecules in the medium, 3-oxo-C_{12}-homoserine lactone (3-oxo-C_{12}-HSL) and C_4-HSL, which induce the *las* and *rhl* quorum-sensing systems when the bacterial cell density reaches a certain threshold (quorum), leading to the transcription of specific genes and the production of the virulence factors cited above.

Both systems involve a transcriptional regulator (r*hlR* and *lasR*, respectively) and an AI synthase (RhlI and LasI, respectively). A hierarchy was proposed in which the *las* system activates the *rhl* system by inducing the transcription of the activator gene *rhlR* (Pesci *et al.*, 1997).

During this study, the quorum sensing repressor *rsaL* was repressed in both mutants when exposed to hydrogen peroxide (data not shown). So we could expect the up-regulation of quorum sensing dependent genes. Here, alkaline proteases (*aprA*, *aprE*), LasA protease precursor (*lasA*), elastase (*lasB*), phenazine biosynthesis genes (*phzA1*, *phzB1*, *phzC2*, *phzD2*, *phzE2*, *phzF2*, *phzG2*), hydrogen cyanide biosynthesis genes (*hcnB*, *hcnC*), rhamnolipid biosynthesis genes (*rhlA*, *rhlB*) and pyochelin biosynthesis genes (*pchA*, *pchB*, *pchC*, *pchD*, *pchE*, *pchF*, *pchG*) were significantly up-regulated in both the mutants after exposure to hydrogen peroxide. All these gene products belong to both quorum sensing (*rhlR-rhlI* and *lasR-lasI*) systems. These results suggest that the transposon mutant could be handicapped in certain known or unknown virulence factors and then up-regulate the quorum sensing dependent virulence factors in presence of hydrogen peroxide as a rescue mechanism.

Iron is one of the essential elements for almost all bacteria, and the ability of pathogenic bacteria to acquire iron in hosts is essential for their growth and infection (Bullen, 1981; Neilands, 1981). In animal hosts, iron is usually bound to proteins such as transferrin, lactoferrin, and ferritin and bound as heme to hemoglobin (Hb) and various enzymes (Otto *et al.*, 1992; Wooldridge and Williams, 1993). To utilize such complexes as iron sources, bacteria possess some sophisticated mechanisms, including an iron uptake system mediated by high-affinity iron chelators called siderophores such as pyoverdin and pyochelin and a heme uptake system, which involve specific receptors (Marinez *et al.*, 1990; Otto *et al.*, 1992). Most of the pyochelin biosynthesis genes (*pchA*, *pchB*, *pchC*, *pchD*, *pchE*, *pchF* and *pchG*) were up-regulated in both the mutants when exposed to hydrogen peroxide. It is known that iron and iron-containing proteins play important roles in the growth and pathogenesis of *P. aeruginosa*, especially in its defense against oxidative stress (Vasil and Ochsner, 1999). Many proteins involved in respiration (e.g. ferredoxins and other iron–sulphur proteins) and degradation of H_2O_2 and (e.g. haem catalase, iron

superoxide dismutase and peroxidase) require iron for functionality. Therefore, this up-regulation in pyochelin biosynthesis genes in both the non-PAO1 mutants would help to combat the oxidative stress response generated by hydrogen peroxide.

The microarray (GeneChip) results from these two mutants can be summarized in the following way. Inspite of constitutive expression of these non-PAO1 ORFs in all the tested conditions, the microarray data showed that there were only subtle changes in the expression of the PAO1 defined core genome in both the mutants under the standard growth conditions. Only one mutant, 25A12, exhibited a modest down-regulation of arginine utilization. This shows that there is virtually no interference with the core *P. aeruginosa* genome. The down-regulation of large number of denitrification genes in both the mutants when exposed to hydrogen peroxide and PMNs suggests that *P. aeruginosa* TB could switch off the aerobic respiration during the intracellular survival and growth in PMNs. The type III secretion system and quorum sensing systems were up-regulated in both the mutants suggesting that this derepression of virulence factor expression may be critical for minimizing the stress exerted by hydrogen peroxide and PMNs by over-expressing the virulence factors. The GeneChip experiments showed that these two non-PAO1 ORFs are regulated by their own without severely affecting the core metabolism of *P. aeruginosa*. These two sequences have a low G+C content (60 %) as compared to the average G+C content of *P. aeruginosa* which is 67 %. This suggests that they could be a part of extrachromosomal genetic elements and acquired through horizontal gene transfer. Out of these two ORFs, the ORF from 25A15 is expressed at very low levels in wild type while the other ORF from 45A7 is highly expressed in the wild type *P. aeruginosa* TB. The GeneChip data revealed that transposon insertion into the non-PAO1 ORFs leads to the up-regulation of the type III secretion and quorum sensing systems. The up-regulation of virulence factors suggests that these transposon mutants are more virulent than the *P. aeruginosa* TB wild type but impaired for intracellular survival and growth in PMNs. This suggests that these ORFs stabilizes the expression of virulence genes in *P. aeruginosa* TB wild type but are necessary for the intracellular survival and growth in PMNs. The similar pattern of differential expression on microarray in both the mutants indicate that they have a common phenotypic and genotypic effect on the normal metabolism and virulence factor synthesis *in P. aeruginosa* TB.

3.4.3. Transcriptional analysis of sigma-70 factor

The mutant 23B9 that carries a mutation in the gene PA2468 (probable sigma-70 factor, ECF subfamily) was identified as being impaired for intracellular survival in the granulocytes. It has 70 % homology with the *fecI* from *E. coli* but the function of this sigma factor is not well characterized so far. The upstream (PA2469) and downstream (PA2467) genes are a transcriptional regulator and a probable transmembrane sensor (53 % *pupR*, *P. putida*) respectively. We used *P. aeruginosa* microarrays to uncover the regulatory mechanism of this gene.

3.4.2.1. Expression of sigma-70 factor under various growth conditions

Several standard as well as stress growth conditions (see 2.6.3) were used to see the expression of this gene on Northern blot. The RNA from several growth conditions was isolated from *P. aeruginosa* TB wild type and 23B9 mutant and Northern blot was prepared. Then the probe spanning the whole gene of 23B9 was generated and hybridized on the Northern blot for overnight. The hybridized blot was

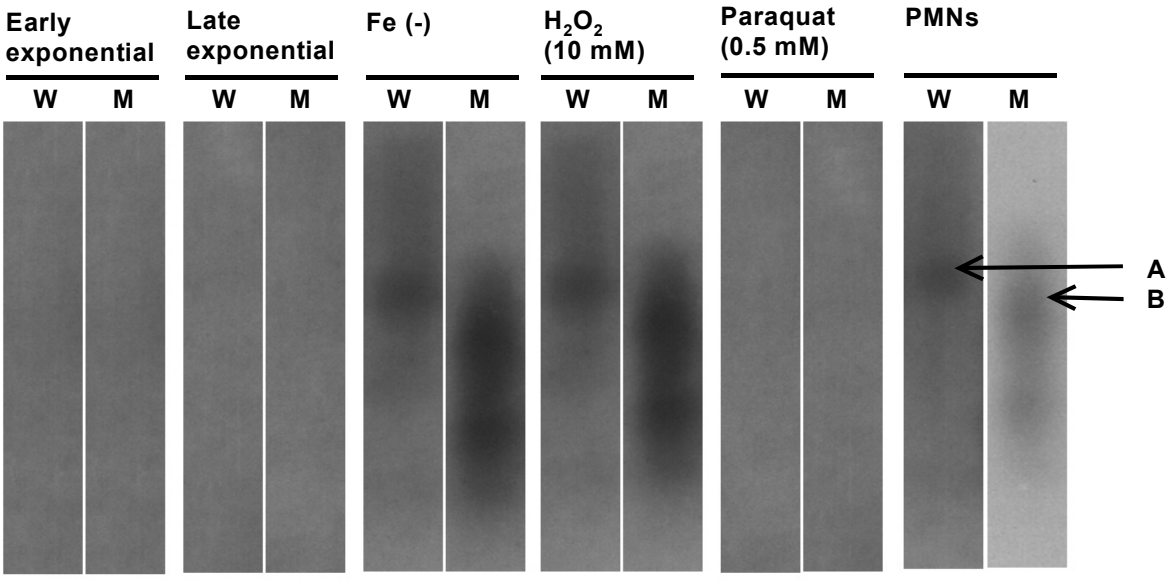


Fig. 3.18. Expression profile of sigma-70 factor on Northern blot under various growth conditions. RNA from various growth conditions was hybridized with the probe prepared from the PA2468 gene. (A: functional ORF; B: truncated and non-functional ORF).

detected as described previously (see 2.6.5.3). We could see the expression of this gene under iron limiting conditions, in presence of hydrogen peroxide and PMNs (figure 3.18), while the expression of this gene under other growth conditions was not observed. Northern blot analysis showed that the signal is weak for *P. aeruginosa* TB wild type, while it is stronger for the mutant. This could be the lowly expressed gene and the mutant tries to produce more and more since it is a truncated and non-functional ORF.

3.4.2.2. GeneChip analysis of sigma-70 factor

GeneChip expression analysis was performed for the mutant 23B9 having a mutation in the probable sigma-70 factor gene. We observed on the Northern blot that this sigma factor was expressed under three growth conditions i.e. when *P. aeruginosa* TB wild type and mutant strains were grown in iron limiting LB medium or when exposed to hydrogen peroxide or PMNs. The RNA from these three growth conditions was isolated and processed further for GeneChip hybridization as previously described.

The differential expression pattern of the wild type was compared with the mutant from all the three growth conditions. Then the differentially regulated genes were classified based on the known or predicted function (Table 3.18). The other genes with unknown function were not included in this table.

Table 3.18. Differentially regulated genes in the sigma-70 factor (PA2468) grown under stress conditions (iron limitation, in presence of hydrogen peroxide and PMNs).

PAO No.[ab]	Average fold change			Protein name[c]
	Fe (-)	H_2O_2	PMNs	
Down-regulated genes in mutant				
Aerobic/anaerobic growth				
PA0024		2.47		coproporphyrinogen III oxidase, *hemF*
PA0517		2.26		probable c-type cytochrome precursor, *nirC*
PA0519		21.32		nitrite reductase precursor, *nirS*
PA0527		61.15		transcriptional regulator, *dnr*
PA1173[b]			2.43	cytochrome c-type protein NapB precursor , *napB*
PA1174			2.01	periplasmic nitrate reductase protein, *napA*
PA1175[b]			2.04	napD protein of periplasmic nitrate reductase, *napD*

PAO No.[ab]	Average fold change			Protein name[c]
	Fe (-)	H_2O_2	PMNs	
Down-regulated genes in mutant				
Aerobic/anaerobic growth				
PA1505		1.90		molybdopterin biosynthetic protein A2, *moaA2*
PA1544	1.50	2.02		transcriptional regulator, *anr*
PA1546		14.85		oxygen-independent coproporphyrinogen III oxidase, *hemN*
PA1556		10.01		probable cytochrome c oxidase subunit
PA1557		43.79		probable cytochrome oxidase subunit (cbb3-type)
PA2664		35.81		flavohemoprotein, *fhp*
PA3877		14.69		nitrite extrusion protein 1, *narK1*
PA3878		3.12		two-component sensor, *narX*
PA3879		3.40		two-component response regulator, *narL*
PA4130[b]	1.98		2.30	probable sulfite or nitrite reductase
PA4133[b]	2.24		3.28	cytochrome c oxidase subunit (cbb3-type)
PA4464		2.51		nitrogen regulatory IIA protein, *ptsN*
PA4587		2.68		cytochrome c551 peroxidase precursor, *ccpR*
PA5170	1.96	36.80		arginine/ornithine antiporter, *arcD*
PA5171	1.88	2.69		arginine deiminase, *arcA*
PA5172	2.12			ornithine carbamoyltransferase, *arcB*
PA5173	1.91			carbamate kinase, *arcC*
Quorum sensing				
PA0026		1.84		hypothetical protein
PA0109[a]			1.87	hypothetical protein
PA0122			2.20	conserved hypothetical protein
PA0176	1.52			probable chemotaxis transducer
PA0179			1.43	probable two-component response regulator
PA0263		4.03	2.69	secreted protein Hcp, *hcpC*
PA0567	1.91			conserved hypothetical protein
PA0586	1.66			conserved hypothetical protein
PA0588	1.60			conserved hypothetical protein
PA0652[a]		1.41		transcriptional regulator, *vfr*
PA1003[a]			2.19	transcriptional regulator, *mvfR*
PA1004			1.59	quinolinate synthetase A, *nadA*
PA1323	1.50			hypothetical protein
PA1430[a]		1.41		transcriptional regulator, *lasR*
PA1431[a]	1.68	4.06	1.26	regulatory protein, *rsaL*
PA1432[a]		3.03		autoinducer synthesis protein, *lasI*
PA1659			1.78	hypothetical protein
PA1664			15.95	hypothetical protein

PAO No.[ab]	Average fold change			Protein name[c]
	Fe (-)	H_2O_2	PMNs	
Down-regulated genes in mutant				
Quorum sensing				
PA1667			2.71	hypothetical protein
PA1869 [a]			1.96	hypothetical protein
PA1871 [a]	1.40			lasA protease precursor, *lasA*
PA1901 [a]			3.02	phenazine biosynthesis protein, *phzC2*
PA1902			2.85	phenazine biosynthesis protein, *phzD2*
PA1903			2.58	phenazine biosynthesis protein, *phzE2*
PA1904			3.12	probable phenazine biosynthesis protein, *phzF2*
PA1905			2.94	probable pyridoxamine 5'-phosphate oxidase, *phzG2*
PA2031	1.52			hypothetical protein
PA2066	1.60			hypothetical protein
PA2067	1.52			probable hydrolase
PA2068	1.53			probable MFS transporter
PA2069	1.50			probable carbamoyl transferase
PA2300 [a]	2.76			chitinase, *chiC*
PA2331	2.43			hypothetical protein
PA2366			2.18	conserved hypothetical protein
PA2426 [a]		1.70		sigma factor, *pvdS*
PA2570 [a]	1.99		2.81	PA-I galactophilic lectin, *pa1L*
PA2586 [a]			1.40	response regulator, *gacA*
PA2588			1.40	probable transcriptional regulator
PA2747	2.28		2.20	hypothetical protein
PA2939	2.36		3.44	probable aminopeptidase
PA3006		2.88	2.57	transcriptional regulator, *psrA*
PA3326 [a]			1.85	probable Clp-family ATP-dependent protease
PA3329 [a]			3.23	hypothetical protein
PA3331 [a]			2.48	cytochrome P450
PA3332			1.68	conserved hypothetical protein
PA3334			6.48	probable acyl carrier protein
PA3418			1.77	leucine dehydrogenase, *ldh*
PA3478 [a]	1.50		1.71	rhamnosyltransferase chain, *rhlB*
PA3479 [a]			1.79	rhamnosyltransferase chain, *rhlA*
PA3520	1.81			hypothetical protein
PA3691			1.60	hypothetical protein
PA3692	1.40			probable outer membrane protein precursor
PA3724			1.40	elastase, *lasB*
PA3904			3.00	hypothetical protein
PA4129	2.31			hypothetical protein

PAO No.[ab]	Average fold change			Protein name[c]
	Fe (-)	H_2O_2	PMNs	
Down-regulated genes in mutant				
Quorum sensing				
PA4134	11.96			hypothetical protein
PA4139		1.64		hypothetical protein
PA4141			2.62	hypothetical protein
PA4210 [a]			7.62	probable phenazine biosynthesis protein, *phzA1*
PA4211			6.49	probable phenazine biosynthesis protein, *phzB1*
PA4217 [a]			5.70	flavin-containing monooxygenase, *phzS*
PA4296	1.84	2.06	3.11	probable two-component response regulator
PA4311	1.50			conserved hypothetical protein
PA4587		2.68		cytochrome c551 peroxidase precursor, *ccpR*
PA4739			2.17	conserved hypothetical protein
PA4770		5.08		L-lactate permease, *lldP*
PA4778	1.50			probable transcriptional regulator
PA4880			2.14	probable bacterioferritin
PA5220 [a]	1.52			hypothetical protein
PA5356 [a]		1.75		transcriptional regulator, *glcC*
PA5482	2.25		6.80	hypothetical protein
Regulation				
PA2469			2.43	probable transcriptional regulator
Type III secretion system				
PA0044		1.76		exoenzyme T, *exoT*
PA1694		1.69		translocation protein in type III secretion, *pscQ*
PA1701		1.87		conserved hypothetical protein in type III secretion
PA1706		3.90		type III secretion protein, *pcrV*
PA1707		2.93		regulatory protein, *pcrH*
PA1708		1.83		translocator protein, *popB*
PA1714		1.49		hypothetical protein
PA1718		5.55		type III export protein, *pscE*
PA1719		4.02		type III export protein, *pscF*
PA3841		3.82		exoenzyme S, *exoS*
Iron homeostasis				
PA4221			11.49	fe(III)-pyochelin receptor precursor, *fptA*
PA4224			3.15	pyochelin biosynthetic protein, *pchG*
PA4225			44.18	pyochelin synthetase, *pchF*
PA4226			31.43	dihydroaeruginoic acid synthetase, *pchE*
PA4228			3.62	pyochelin biosynthesis protein, *pchD*
PA4229			4.98	pyochelin biosynthetic protein, *pchC*
PA4230			8.90	salicylate biosynthesis protein, *pchB*

PAO No.[ab]	Average fold change			Protein name[c]
	Fe (-)	H_2O_2	PMNs	
Down-regulated genes in mutant				
Iron homeostasis				
PA4231			4.27	salicylate biosynthesis isochorismate synthase, *pchA*
PA4880			2.14	probable bacterioferritin
Antimicrobial peptide resistance				
PA1178			13.48	PhoP/Q and low Mg2+ inducible outer membrane protein H1 precursor, *oprH*
PA1179			8.48	two-component response regulator, *phoP*
Up-regulated genes in mutant				
Regulation				
PA2467		4.81	4.01	probable transmembrane sensor
Quorum sensing				
PA0852 [a]		4.54		chitin-binding protein CbpD precursor, *cbpD*
PA0996	2.12			probable coenzyme A ligase, *pqsA*
PA0997	1.78			homologous to beta-keto-acyl-acyl-carrier protein synthase, *pqsB*
PA0998	1.78			beta-keto-acyl-acyl-carrier protein synthase, *pqsC*
PA0999	1.60			3-oxoacyl-(acyl-carrier-protein) synthase III, *pqsD*
PA1000	1.70			quinolone signal response protein, *pqsE*
PA1001 [a]	1.72			anthranilate synthase component I, *phnA*
PA1317		2.26		cytochrome o ubiquinol oxidase subunit II, *cyoA*
PA1318		2.19		cytochrome o ubiquinol oxidase subunit I, *cyoB*
PA1901 [a]		26.12		phenazine biosynthesis protein, *phzC2*
PA1903		3.18		phenazine biosynthesis protein, *phzE2*
PA1904		18.96		probable phenazine biosynthesis protein, *phzF2*
PA1905		4.05		pyridoxamine 5'-phosphate oxidase, *phzG2*
PA3478 [a]		3.31		rhamnosyltransferase chain, *rhlB*
PA3724 [a]		6.96		elastase, *lasB*
Iron homeostasis				
PA3407	1.98			heme acquisition protein, *hasAp*
PA3408	1.70			haem uptake outer membrane receptor, *hasR*
PA4224		3.27		pyochelin biosynthetic protein, *pchG*
PA4225		2.67		pyochelin synthetase, *pchF*
PA4229		3.05		pyochelin biosynthetic protein, *pchC*
PA4231		3.66		salicylate biosynthesis isochorismate synthase, *pchA*

[a]: Genes previously identified as being QS regulated (de Kievit *et al.*, 1999; Gambello *et al.*, 1993; Pearson *et al.*, 1997; Pesci *et al.*, 1997; Pessi and Haas, 2000; Toder *et al.*, 1991; Toder *et al.*, 1994; Whitely *et al.*, 1999).

[b]: Genes identified as being QS regulated by microarray analysis (Wagner *et al.*, 2003; Schuster *et al.*, 2003).

[c]: Gene names are indicated in italics.

We could see that the genes involved in aerobic and anaerobic growth, quorum sensing, type III secretion system, iron homeostasis, antimicrobial peptide resistance were down-regulated in the mutant in either one or two growth conditions. The genes involved in oxidative phosphorylation (PA1556 and PA1557) were severely down-regulated in the mutant after exposure to hydrogen peroxide. Most of the genes (*hemF*, *nirC*, *nirS*, *dnr*, *moaA2*, *anr*, *hemN*, *fhp*, *narK1*, *narX*, *narL*, *ptsN*, *ccpR*) involved in denitrification were down-regulated in the mutant when exposed to hydrogen peroxide, while the other genes *napA*, *napB*, *napD*, *anr*, PA4130 and PA4133 were down-regulated during the growth in iron limiting condition or when exposed to PMNs. The *nirS* and *dnr* regulators in denitrification were severely down-regulated in the mutant after exposure to hydrogen peroxide. The other genes (*arcA*, *arcB*, *arcC* and *arcD*) for arginine fermentation were repressed during the growth in iron limiting condition and when exposed to hydrogen peroxide. We could not see the repression of *arcB* and *arcC* when exposed to hydrogen peroxide. In some instances, for genes that formed an operon, such as *arcABCD,* the changes for the first gene (*arcD*) in the operon were much greater than the changes for the downstream genes. This phenomenon has also been noted by the other scientists and has been attributed to RNA degradation from the 3‘ end of the transcript (Whiteley *et al.*, 2000). During microaerophilic or anaerobic growth, it is known that *P. aeruginosa* generates energy through the arginine fermentation or nitrate respiration respectively. The GeneChip data revealed that the mutant is unable to generate energy via oxidative phosphorylation under aerobic growth condition, via arginine fermentation under microaerophilic condition and through denitrification under anaerobic condition. This suggests that the mutant is handicapped for the metabolic energy generation in all the growth conditions. We also found that lot of genes involved in quorum sensing were down-regulated in the mutant in either one or multiple growth conditions (Table 3.18). Some of these genes are already confirmed as being quorum sensing regulated by experimental evidence, while the other genes from the quorum sensing category were identified by using the microarray approach used by us and others (see table 3.19) (Wagner *et al.*, 2003; Schuster *et al.*, 2003). So such genes are also included in this table 3.18. Twenty seven, thirteen and forty three known or predicted

genes involved in quorum sensing were repressed in the mutant when grown in iron limitation, in presence of hydrogen peroxide and in presence of PMNs respectively. Only two genes (*rsaL* and PA4296) identified as being QS regulated were down-regulated in the mutant under all the growth conditions. Interestingly, most of the known genes (*mvfR*, *rsaL*, *phzCDEFG2*, *pa1L*, *gacA*, *rhlAB*, *lasB*, *phzAB1*, *phzS*) identified as being quorum sensing regulated are repressed when the mutant was exposed to PMNs. Some of these genes act as virulence factors. Some investigators have presented evidence showing that additional genes are controlled by quorum sensing in *P. aeruginosa* (Wagner *et al.*, 2003; Schuster *et al.*, 2003). Furthermore, several reports have shown that a variety of regulatory proteins can influence the expression of quorum sensing controlled genes (Schuster *et al.*, 2003). The other transcriptional regulator PA2469 with unknown function was also down-regulated in the mutant. This gene is upstream of the sigma factor (PA2468) and could form an operon structure. So the sigma factor could be involved in the stimulation of this transcriptional regulator by transferring the signals. We also identified that the genes involved in building the type three secretion apparatus and the effector proteins (exoT, exoS) were down-regulated in the mutant when exposed to hydrogen peroxide. The type III secretion system in *P. aeruginosa* is very well characterized and contributes to the pathogenesis. The opportunistic human pathogen *P. aeruginosa* responds to iron-limiting growth conditions by the production of two major siderophores, pyoverdin (Meyer *et al.*, 1996) and pyochelin (Cox *et al.*, 1981). These compounds are released to the extracellular environment, where they form a complex with iron and deliver it to the bacterial cell via their specific membrane receptors, FpvA (Poole *et al.*, 1993) and FptA (Ankenbauer and Quan, 1994), respectively. Both siderophores contribute to the virulence of *P. aeruginosa* (Meyer *et al.*, 1996; Takase *et al.*, 2000). During our studies, the *fptA*, *pchABCDEFG* genes involved in iron acquisition were significantly down-regulated in the mutant when exposed to PMNs. Under the same growth condition, the genes (*oprH*, *phoP*) which are expressed under low magnesium ion concentration and involved in antimicrobial peptide resistance were down-regulated in the mutant. PMNs are known to produce antimicrobial peptides as a defense mechanism for microbial killing.

Several genes were up-regulated in the mutant, which could be the rescue mechanism. We found that the downstream gene (PA2467) which is a

transmembrane sensor with unknown function was significantly up-regulated in the mutant in presence of hydrogen peroxide and PMNs. This could be up-regulated due to the feedback mechanism in the mutant due to a non-functional PA2468 (sigma-70 factor). Six and nine genes involved in quorum sensing were also up-regulated in the mutant when grown in iron limiting LB medium and in presence of hydrogen peroxide, while we could not see a derepression of quorum sensing regulated genes in the mutant when exposed to PMNs. However, significant number of QS regulated genes were down-regulated in the mutant when exposed to PMNs. The bacterium could encounter extremely low concentrations of free iron during growth in iron limiting LB medium. Iron restriction plays a central role in the stress response in *P. aeruginosa*, which has thus evolved numerous iron-acquisition systems. These include the release of siderophores, production of extracellular proteases, secretion of cytotoxic exotoxin A, and, the capacity to utilize haem and haemoglobin from exogenous sources (Ochsner *et al.*, 2000a). The up-regulation of *hasAp*, *hasR* and some pyochelin biosynthesis genes (*pchA*, *pchC*, *pchF*, *pchG*) could rescue the mutant during growth under iron limitation. Interestingly, the pyochelin biosynthesis cluster was down-regulated when the mutant was grown in the presence of PMNs, while it is up-regulated during the growth in presence of hydrogen peroxide. These results suggests that this sigma factor respond to the environmental conditions and influences the gene regulation in various growth conditions.

Summary

The ECF family of sigma factors constitutes a group of environmentally responsive transcription factors of the RpoD (σ^{70}) family (Lonetto *et al.*, 1994; Missiakas and Raina, 1998). These proteins are found in a diverse spectrum of bacteria and control a very wide range of functions such as response to heat, osmotic and oxidative stresses, virulence, motility, transport of metal ions and synthesis of alginate or carotenoids (Missiakas and Raina, 1998). Multiple members of the ECF family can be present in the same species. The ECF sigma factors PvdS from *P. aeruginosa*, PbrA from *P. fluorescens*, PfrI and PupI *from P. putida* and FecI from *E. coli* K-12 are involved in iron uptake and their production is negatively regulated by Fur (Visca *et al.*, 2002). Here, we investigated the putative sigma-70 factor which also responds to the iron limiting growth condition. The GeneChip data revealed that the mutant is

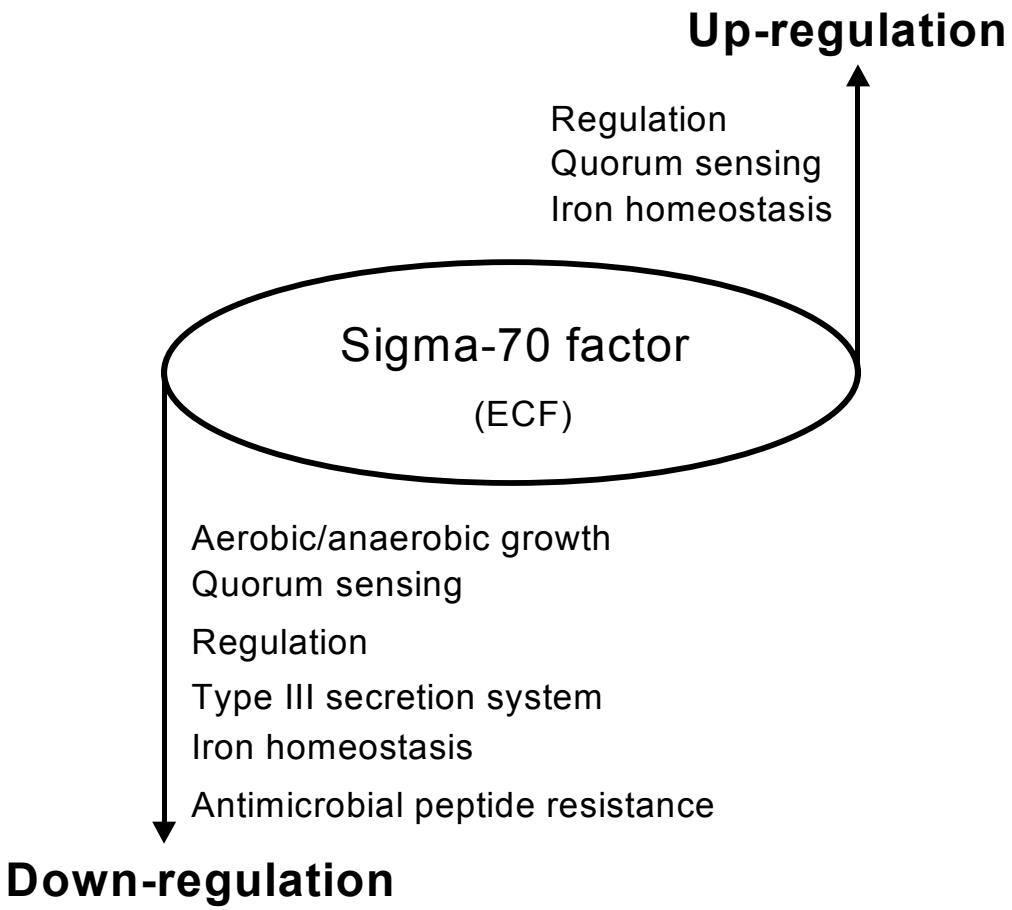


Fig. 3.19. Schematic representation showing differentially regulated genes from different metabolic categories in the sigma-70 factor mutant.

hampered for energy generation in aerobic and anaerobic growth conditions. Energy is generated through oxidative phosphorylation during aerobic process and through arginine fermentation and denitrification during microaerophilic and anaerobic processes. The GeneChip experiment showed that the mutant is handicapped for energy generation via all the three processes and can be converted to the dormant metabolic stage. In addition to the energy conservation, the mutant was also defective for the expression of quorum sensing and type III secretion system dependent virulence factors. Some of the quorum sensing dependent and iron acquisition genes were up-regulated in the mutant as a rescue mechanism. Our GeneChip results suggest that this sigma-70 factor could be involved in the regulation of genes involved in aerobic/anaerobic growth, quorum sensing, type III secretion system, iron uptake and antimicrobial peptide resistance through unknown regulatory mechanisms and these affected genes can contribute to the intracellular survival of *P. aeruginosa* in PMNs. The observed up- or down-regulation of genes

necessary for iron acquisition and quorum sensing in the mutant indicate that the sigma-70 factor can modulate gene expression depending on the environmental stimuli during the diverse growth conditions. The differentially regulated genes from different metabolic categories are summarized in the following model (figure 3.19).

3.4.4. Transcriptional analysis of C47 gene involved in quorum sensing

During the screening of the transposon library for mutants defective in protease secretion, a previous Ph. D. student (Wiehlmann, 2001) found the mutant D8A6 which is completely defective in protease secretion on casein agar plate (D8 is the number of micro titer plate and A6 stands for the position of the mutant in this plate). Latter, this mutant has been confirmed as attenuated in the quorum sensing assays by luminescence production on TLC plate in Dr. Leo Eberl‘s lab.

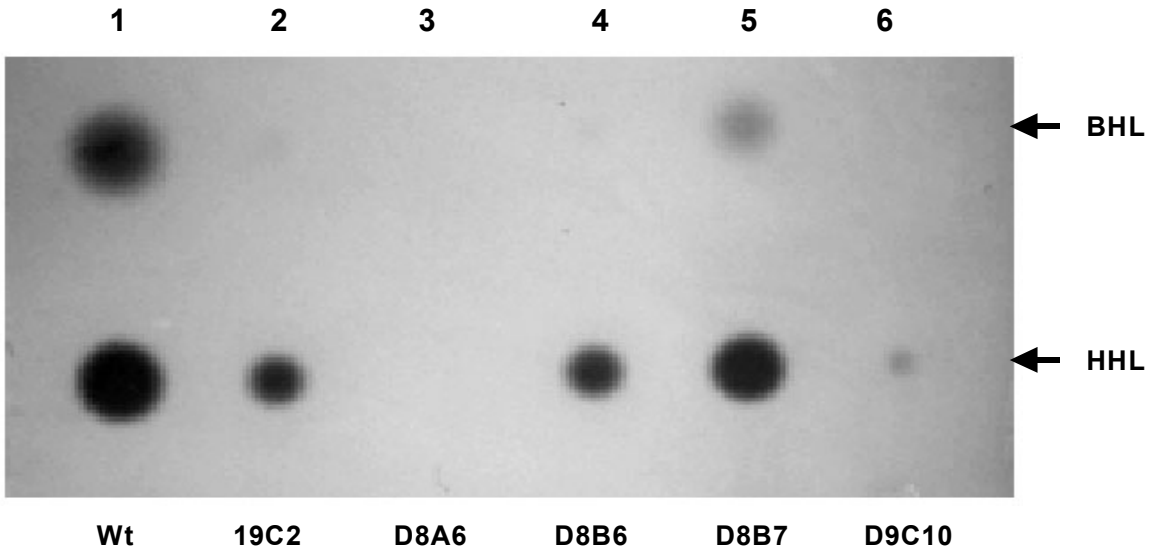


Fig. 3.20. TLC analyses of N-butanoyl homoserine lactone (BHL) and N-hexanoyl-L-homoserine lactones (HHL) produced by *P. aeruginosa* TB. Samples were chromatographed on C18 reversed-phase thin-layer plates, developed with methanol/water (60:40, v/v) and spots were visualized by overlaying the TLC plates with *C. violaceum* CV026. AHL profiles of *P. aeruginosa* TB wild type (Wt) and selected mutants are shown (lanes 1-6). The positions of identified AHLs (BHL & HHL) are marked.

It has been observed that the D8A6 mutant was completely attenuated for the production of both N-butanoyl homoserine lactone (BHL) and N-hexanoyl-L-homoserine lactone (HHL) quorum sensing molecules (figure 3.20). While the other mutants 19C2 and D8B6 showed production of HHL only and BHL was slightly produced in both mutants. D9C10 mutant was also attenuated for the production of BHL, while it produced very less amounts of HHL. These results indicated that these mutants were attenuated in the quorum sensing at various regulatory levels.

Then the genomic insertion in D8A6 mutant was identified by plasmid rescue. It observed that the 800 bp sequence did not show homology with the other DNA sequences on the available database. The other graduate student from our laboratory is analyzing the hyper variable regions in different clinical and environmental *P. aeruginosa* isolates. It observed that this sequence showed signals on the Southern blot prepared from the *P. aeruginosa* strain C (CF isolate) and SG17M (river isolate). He identified that the homologous sequence is present in the mobile gene island inserted in C and SG17M strains. Then this gene was named as C47 (C for strain C and 47 number ORF from the 105 Kb gene island).

Since this C47 mutant was unable to produce both BHL and HHL quorum sensing molecules, we were interested to uncover the regulatory function of this gene in the quorum sensing cascade. For this, the *P. aeruginosa* PAO1 GeneChip approach has been persued.

3.4.4.1. Expression of C47 gene on Northern blot

ABC minimal medium was used for the detection of homoserine lactone molecules in the quorum sensing assays. So we used the same medium to see the expression of this unknown gene (C47) on the Northern blot. It was observed that we could not see the visible signals for the wild type mRNA, while the signal for the mutant was comparatively stronger on the Northern blot (figure 3.21). It suggests that the gene is lowly expressed but essential since the mutant tries to produce more and more. However, the mRNA produced is non-functional since it is truncated.

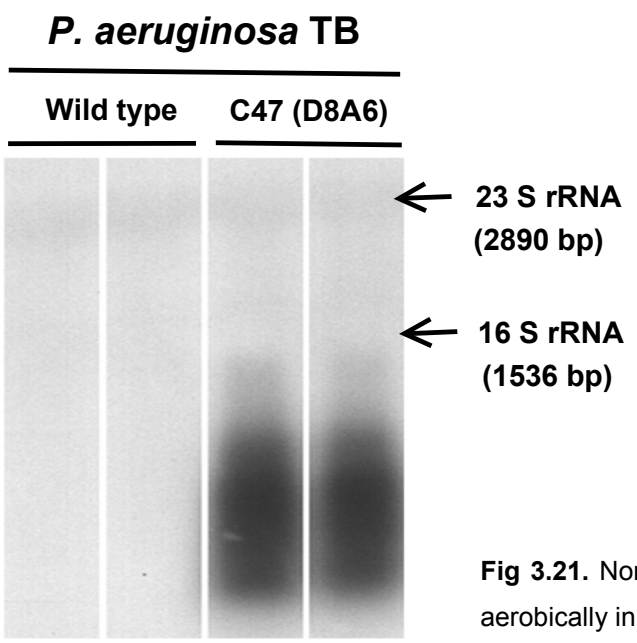


Fig 3.21. Northern blot expression for C47 mutant grown aerobically in the ABC minimal medium.

3.4.4.2. GeneChip analysis for C47 mutant

In order to determine the function of C47 gene in quorum sensing cascade, samples of total RNA were extracted from two independent cultures (wild type and D8A6 mutant) grown aerobically in ABC minimal medium. RNA samples were processed and hybridized to *P. aeruginosa* PAO1 GeneChips. Altogether, 4 GeneChips were used, 2 per strain (wild type and mutant respectively). Then the data obtained was sorted as described previously. The gene expression pattern of *P. aeruginosa* TB wild type and C47 mutant was compared. By using this approach, 400 genes were identified as being QS regulated. Table 3.19 shows genes that were differentially regulated in the mutant C47 (D8A6) and were identified as being QS regulated.

Of the 400 genes whose expression was identified as being QS regulated, 285 genes were down-regulated, while 115 genes were up-regulated in the mutant strain (Table 3.19). The expression of 53 genes was decreased more than 15-fold, and 11 genes exhibited decrease of more than 100-fold in the C47 mutant. In comparison, only 4 genes showed a increase in expression of more than 15-fold, and the expression of 9 genes was increased in between 5 to 15 fold in the mutant. In the meantime, Wagner *et al.* (2003) and Schuster *et al.* (2003) have also performed experiments using *P. aeruginosa* PAO1 microarrays for the identification of quorum sensing regulated genes. So our data obtained from this mutant was compared with the data from Wagner *et al.* (2003) and Schuster *et al.* (2003) even though different experimental

conditions were used in all the three investigations. Both groups of investigators (Wagner *et al.*, 2003; Schuster *et al.*, 2003) employed mutants deficient in AHL production (defective in *luxI* and *rhlI*) and compared the transcriptional responses in the mutant to those in the parental strain in the presence and absence of AHL. Schuster *et al.* (2003) also compared the transcript levels in a strain lacking the AHL receptors (deficient in *lasR* and *rhlR*), while Wagner *et al.* (2003) explored the responses in the presence of a range of growth media and other environmental conditions. Both groups reported that a striking number of genes were induced and repressed by AHL-mediated signaling. Schuster *et al.* (2003) found 315 and 38 such genes, respectively, and Wagner *et al.* (2003) found 394 and 222 such genes, respectively. The quorum-sensing regulon thus comprises about 6 % of the *P. aeruginosa* genome. Many of the genes that were previously identified as being quorum sensing regulated were verified in the data obtained from these three independent investigations (Table 3.19). Wagner *et al.* (2003) and Schuster *et al.* (2003) were frequently agreed in the assignment of many genes or operons with known or unknown functions which had not previously been identified as responsive to AHL quorum sensing. We have included such genes in the following table (Table 3.19) and the other unknown genes were omitted due to the space limitations.

Table 3.19. Genes which are differentially regulated in C47 mutant and whose expression was identified as being QS regulated by using microarrays.

PAO No.[a]	Protein name[b]	Fold change	Wagner *et al.*	Schuster *et al.*
Down-regulated (QS-promoted) genes in C47				
PA0026	hypothetical protein	2,79		+
PA0050	hypothetical protein	4,72		+
PA0059	osmotically inducible protein, *osmC*	5,57		+
PA0106	cytochrome c oxidase, subunit I, *coxA*	9,86	+	+
PA0108	cytochrome c oxidase, subunit III, *coIII*	2,61	+	+
PA0109[a]	hypothetical protein	2,60	+	+
PA0122	conserved hypothetical protein	17,90	+	+
PA0143	nonspecific ribonucleoside hydrolase, *nuh*	4,88		+
PA0176	probable chemotaxis transducer	2,11		+
PA0179	probable two-component response regulator	2,94	+	+
PA0263	secreted protein Hcp, *hcpC*	37,47		+
PA0355	protease PfpI, *pfpI*	4,96		+

PAO No.[a]	Protein name[b]	Fold change	Wagner *et al.*	Schuster *et al.*
PA0365	hypothetical protein	1,74		+
PA0399	cystathionine beta-synthase	3,81	+	
PA0567	conserved hypothetical protein	10,54		+
PA0586	conserved hypothetical protein	3,54		+
PA0588	conserved hypothetical protein	6,24	+	
PA0652[a]	transcriptional regulator, *vfr*	1,60		
PA0852[a]	chitin-binding protein CbpD precursor, *cbpD*	10,62	+	+
PA0855[a]	hypothetical protein	5,60	+	+
PA0996	probable coenzyme A ligase, *pqsA*	158,15		+
PA0997	homologous to beta-keto-acyl-acyl-carrier protein synthase, *pqsB*	76,12	+	+
PA0998	homologous to beta-keto-acyl-acyl-carrier protein synthase, *pqsC*	955,80	+	+
PA0999	3-oxoacyl-(acyl-carrier-protein) synthase III, *pqsD*	99,20	+	+
PA1000	quinolone signal response protein, *pqsE*	157,07		+
PA1001[a]	anthranilate synthase component I, *phnA*	73,04		+
PA1002[a]	anthranilate synthase component II, *phnB*	62,02		+
PA1003[a]	transcriptional regulator, *mvfR*	7,28	+	+
PA1173	cytochrome c-type protein NapB precursor, *napB*	4,41		+
PA1174	periplasmic nitrate reductase protein, *napA*	2,98		
PA1175	NapD protein of periplasmic nitrate reductase, *napD*	3,03		+
PA1176	ferredoxin protein, *napF*	9,14		+
PA1177	periplasmic nitrate reductase protein, *napE*	5,74		+
PA1216	hypothetical protein	4,54		+
PA1245	hypothetical protein	12,08	+	+
PA1250	alkaline proteinase inhibitor, aprI	13,56	+	+
PA1289	hypothetical protein	2,12		+
PA1317	cytochrome o ubiquinol oxidase subunit II, *cyoA*	2,07		+
PA1318	cytochrome o ubiquinol oxidase subunit I, *cyoB*	2,35	+	+
PA1320	cytochrome o ubiquinol oxidase subunit IV, *cyoD*	3,00		+
PA1323	hypothetical protein	7,94		+
PA1324	hypothetical protein	8,71		+
PA1431[a]	regulatory protein, *rsaL*	622,83	+	+
PA1432[a]	autoinducer synthesis protein, *lasI*	14,48		+
PA1656	hypothetical protein	15,42		+
PA1657	conserved hypothetical protein	53,69	+	+
PA1658	conserved hypothetical protein	24,75		+
PA1659	hypothetical protein	11,51		+
PA1660	hypothetical protein	6,25		+

PAO No.[a]	Protein name[b]	Fold change	Wagner *et al.*	Schuster *et al.*
PA1662	probable ClpA/B-type protease	15,30		+
PA1663	probable transcriptional regulator	21,44		+
PA1664	hypothetical protein	27,51		+
PA1665	hypothetical protein	98,14		+
PA1666	hypothetical protein	10,11		+
PA1667	hypothetical protein	66,77		+
PA1668	hypothetical protein	5,32		+
PA1669	hypothetical protein	8,84		+
PA1670	serine/threonine phosphoprotein phosphatase, *stp1*	10,19		+
PA1745	hypothetical protein	4,13		+
PA1869[a]	probable acyl carrier protein	76,24	+	+
PA1881	probable oxidoreductase	4,51		+
PA1894[a]	hypothetical protein	15,06	+	+
PA1895	hypothetical protein	4,13	+	+
PA1896[a]	hypothetical protein	9,75	+	+
PA1897[a]	hypothetical protein	13,07	+	+
PA4210	probable phenazine biosynthesis protein, *phzA1*	11,95	+	+
PA4211	probable phenazine biosynthesis protein, *phzB1*	53,66	+	+
PA1901[a]	phenazine biosynthesis protein, *phzC2*	46,30	+	+
PA1903	phenazine biosynthesis protein, *phzE2*	7,46	+	+
PA1904	probable phenazine biosynthesis protein, *phzF2*	18,93	+	+
PA1905	probable pyridoxamine 5'-phosphate oxidase, *phzG2*	5,91	+	+
PA1987	pyrroloquinoline quinone biosynthesis protein, *pqq C*	2,97		
PA2030	hypothetical protein	3,05	+	+
PA2031	hypothetical protein	3,63		+
PA2067	probable hydrolase	3,81		+
PA2068	probable MFS transporter	13,58	+	+
PA2069	probable carbamoyl transferase	12,67	+	+
PA2080	hypothetical protein	7,69		+
PA2193[a]	hydrogen cyanide synthase, *hcnA*	30,22	+	+
PA2194[a]	hydrogen cyanide synthase, *hcnB*	14,98	+	+
PA2195[a]	hydrogen cyanide synthase, *hcnC*	12,24	+	+
PA2300[a]	chitinase, *chiC*	5,21	+	+
PA2305	probable non-ribosomal peptide synthetase	10,09	+	+
PA2306	conserved hypothetical protein	21,62	+	
PA2331	hypothetical protein	3,37	+	+
PA2365	conserved hypothetical protein	4,45		+
PA2366	conserved hypothetical protein	6,99	+	+
PA2367	hypothetical protein	20,33	+	+

PAO No.[a]	Protein name[b]	Fold change	Wagner *et al.*	Schuster *et al.*
PA2375	hypothetical protein	12,95		
PA2423	hypothetical protein	7,02	+	+
PA2587[a]	probable FAD-dependent monooxygenase, *pqsH*	20,83	+	+
PA2588	probable transcriptional regulator	3,57	+	+
PA2591[a]	probable transcriptional regulator	6,34	+	+
PA2592[a]	probable periplasmic spermidine/putrescine-binding protein	20,18	+	+
PA2593	hypothetical protein	44,06		+
PA2747	hypothetical protein	7,08		+
PA2939	probable aminopeptidase	16,64	+	
PA3022	hypothetical protein	1,84		+
PA3100	general secretion pathway protein H, *xcpU*	1,75	+	
PA3101	general secretion pathway protein G, *xcpT*	1,60	+	
PA3102	general secretion pathway protein F, *xcpS*	1,69	+	
PA3103	general secretion pathway protein E, *xcpR*	2,93	+	
PA3104[a]	secretion protein, *xcpP*	4,48	+	+
PA3105	general secretion pathway protein D, *xcpQ*	3,80	+	
PA3326[a]	probable Clp-family ATP-dependent protease	24,25	+	+
PA3327[a]	probable non-ribosomal peptide synthetase	10,99		+
PA3328[a]	probable FAD-dependent monooxygenase	50,81	+	+
PA3329[a]	hypothetical protein	131,53	+	+
PA3330[a]	probable short chain dehydrogenase	67,69	+	+
PA3331[a]	cytochrome P450	52,31	+	+
PA3332	conserved hypothetical protein	45,54	+	+
PA3333[a]	3-oxoacyl-(acyl-carrier-protein) synthase III, *fabH2*	24,53	+	+
PA3334	probable acyl carrier protein	91,28	+	+
PA3335	hypothetical protein	33,27	+	+
PA3336[a]	probable MFS transporter	11,63		+
PA3346	probable two-component response regulator	4,32		+
PA3347	hypothetical protein	2,97		+
PA3369	hypothetical protein	3,91		+
PA3370	hypothetical protein	2,86		+
PA3418	leucine dehydrogenase, *ldh*	5,40	+	+
PA3476[a]	autoinducer synthesis protein, *rhlI*	10,76		+
PA3477[a]	transcriptional regulator, *rhlR*	13,17	+	+
PA3478[a]	rhamnosyltransferase chain, *rhlB*	6,61	+	+
PA3479[a]	rhamnosyltransferase chain, *rhlA*	18,14	+	+
PA3520	hypothetical protein	4,56	+	+
PA3535	probable serine protease	9,97		+

PAO No.[a]	Protein name[b]	Fold change	Wagner *et al.*	Schuster *et al.*
PA3622	sigma factor, *rpoS*	3,20	+	
PA3691	hypothetical protein	9,88		+
PA3692	probable outer membrane protein precursor	7,32		+
PA3724[a]	elastase, *lasB*	22,67	+	+
PA3904	hypothetical protein	48,53	+	+
PA3906	hypothetical protein	107,11	+	+
PA3907[a]	hypothetical protein	26,56		+
PA3908	hypothetical protein	142,63	+	+
PA3904	hypothetical protein	48,53	+	
PA3923	hypothetical protein	3,10	+	
PA4117	probable bacteriophytochrome	2,19		+
PA4129	hypothetical protein	17,58	+	+
PA4130	probable sulfite or nitrite reductase	22,93	+	+
PA4131	probable iron-sulfur protein	141,31	+	+
PA4132	conserved hypothetical protein	9,02	+	+
PA4133	cytochrome c oxidase subunit (cbb3-type)	39,24	+	+
PA4134	hypothetical protein	22,42	+	+
PA4139	hypothetical protein	126,01		+
PA4141	hypothetical protein	66,46	+	+
PA4142	probable secretion protein	16,59	+	+
PA4143	probable toxin transporter	6,31	+	
PA4144	probable outer membrane protein precursor	8,24	+	
PA4175	Pvds-regulated endoprotease, lysyl class, *prpL*	4,87	+	+
PA4209	probable phenazine-specific methyltransferase, *phzM*	35,90	+	+
PA4217[a]	flavin-containing monooxygenase, *phzS*	9,61	+	+
PA4296	probable two-component response regulator	3,37	+	+
PA4311	conserved hypothetical protein	2,24		+
PA4496	probable binding protein component of ABC transporter	5,37	+	
PA4590	protein activator, *pra*	4,27		+
PA4677	hypothetical protein	15,52	+	+
PA4738	conserved hypothetical protein	13,70		+
PA4739	conserved hypothetical protein	19,45		+
PA4778	probable transcriptional regulator	5,48		+
PA4869[a]	hypothetical protein	4,52		+
PA4876	osmotically inducible lipoprotein, *osmE*	5,09		+
PA4880	probable bacterioferritin	6,69		+
PA5058	poly(3-hydroxyalkanoic acid) synthase 2, *phaC2*	2,45		+
PA5220[a]	hypothetical protein	11,36	+	+
PA5356[a]	transcriptional regulator, *glcC*	2,12	+	+

PAO No.[a]	Protein name[b]	Fold change	Wagner *et al.*	Schuster *et al.*
PA5481	hypothetical protein	25,58		+
PA5482	hypothetical protein	17,19		+
Up-regulated (QS-repressed) genes in C47 mutant				
PA0165	hypothetical protein	3,01		+
PA0413[a]	still frameshift probable component of chemotactic signal transduction system	2,01	+	
PA0510[a]	probable uroporphyrin-III c-methyltransferase	9,68	+	
PA0511[a]	heme d1 biosynthesis protein, *nirJ*	3,95	+	
PA0512[a]	conserved hypothetical protein	14,68	+	
PA0513[a]	probable transcriptional regulator	4,18	+	
PA0515[a]	probable transcriptional regulator	17,06	+	
PA0516[a]	heme d1 biosynthesis protein, *nirF*	15,00	+	
PA0517[a]	probable c-type cytochrome precursor, *nirC*	7,07	+	
PA0518[a]	cytochrome c-551 precursor, *nirM*	4,92	+	
PA0519	nitrite reductase precursor, *nirS*	2,66		
PA0527	transcriptional regulator, *dnr*	2,74		
PA1458[a]	probable two-component sensor	1,41	+	
PA2540	conserved hypothetical protein	1,75	+	
PA4587	cytochrome c551 peroxidase precursor, *ccpR*	5,48	+	
PA4770	L-lactate permease, *lldP*	3,84		+

[a]: Genes previously identified as being QS regulated (de Kievit *et al.*, 1999; Gambello *et al.*, 1993; Pearson *et al.*, 1997; Pesci *et al.*, 1997; Pessi and Haas, 2000; Toder *et al.*, 1991; Toder *et al.*, 1994; Whitely *et al.*, 1999).

[b]: Gene names are indicated in italics.

3.4.4.3. Correlation of GeneChip data to previously QS-regulated genes

P. aeruginosa is a highly adaptable bacterium that can colonize various environmental niches, including soil and marine habitats, plants, animals, and humans (de Kievit and Iglewski, 2000). *P. aeruginosa* possesses one of the best-studied models of QS, and two complete *lux*-like QS systems, *las* and *rhl*, have been identified (Pesci *et al.*, 1997). The *las* system consists of the transcriptional regulatory protein LasR and its cognate signaling molecule, *N*-(3-oxododecanoyl) homoserine lactone (3O-C_{12}-HSL), whose production is directed by the autoinducer synthase encoded by *lasI*. The *rhl* system consists of the RhlR protein and an autoinducer

synthase (RhlI), which is involved in production of the cognate autoinducer *N*-butyryl homoserine lactone (C_4-HSL). These systems are intertwined in a hierarchical manner, and the *las* system controls the *rhl* system at both the transcriptional and post-translational levels. QS has been shown to regulate the production of *P. aeruginosa* virulence factors (such as proteases, exotoxin A, rhamnolipids, and pyocyanin) and to be involved in biofilm formation and development, and it has been implicated in antibiotic resistance (Davies *et al.*, 1998).

Of 52 previously reported QS-regulated genes, 49 were confirmed by using microarrays (Table 3.19). These 49 genes are PA0109, PA0413, PA0511 (*nirJ*), PA0512, PA0513, PA0515, PA0516 (*nirF*), PA0517 (*nirC*), PA0518 (*nirM*), PA0652 (*vfr*), PA0855 (*cbpD*), PA0855, PA1001 (*phnA*), PA1002 (*phnB*), PA1003 (*mvfR*), PA1431 (*rsaL*), PA1432 (*lasI*), PA1458, PA1869, PA1894, PA1896, PA1897, PA1901 (*phzC2*), PA2193 (*hcnA*), PA2194 (*hcnB*), PA2195 (*hcnC*), PA2300 (*chiC*), PA2587 (*pqsH*), PA2591, PA2592, PA3104 (*xcpP*), PA3326, PA3327, PA3328, PA3329, PA3330, PA3331, PA3333 (*fabH2*), PA3336, PA3476 (*rhlI*), PA3477 (*rhlK*), PA3478 (*rhlB*), PA3479 (*rhlA*), PA3724 (*lasB*), PA3907, PA4217 (*phzS*), PA4869, PA5220, PA5356 (*glcC*) (Wagner *et al.*, 2003; de Kievit *et al.*, 1999; Gambello *et al.*, 1993; Pearson *et al.*, 1997; Pesci *et al.*, 1997; Pessi and Haas, 2000; Toder *et al.*, 1991; Toder *et al.*, 1994; Whitely *et al.*, 1999). However, 37 and 43 known genes were confirmed by Wagner *et al.* and Schuster *et al.* respectively in the experiments performed for AHL-mediated quorum sensing (Wagner *et al.*, 2003; Schuster *et al.*, 2003). We found that PA3622 (*rpoS*) is QS promoted (3.20−fold change) which agreed with several previous reports (Latifi *et al.*, 1996; van Delden *et al.*, 2001) but differed from one study in which it was concluded that *rpoS* was not QS-regulated (Whiteley *et al.*, 2000). Wagner *et al.* (2003) also detected that *rpoS* was QS-promoted (2.7 fold change) during the AHL-mediated quorum sensing experiments. They have also recently identified that PA0179 and PA3904 are being QS-promoted by using a fluorescence-activated cell sorting approach (Wagner *et al.*, 2003). We also confirmed the up-regulation of these two genes. We also confirmed the QS-repression of 10 known genes (Table 3.19) but Wagner *et al.* (2003) and Schuster *et al.* (2003) could detect only 3 genes (PA308, PA3234, PA3235) as being QS-repressed. However, we could not see these genes as being QS-repressed during our experiments. We also included the genes those are identified by Wagner *et al.*

(2003) and Schuster *et al.* (2003) in the above table (Table 3.19). Our data showed that there is 43 % and 69 % agreement regarding the QS inducible genes, while there is 10 % and 2 % concordance regarding the genes listed as being QS repressible that are identified by Wagner *et al.* (2003) and Schuster *et al.* (2003) respectively. This microarray experiment showed that the quorum-sensing regulon comprises about 6 % of the genome. In the present study with *P. aeruginosa* TB, the largest percentage of QS-regulated transcripts (50 %) encoded proteins having hypothetical, unclassified and unknown function. Microbial pathogenesis has been intimately linked to QS regulatory circuits, and several *P. aeruginosa* virulence factors are known to be quorum sensing regulated (de kievit and Iglewski, 2000). More than 50 probable or known virulence genes such as those that may be involved in tissue destruction (PA4142–PA4144), were identified as being QS regulated. Expression of the transcripts of several well characterized genes involved in the denitrification system (PA510–PA0519 and PA0527) were repressed by quorum sensing. It suggests that QS has impact on the anaerobic growth of *P. aeruginosa*.

It is already known that the two QS systems (*lasI-lasR* and *rhlI-rhlR*) are regulated in a hierarchical manner in *P. aeruginosa* responsible for regulating the expression of many virulence determinants, secondary metabolites, stationary phase genes and genes involved in biofilm formation (Withers *et al*., 2001). In *P. aeruginosa*, there is an interesting co-ordination of the two QS systems (figure 3.22), the LasR protein regulating expression of the *rhl*R and the *rhlI* genes (Latifi *et al*., 1995). The *lasR* gene itself is positively controlled by the *gacA* two-component response regulator, a common regulator of virulence in *P. aeruginosa* (Reimmann *et al*., 1997) and also by a CRP homologue called *vfr* (Albus *et al*., 1997). The environmental conditions to which these regulators respond and their influence are not yet fully understood. The *lasI*, *lasR*, *rhlI*, *rhlR*, *rsaL*, *gacA* and *vfr* are the major hierarchical regulators in *Pseudomonas aeruginosa* quorum sensing system. The GeneChip experiment revealed that all these major regulators except *lasR* and *gacA* were significantly down-regulated in the C47 mutant. Then we checked the absolute signal intensities for *lasR* and *gacA* and found that they were not changed in mutant as compared to the wild type *P. aeruginosa* TB (data not shown). From this microarray data, it observed that nearly all the known genes including major regulators, involved in quorum sensing were differentially regulated by the C47 mutant. However, *lasR* and

gacA were not up- or down-regulated in the mutant strain. If we believe that the quorum sensing system in *P. aeruginosa* TB is regulated by the same hierarchical manner like PAO1 strain. Then we should not find differentially regulated genes in the mutant since *lasR*, the major regulator for both quorum sensing systems is not affected. So the plausible hypothesis is that the gene regulation for quorum sensing hierarchy is different in *P. aeruginosa* TB unlike to the well studied quorum sensing system in PAO1 strain. We would like to say that the gene product from C47 makes *lasR* boxes accessible due to the DNA-protein interactions for the expression of quorum sensing dependent regulated genes in *P. aeruginosa* TB. So we would conclude that this gene product is necessary for the functioning of quorum sensing hierarchy and the whole quorum sensing dependent gene expression cascade. The large number of quorum sensing regulated genes that encode known or probable virulence genes identified in the present investigation further emphasizes the role of QS in *P. aeruginosa* pathogenesis.

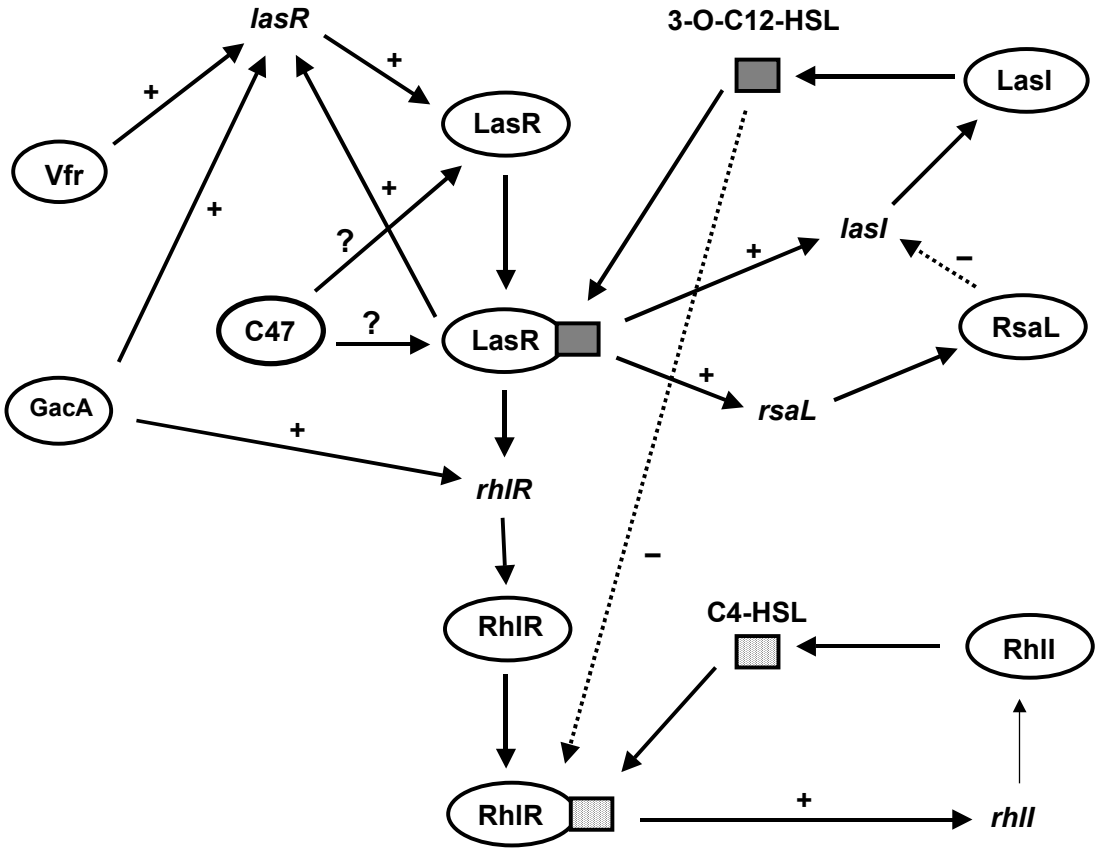


Fig. 3.22. Schematic diagram showing the proposed hierarchical position of C47 gene in the *las-rhl* quorum sensing system in *P. aeruginosa* TB.

3.5. Examination of *P. aeruginosa* pathogenicity in murine infection model

We used a murine lung infection model for the examination of STM knockout mutants which were obtained during the screening for intracellular survival in PMNs and quorum sensing. The subset of mutants was examined in the murine infection lung model which is already established in Dr. Hoiby's Laboratory at the Copenhegan University. This model has been used in their laboratory for more than 10 years for different purposes (Johansen *et al.*, 1993; Johansen *et al.*, 1995; Song *et al.*, 1997). Since Dr. Hoiby is a member of European Graduate Program 'Pseudomonas: Pathogenicity and Biotechnology', we had the opportunity to exploit this model during our studies.

Since each *P. aeruginosa* strain differs in its degree of virulence, we optimized the following different parameters to test the pathogenicity of *P. aeruginosa* TB mutants.

3.5.1. Optimization of challenge procedure

P. aeruginosa must be entrapped into immobilizing agents such as agar, agarose or seaweed alginate to limit bacterial clearance in order to achieve a chronic infection. However, other challenge procedure has been established in Dr. Hoiby's Laboratory where they have used free bacteria without entrapment in alginate. For this, the free bacterial cells of *P. aeruginosa* clinical isolate which is alginate hyper-producer, were used to infect the mice (Dr. Hoiby, unpublished data). Therefore we used both methods to establish the chronic infection with our CF isolate *P. aeruginosa* TB.

In order to optimize the challenge procedure and inoculum size, the 11–12 week old C3H/HeN mice were intratracheally challenged with various doses of free as well as alginate embedded *P. aeruginosa* TB cells directly in the left lung. After challenge, mortality was monitored for 7 days. All the mice challenged with 10^7–10^9 cfu of alginate embedded bacteria and with 10^8 cfu of free bacteria died within 2–3 days (figure 3.23). Seven and two mice were alive up to 7 days when challenged with free bacterial suspension of 10^6 cfu and 10^7 cfu respectively. But we could not observe *P. aeruginosa* TB cells in the lung homogenate on the seventh day from these mice. These results indicated that the free bacteria were cleared by the mice in less than 7

days. In case of alginate embedded *P. aeruginosa* cells, eight and two mice were alive up to 7 days when challenged with 10^5 cfu and 10^6 cfu respectively. We could able to detect 1–10 bacteria per mice on the seventh day when they were infected only with 10^6 cfu of alginate embedded *P. aeruginosa* TB. This data suggested that the optimum dose could be 10^6 cfu or less of the alginate embedded bacterial cells with lesser days of incubation period. Since we were interested to test the gain of function mutants (more virulent than TB wild type from PMN assay) in addition to loss of function mutants, we decided to use 8 x 10^5 cfu/mice as a inoculum size in the further experiments.

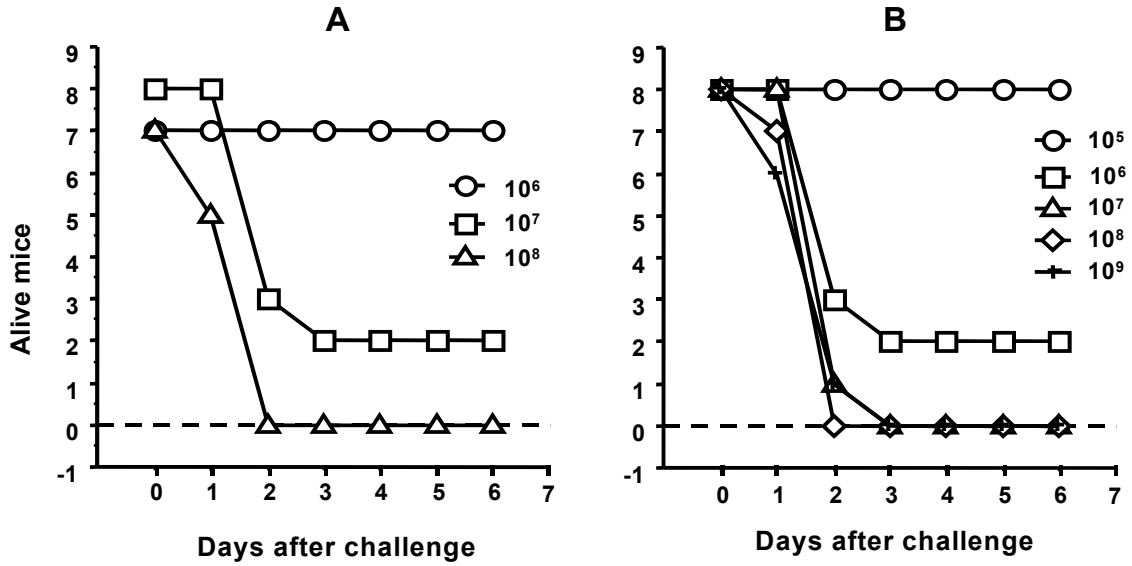


Fig. 3.23. Kinetics of killing of 11–12 week old C3H/HeN mice (n=7 or 8) infected intratracheally with free (A) or alginate embedded (B) *P. aeruginosa* TB wild type. The mice were challenged with 40 µl of different initial CFU concentration directly in the left lung and mortality was monitored up to 7 days.

We were not able to detect enough bacteria in the lung in the previous experiment. So to determine the suitable time point, mice were intratracheally challenged with *P. aeruginosa* TB (8 x 10^5 cfu/mice) and group of mice were sacrified on each day. Then the bacteriology was performed from the lung homogenate. We found 1 x 10^3 bacterial cells/lung on the day 4. After that the bacteria were cleared rapidly by the mice (data not shown). In order to check the various parameters of inflammatory response, we used day 3 and day 4 to sacrify the mice after bacterial challenge.

We selected mutants from various groups such as having defect in flagella biogenesis (*flhA*, *fliK*), pili biogenesis (*pilQ*, *pilY1*), quorum sensing (C47) and non-

PAO1 gene (25A12 mutant) to test their virulence in the mouse infection model. All further experiments were performed with 8 x 10^5 cfu/mice and they were sacrified on day 3 and 4 and the following parameters were evaluated.

3.5.2. Leukocyte subpopulation from blood

The mice were challenged with 0.04 ml of 8 x 10^5 cfu of alginate embedded mutants and wild type and leukocyte subpopulation was determined from blood on day 3 and 4. We examined the less virulent mutants (*flhA*, *fliK*-flagella biogenesis; C47- quorum sensing; 25A12- non-PAO1 gene) as well as more virulent mutants (*pilQ*, *pilY1*- type IV pili biogenesis) from the PMN phagocytosis assay. On day 3, there were significantly less number of white blood cells (WBCs) when the mice were challenged with *flhA* and C47 mutant as compared to the mice challenged with *P. aeruginosa* TB wild type (figure 3.24). The differences were not significant for *fliK* mutant on day 3 and *pilY1* and *flhA* mutants on day 4. There were less number of WBCs observed on day 4 when the mice were challenged with *pilQ* mutant. However, significantly more number of WBCs were found in the blood when the mice were infected with 25A12 mutant.

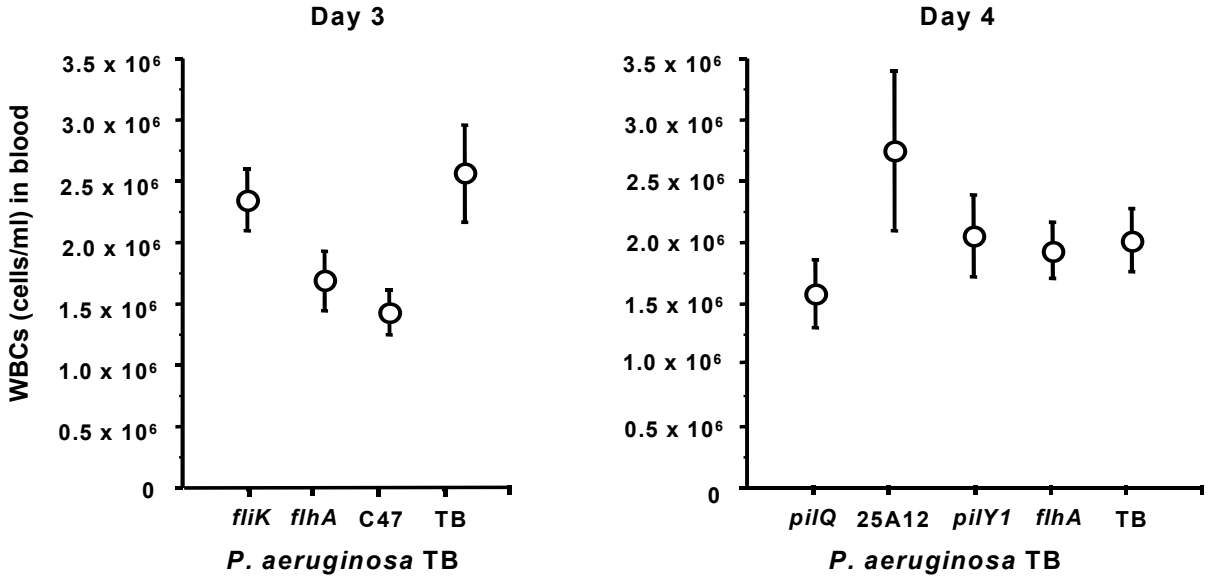


Fig. 3.24. The concentration of WBCs in the peripheral blood from C3H/HeN mice on day 3 and 4 after intratracheal installation of 8 x 10^5 cfu of alginate embedded *P. aeruginosa* TB wild type and mutants.

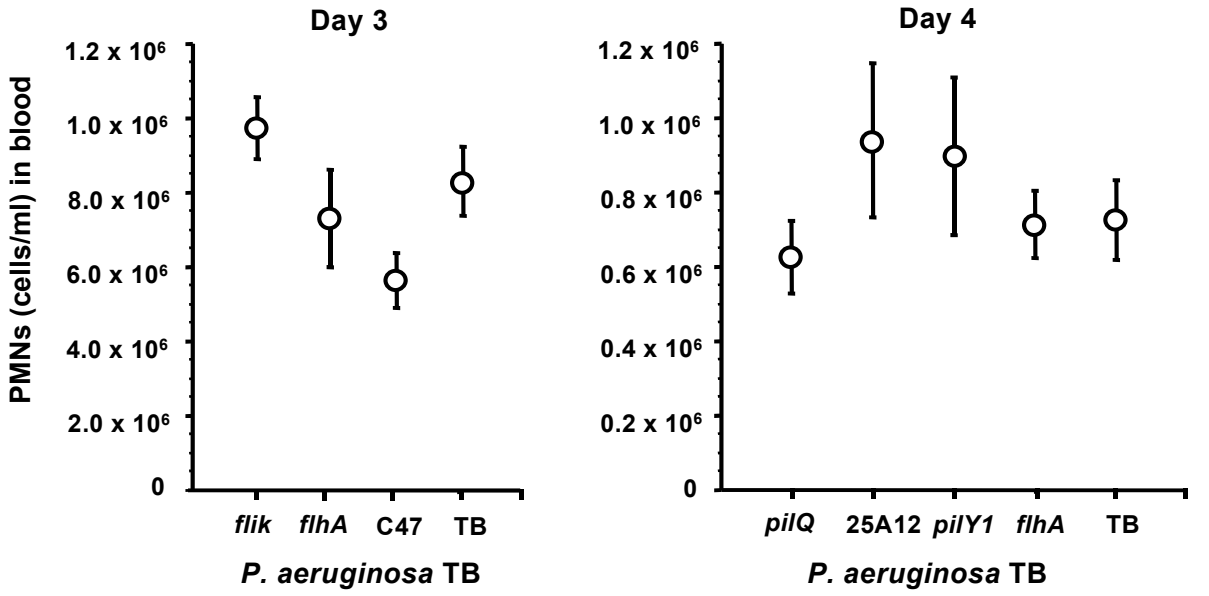


Fig. 3.25. The concentration of PMNs in the peripheral blood from C3H/HeN mice on day 3 and 4 after intratracheal installation of 8 x 10^5 cfu of alginate embedded *P. aeruginosa* TB wild type and mutants.

The PMNs are normally the first phagocytes to appear in high numbers at the site of bacterial infections. In the present study, the early involvement of the PMNs in the antibacterial defense was indicated by the increased number of PMNs in the peripheral blood. Similar patterns were observed for PMNs from the peripheral blood with exceptions of high number of PMNs for *flik* on day 3 and for *pilY1* on day 4 (figure 3.25). The high number of WBCs and PMNs in the blood is due to the inflammatory response exerted by the mice against *P. aeruginosa* lung infection. The difference for WBCs and PMNs in the peripheral blood was not significant on day 4 when the mice were challenged with *flhA* mutant. This finding could be attributed due to the faster elimination of the *flhA* mutant as compared to the *P. aeruginosa* TB wild type. These results suggests that there was not severe lung inflammation observed when the mice were challenged with *flhA* and C47 mutants indicating that these mutants were unable to persist for longer time in the murine lungs. In contrast, there could be severe lung inflammation when the mice were challenged with 25A12 and *pilY1* mutants suggesting for the longer persistence in the mice lung as compared to the TB wild type. From above results it was observed that *flhA* and C47 mutants are less virulent and *pilY1* and 25A12 mutants are more virulent than the TB wild type in a murine infection model.

3.5.3. Leukocyte subpopulation from BAL-fluid

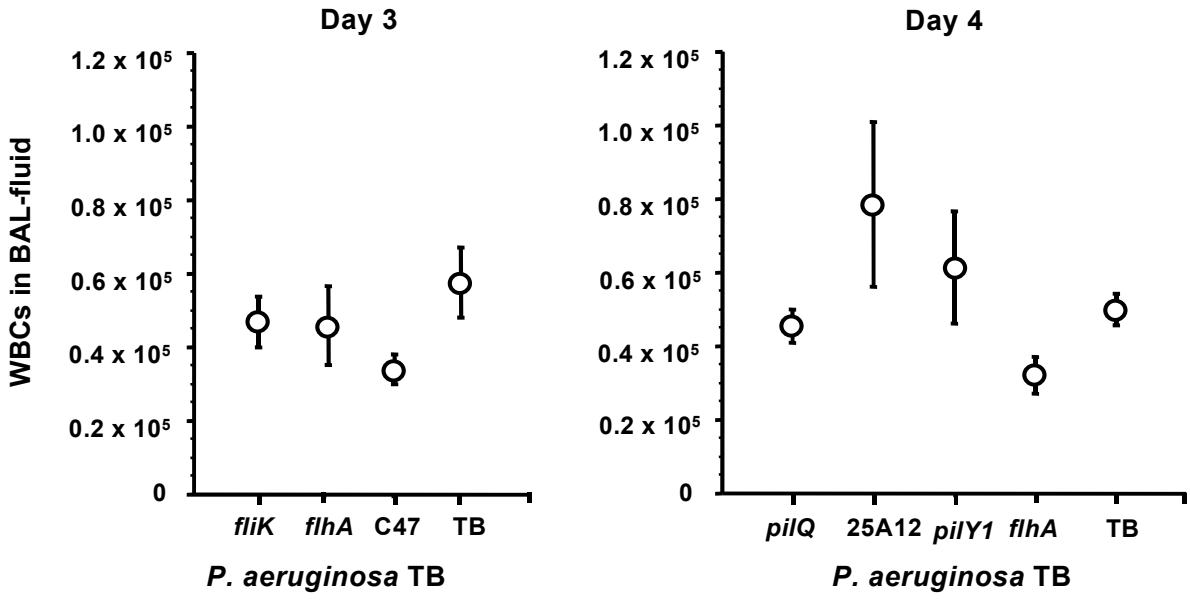
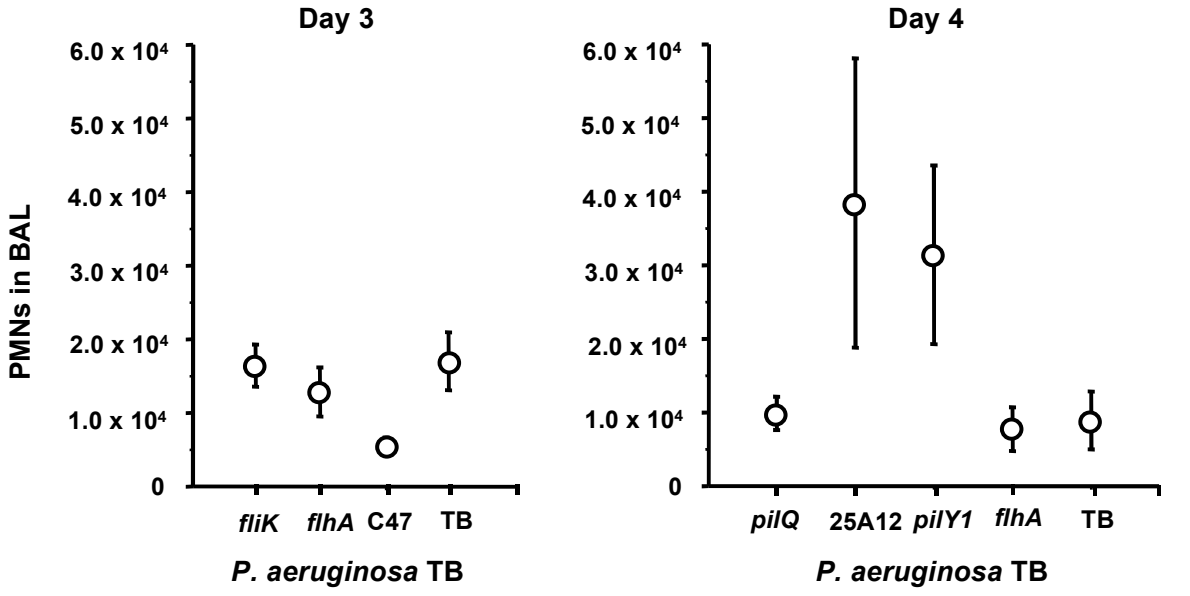


Fig. 3.26. The concentration of WBCs in the BAL-fluid from C3H/HeN mice on day 3 and 4 after intratracheal installation of 8 x 10^5 cfu of alginate embedded *P. aeruginosa* TB wild type and mutants.



3.27. The concentration of PMNs in the BAL-fluid from C3H/HeN mice on day 3 and 4 after intratracheal installation of 8 x 10^5 cfu of alginate embedded *P. aeruginosa* TB wild type and mutants.

On day 3 and 4, we found less WBCs in the BAL-fluid when the mice were challenged with loss of virulence mutants: *flik*, *flhA* and C47 as compared to the *P. aeruginosa* TB wild type (figure 3.26), however, there were more WBCs found in the BAL-fluid when the mice were infected with *pilY1* and 25A12 mutants. As an estimate of the intrapulmonary content of PMNs, the number of PMNs in the BAL-fluid were measured. We found that there were less PMNs in the BAL-fluid when the mice were challenged with the quorum sensing knockout mutant C47, but more PMNs were observed when mice challenged with 25A12 and *pilY1* mutants (figure 3.27). The PMNs from BAL-fluid were not significantly different on day 3 and day 4 (figure 3.27) when the mice were challenged with *fliK*, *flhA* and *pilQ* mutants. These results support the findings that C47 mutant is less virulent and unable to persist for longer period, but 25A12 and *pilY1* mutants are more virulent and persist for longer time in the mice lungs than the TB wild type.

3.5.4. Respiratory burst from BAL-fluid

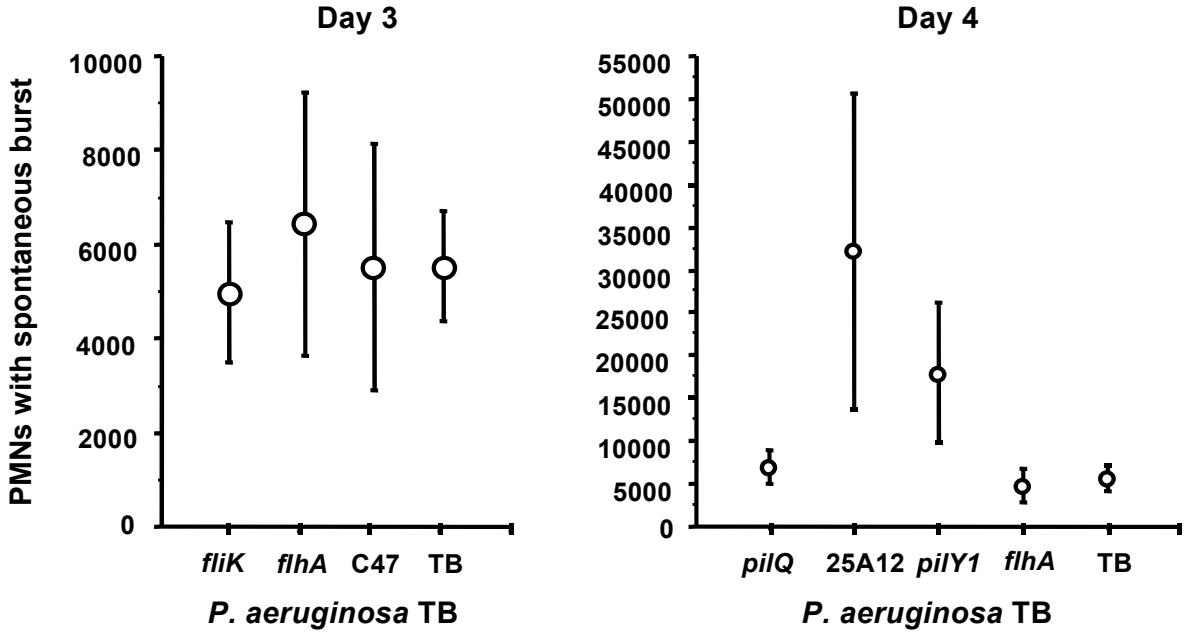


Fig. 3.28. The number of PMNs with spontaneous respiratory burst recovered in the BAL-fluid from C3H/HeN mice on day 3 and day 4 after intratracheal installation of 8 x 10^5 cfu of alginate embedded *P. aeruginosa* TB wild type and mutants.

In the BAL-fluid, the number of PMNs with a spontaneous respiratory burst were increased to 3 and 5 fold on day 4 when the mice were infected with *pilY1* and 25A12 mutants as compared to the *P. aeruginosa* TB wild type (figure 3.28). However, there

were not significantly different number of PMNs with a spontaneous respiratory burst for *flik*, *flhA*, C47, *pilQ* mutants as compared to the TB wild type. The more number of PMNs with spontaneous burst is the sign of bacterial infection in the lung. PMNs are known to produce oxidative radicals for microbial killing by spontaneous respiratory burst in the lungs. So above results emphasizes that there could be more number of bacterial cells for *pilY1* and 25A12 mutant persisting for the longer infection as compared to the TB wild type. The small differences for the other mutants may not be observed because we had sacrified mice on day 3 and day 4. It would be necessary to sacrify the mice on day 1 and day 2 to observe the differences in the spontaneous respiratory burst rate with the less virulent mutants.

3.5.5. Other Parameters

We also measured the other parameters such as lung bacteriology and pro-inflammatory response in terms of various cytokines. There were very less number of bacteria (average 10−100 cfu/lung) recovered from the lung homogenate so we could not see significantly different number of bacteria when the mice were infected with mutants and *P. aeruginosa* TB wild type (data not shown). At the same time we also measured cytokines such as GM-CSF, KC, IL-12, MIP-2, TNF-α, and IFN-γ from the lung homogenate on day 3 and 4 (Table 3.20 and 3.21). The cytokines are mediators of pro-inflammatory response and increased levels of these cytokines provide an explanation for the faster clearance of the bacteria in the lungs of the C3H/HeN mice. However, the cytokine levels were not significantly changed when the mice were challenged with *P. aeruginosa* TB wild type and mutants. In conclusion, we could not found the correlation between the cytokine concentration from the mice lung and bacterial virulence on day 3 and 4.

Table 3.20. The concentration of cytokines in the lungs from C3H/HeN mice on day 3 after intratracheal installation of 8 x 10^5 cfu of alginate embedded *P. aeruginosa* TB wild type and mutants.

Bacterial strain/mutant	GM-CSF	IL-12	TNF-α	KC	MIP-2	IFN-γ
flik	749	1279	1042	649	821	1175
flhA	735	1418	910	690	739	1037
C47	538	1532	1024	585	840	1097
TB wild type	483	1697	892	610	830	1125

Table 3.21. The concentration of cytokines in the lungs from C3H/HeN mice on day 4 after intratracheal installation of 8 x 10^5 cfu of alginate embedded *P. aeruginosa* TB wild type and mutants.

Bacterial strain/mutant	GM-CSF	IL-12	TNF-α	KC	MIP-2	IFN-γ
pilQ	440	1250	1022	495	736	1097
25A12	460	1369	1070	560	755	1085
wspE	382	1121	947	610	681	1145
flhA	415	1256	976	490	735	1035
TB wild type	373	1146	895	520	787	1117

3.5.6. Conclusions from murine infection experiments

Above results indicated that the loss of function mutants *flhA* and C47 were found to be less virulent in the murine lung infection model as compared to the TB wild type. This suggest that *flhA* gene is necessary for the *P. aeruginosa* TB virulence in the ex vivo PMN assay as well as in the murine lung infection model. It has been reported that QS is involved in the pathogenesis of *P. aeruginosa* during lung, corneal and burn wound infections (Tang *et al.*, 1996; Wu *et al.*, 2001; Rumbaugh *et al.*, 1999). This C47 mutant is attenuated for nearly all the known *lasI-lasR* and *rhlI-rhlR* regulated genes (see table 3.19). So it was worthwhile to test the pathogenicity of this mutant in the murine lung infection model. Our finding confirms the results that quorum sensing regulated gene products are essential for *P. aeruginosa* virulence in the mouse infection model. The gain of function mutant *pilY1* from PMN assay was also found to be more virulent in this mouse infection model. These results for *flhA*, C47 and *pilY1* mutants are consistent with the results obtained from ex vivo PMN phagocytosis assay. All the parameters from above studies show that the 25A12 mutant is even more virulent than the *P. aeruginosa* TB wild type and gain of function mutant *pilY1*. This mutant is strongly attenuated for intracellular survival in PMN assay. This discrepancy in the results from two different model systems could not be explained since both models can use primarily the same mechanisms for microbial killing. But these results of mouse infection model correlates with the microarray data obtained for this mutant under various growth conditions. Lots of genes involved in quorum sensing and QS dependent virulent factors and type III secretion system were up-regulated in the mutant when grown in presence of hydrogen peroxide and PMNs (see 3.4.2). The microarray data and mouse infection results indicated that the mutation in non-PAO1 gene changes the phenotype to the more virulent strain than

the TB wild type. The pathogenicity of non-PAO1 mutant 25A12 has been examined in the *Caenorhabditis elegans* model in co-operation with Dr. Ivo Steinmetz in the Institute of microbiology at the Medizinische Hochschule Hannover. *P. aeruginosa* TB wild type killed 45 % worms, while 98 % and 60 % of worms were killed by 25A12 and 45A7 mutant in the first 3 h incubation period (data not shown). From these results, it could observed that 25A12 achieved nematode killing maxima within 3 h incubation period, while TB wild type took more than 5 hours. This indicated that 25A12 mutant is about two times virulent than the TB wild type in the nematode worm killing model. The transcriptome data from 25A12 mutant revealed that the type III secretion and quorum sensing dependent virulence factors were up-regulated in the 25A12 mutant. This GeneChip experiment uncovered that the 25A12 mutant is more virulent as compared to the *P. aeruginosa TB* wild type. The results from the mouse infection experiments supports the transcriptome finding that the 25A12 mutant alters its phenotype to the more virulent.

We could not see the same co-relation of PMN phagocytosis assay with a murine infection model for *fliK* and *pilQ* mutants. This could be due to the fact that the subtle changes in a murine infection model are difficult to notice. The PMN phagocytosis assay is more sensitive and the signals were enriched several times, so it was possible to find out the small differences in this assay as compared to the mouse infection model.

4. Conclusions and perspectives

The aim of this study was to identify genes that enable the opportunistic pathogen *P. aeruginosa* TB to survive intracellularly. For this, the work presented in this thesis followed the screening of the STM library for intracellular survival and quorum sensing, transcriptional analysis of *P. aeruginosa* TB and knockout mutants and examination of the virulence attenuated mutants in a murine lung infection model.

4.1. Screening of STM library for intracellular survival in PMNs

A previous Ph. D. student had constructed the STM library of *P. aeruginosa* TB and he screened 960 transposon mutants for intracellular survival in PMNs during his Ph. D. studies (Wiehlmann, 2001). The rest of the library consisting of 1344 mutants was screened during this study. In each assay, 4–5 mutants were found to be attenuated for virulence, then these mutants were retested in several phagocytosis assays in different combinations of mutants to make sure that the mutant is attenuated independent of others. In this way, total 44 mutants were found which significantly differed in the survival rate in comparison to wild type *P. aeruginosa* TB. Out of these 44 mutants, 34 were impaired (loss of function) for intracellular survival in PMNs, while 9 mutants even survived better (gain of function) as compared to the *P. aeruginosa* TB wild type. This was the most unexpected finding of gain of function mutants during the PMN phagocytosis assays. These mutants were subjected to plasmid rescue to find out the genomic insertions. This was a rather difficult task to get the transformants from these mutants after genomic DNA restriction digestion and ligation. But this problem was solved by using highly competent cells (10^7 transformants/ng DNA). The identified genes were divided into different categories based on the known or predicted function. The results indicated that most of the genes identified during the second screening belong to the same metabolic categories obtained from the first screening. This proves the applicability of the STM procedure for the identification of habitat specific genes. The major identified metabolic categories belong to the oxidative stress response, flagella and type IV pili biogenesis and hypotheticals with unknown function. During this screening, two clone specific or strain specific genes were identified in addition to the previous clone or strain specific gene. Complementation of most of the genes is required to rule out the

possibility of the effect of neighbouring genes. It has been found that many genes control the complex phenotype of intracellular survival in PMNs and almost all genes that are needed by the bacterium are present in the sequenced PAO1 core genome, only very few are strain-or clone-specific. This indicates that subtle shifts in the genetic repertoire are sufficient to cause a smooth transition from extracellular lifestyle to intracellular survival and growth in the major antipseudomonal host defense cell. Unexpectedly, quite a few mutants gained better survival rates due to transposon insertion. Some of the genes knocked out in these mutants are also the targets that are modulated in wild type bacteria during the adaptation to the CF lung habitat (Govan and Deretic, 1996; Tümmler and Kiewitz, 1999).

The genes involved in oxidative stress response and flagella biogenesis were further characterized. It has been observed that some of the unknown genes (*betB*, *napA*, PA0880, PA1288, PA4621, PA5349) and known genes (*recQ*, *katB*) for oxidative stress response were also identified on hydrogen peroxide agar assay and found necessary for intracellular survival of *P. aeruginosa* TB in PMNs. Further biochemical and genetic characterization of these mutants is required to know their exact mechanism during the intracellular survival in PMNs. Three mutants carrying defects in flagella biogenesis were identified during the STM screening. The cellular morphotypes and structure of flagellum were characterized by electron microscopy. The *flhA* mutant was found to be non-flagellated, the *fliK* mutants showed elongated flagella, while the *fliI* mutant was having multiple flagellas. The flagellin monomers of the *fliK* mutant were assembled to a flagellar structure with a hook- like relief that exposes more epitopes on its surface than the wild- type flagella. The cytotoxic assay showed that the *fliK* and *flhA* mutants were less cytotoxic than the TB wild type. All the three identified flagella genes are located in the center of flagellar export apparatus. It has been reported that the flagellar export apparatus is not only involved in the transport of flagellin molecules, but it can also transport virulence factors in the extracellular environment (Young *et al.*, 1999). Further research on the identification and characterization of flagellar apparatus dependent export of virulence factors in TB is required.

4.2. Screening of STM library for quorum sensing

A simple and rapid method of the degradation of casein was used to screen the STM mutants for defect in quorum sensing. Quorum sensing is involved in the secretion of virulence factors in the form of extracellular proteases. After screening the 2304 STM mutants, 21 mutants were attenuated for extracellular protease secretion. Most of these mutants were further confirmed for defect in quorum sensing by the AHL-dependent quorum sensing assay. Unfortunately, plasmid rescue for 7 mutants failed even after successive efforts to find out the genomic disruptions. After sequencing the disrupted genes from 14 mutants, known quorum sensing dependent gene (*rhlR*, *gcdH*) were identified and others are with unknown function. It will be the next task for the characterization of their role in quorum sensing cascade. The results indicated that the screening of mutants for protease secretion is an easy and rapid method for the identification of quorum sensing dependent genes.

4.3. Transcriptional analysis of *P. aeruginosa* TB

The genome-wide transcription profile against the superoxide stress generating agent paraquat and oxidative stress generating agent hydrogen peroxide was measured in the *P. aeruginosa* reference strain PAO1 and the cystic fibrosis isolates TB and 892 by using the PAO1 GeneChips. One operon encoding four proteins of previously unknown function (PA0939–PA0942) was 20–200 fold up-regulated in all three analyzed strains in presence of paraquat. This operon that exhibits the strongest up-regulation and the constitutively highly expressed manganese-dependent superoxide dismutase *sodB*, the catalase *katA* and the alkyl hydroperoxide reductase *ahpC* seem to confer most resistance to paraquat-induced stress in *P. aeruginosa.* The next task is the characterization of this operon (PA0939–PA0942) to uncover how paraquat mediated superoxide stress is handled by this operon in addition to the other known genes. Upon exposure to hydrogen peroxide, 23.5, 17.5, 15 % of ORFs were significantly differentially regulated in *P. aeruginosa* TB, 892 and PAO1 respectively. The total number of genes expressed in the chosen condition were about 75 % from all the three strains indicating that most of the genes were essential to combat the oxidative stress. The up-regulated genes belong to aerobic energy generation, amino acid metabolism, polyamine transport, iron-sulfur metabolism, cell rescue/defense and translation machinery. The down-regulated genes belong to the microaerophilic and anaerobic growth, quorum

sensing and flagella biogenesis and chemotaxis. The transcriptome data indicated that more number of genes were significantly modulated against the hydrogen peroxide mediated oxidative stress response in TB as compared to the 892 and PAO1 strains.

The STM screening identified 3 mutants that harbor insertions in non-PAO1 genes and 2 mutants carrying the transposon in regulatory genes. The sequence from 25A12 mutant was found completely unknown, while the sequence from 45A7 mutant showed slight homologies with proteins of unknown function. The 25A12 and 45A10 non-PAO1 mutants and 23B9 mutant having insertion in sigma-70 factor was considered for GeneChip analysis. It was observed that both non-PAO1 ORFs were expressed in all the tested standard and stress conditions. While the sigma factor was expressed only in iron limiting condition and in presence of hydrogen peroxide and PMNs. The transcriptome data from both non-PAO1 mutants revealed that there were subtle changes in the normal metabolism during standard growth condition. Only 25A12 mutant showed slight down-regulation in the arginine utilization as energy source. This indicated that the mutation in the non-PAO1 genes does not interfere with the *P. aeruginosa* PAO1 defined core genome under standard growth conditions. These two non-PAO1 ORFs expressed constitutively and could be regulated their own without affecting the core metabolism of *P. aeruginosa* TB. It was identified that the mutation in the non-PAO1 genes has impact on the denitrification under hydrogen peroxide and PMNs stimulated growth conditions. This reflects that the genes involved in energy generation during anaerobic condition are necessary for the intracellular survival and growth of *P. aeruginosa* TB. The up-regulation of type III secretion and quorum sensing systems showed that both mutants are more virulent than the TB wild type. However, these mutants are impaired for the intracellular survival and growth in PMNs. This suggested that the transposon insertion in the strain specific DNA destabilizes the expression of virulence factors but it is necessary for the intracellular life style of the bacterium *P. aeruginosa* TB. It was identified that these two non-PAO1 ORFs have less G+C content (60 %) as compared to the average G+C content (67 %) of *P. aeruginosa*. This suggests that they could be a part of extrachromosomal genetic elements and acquired through the horizontal gene transfer. It will be interesting to characterize these unknown gene products and their role for the virulence gene regulation under the stress conditions. The GeneChip data

from sigma-70 factor showed the down-regulation of the genes involved in aerobic or anaerobic growth, quorum sensing, type III secretion system, iron homeostasis and antimicrobial peptide resistance besides the up-regulation of genes involved in quorum sensing and iron homeostasis as a rescue mechanism. The GeneChip data revealed that the mutant is hampered for energy generation via aerobic and anaerobic processes and can be transformed to dormant metabolic stage which is responsible for the bacterial death. The interesting results obtained was that some of the quorum sensing dependent genes were down-regulated as well as some were up-regulated in the mutant. Further work in this direction is necessary to know the exact function of this sigma factor and its role in quorum sensing cascade under various environmental conditions.

The GeneChip experiment from the D8A6 mutant revealed that nearly all the known quorum sensing dependent genes except *lasR* and *gacA* were differentially regulated. The transcriptome data revealed that the gene regulation for quorum sensing hierarchy is different in *P. aeruginosa* TB as compared to the well studied quorum sensing cascade. Most of the genes were also identified as being quorum sensing dependent regulated in the microarray experiments by Wagner *et al.* (2003) and Schuster *et al.* (2003). This mutant has been identified during the screening for defect in protease secretion. The transcriptome data suggest that this gene can get top hierarchical position in the quorum sensing network. It would be interesting to do further experiments as how this non-PAO1 gene regulates both quorum sensing systems and its abundance in other *P. aeruginosa* strains.

4.4. Examination of *P. aeruginosa* pathogenicity in murine lung infection model

The subset of STM knockout mutants were examined for their pathogenicity in a murine lung infection model. The mouse infection experiments indicated that some mutants such as *flhA* and C47 were also less virulent, while *pilY1* and 25A12 were more virulent than the TB wild type. There was good correlation observed in case of *flhA* and *pilY1* mutants from the ex vivo PMN phagocytosis assay with the results from murine lung infection model. The C47 mutant has not been tested in the PMN assays. But there is lot of literature available on quorum sensing and *P. aeruginosa* pathogenesis (Ritchie *et al.*, 2003; Lesprit *et al.*, 2003; Smith and Iglewski, 2003 and

references cited therein). The monitored parameters during mouse infection experiments for other mutants were not significantly different than TB wild type indicating that there could be small difference in the virulence of those mutants and TB which is difficult to observe in the mouse model. There was large variation in the experiments suggesting that these mutants should be retested in this murine lung model. The results from *C. elegans* showed that 25A12 mutant killed twice the number of nematodes killed by TB, while 45A7 killed 10 % more than the TB wild type (data not shown). This supports the results obtained from the mouse infection model and the GeneChip experiments.

The transcriptome data together with murine infection model described in the present study could be useful for well directed experiments to dissect the intracellular survival mechanisms in *P. aeruginosa* TB and the biochemical function of the strain specific (non-PAO1) genes.

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6. Abbreviations

Aa	Amino acid
bp	Base pair
approx.	approximately
^{o}C	Degree Celsius
CDS	Coding sequence
CF	Cystic fibrosis
cfu	Colony forming units
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
DEPC	Diethypyrocarbonate
dGTP	Deoxyguanosine triphosphate
DIG	Digoxigenin
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
et. al.,	*et alteri* (and others)
i.e.	That means
FCS	Fetal calf serum
x *g*	Centrifugal acceleration
h	Hour
I.E.	Injection units
kBp	1000 Base pairs
LB	Luria-Bertani
M	Molar
m	milli- (10^{-3}); Meter, Mass
Max.	Maximum
min	Minute
MOPS	Morpholinopropanesulfonic acid
n	nano- (10^{-9})
OD	Optical Density
ORF	Open reading frame; possible gene
p	pico- (10^{-12})
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
rpm	Revolutions per minute
RNase	Ribonuclease
RT	Room temperature (23 ^{o}C)
s	Second
SDS	Sodium dodecyl sulfate
t	Time
T	Temperature
TE	Tris-EDTA
µ	micro- (10^{-6})
Mb	Megabases
max.	Maximum
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

Following are the molecular weight standards used for DNA and RNA agarose gel electrophoresis and the fragment sizes are given in base pairs.

(1) Lambda DNA-BstE II digest (New England Biolab)

8454	7242	6369	5686	4822	4324	3675
2323	1929	1371	1264	702	224	117

(2) 100 bp DNA ladder (New England Biolab)

1517	1200	1000	900	800	700	600
517	500	400	300	200	100	

(3) 0.16−1.77 Kb RNA ladder (Invitrogen)

1770	1520	1280	780	530	400	280
155						

Appendix II

The following primer pairs (oligonucleotide sequences) were used for sequencing.

Description	**Primer sequence**
Signal sequence	TAG-1: 5‘–GTA CCC CAC TAG TCC AAG C
	TAG-2: 5‘–TAC CTC CAC TCA CCC AAG C
25A12	25A12GL: 5‘–ATG TTA GTG GCT TTT CCC CG
	25A12GRN: 5‘–ATC TGG GCC ATT TCA TCA CC
45A7	45A7GRL: 5‘–GTG GAA GAC GCA ATG AAA GG
	45A7GRR: 5‘–AAG GAC CGA GGT TCT TGA TG
23B9	PA2468L: 5‘–GCC CCG ATC CTT CTC AGC
	PA2468R: 5‘–CGC AGC AGG TAG CAG TGG
D8A6	D8A6L: 5‘–GGC TTG CAT GAT GTT GTA GC
	D8A6R: 5‘–GAG ATG TTC AAT CGC AAA GG

Appendix III

Table 7.1. Differential transcription profile of *P. aeruginosa* TB, 892 and PAO1 exposed to hydrogen peroxide.

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
I) Hydrogen peroxide induced genes				
A) Energy metabolism				
TCA cycle and aerobic respiration				
PA0548	2.35 (0.23)			transketolase, *tktA*
PA0552	2.93 (0.20)	2.97 (0.59)	2.53 (0.43)	phosphoglycerate kinase, *pgk*
PA0555	2.07 (0.08)	2.14 (0.00)		fructose-1.6-bisphosphate aldolase, *fda*
PA1580	3.83 (0.60)	3.75 (0.47)	3.11 (0.46)	citrate synthase, *gltA*
PA1588	3.27 (0.41)	2.31 (0.29)	2.56 (0.23)	succinyl-CoA synthetase beta chain, *sucC*
PA1589	2.88 (0.19)		2.59 (0.09)	succinyl-CoA synthetase alpha chain, *sucD*
PA1787	2.32 (0.37)	2.56 (0.23)	2.49 (0.14)	aconitate hydratase 2, *acnB*
PA2023	2.51 (0.09)			UTP--glucose-1-phosphate uridylyltransferase, *galU*
PA2290	2.11 (0.14)			glucose dehydrogenase, *gcd*
PA2322	3.37 (0.13)	3.06 (0.48)		gluconate permease
PA2624	3.88 (0.35)	3.55 (0.83)	4.09 (0.97)	isocitrate dehydrogenase, *idh*
PA2843	2.99 (0.25)	3.63 (0.52)	4.76 (0.19)	probable aldolase
PA3131			2.22 (0.09)	probable aldolase
PA3181			3.12 (0.57)	2-keto-3-deoxy-6-phosphogluconate aldolase
PA3182			2.80 (0.40)	6-phosphogluconolactonase, *pgl*
PA3183			3.26 (0.26)	glucose-6-phosphate 1-dehydrogenase, *zwf*
PA3452	5.69 (0.72)	3.59 (1.03)		malate:quinone oxidoreductase, *mqoA*
PA3560	2.44 (0.35)			Phosphotransferase system. fructose-specific IIBC component, *fruA*
PA3635	2.42 (0.08)			enolase, *eno*
PA4031	2.74 (0.24)	2.89 (0.26)		inorganic pyrophosphatase, *ppa*
PA4333	3.82 (0.44)	3.55 (0.23)	3.05 (0.38)	probable fumarase
PA4470	3.75 (0.47)	3.22 (0.48)	5.09 (1.07)	fumarate hydratase, *fumC*1
PA4770	6.93 (2.44)	8.73 (3.16)	2.72 (0.49)	L-lactate permease, *lldp*
PA4771	2.30 (0.13)	3.35 (0.93)		L-lactate dehydrogenase, *lldD*
PA4772	3.12 (0.57)	3.96 (1.32)		probable ferredoxin
PA5046	2.98 (0.10)	3.09 (0.20)	3.54 (0.72)	malic enzyme
PA5131	3.67 (0.12)	3.35 (0.63)	3.29 (0.59)	Phosphoglycerate mutase, *pgm*
PA5192	3.11 (0.49)	3.07 (0.55)	3.95 (0.95)	Phosphoenolpyruvate carboxykinase, *pckA*
PA5476	3.32 (0.38)	3.93 (1.17)		citrate transporter, *citA*
Acetyl CoA biosynthesis and acetate degradation				
PA0363	4.54 (0.55)	3.14 (0.69)	2.34 (0.15)	Phosphopantetheine adenylyltransferase, *coaD*
PA0887	3.46 (0.62)			acetyl-coenzyme A synthetase, *acsA*

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
I) Hydrogen peroxide induced genes				
A) Energy metabolism				
Acetyl CoA biosynthesis and acetate degradation				
PA4729	2.43 (0.29)			3-methyl-2-oxobutanoate hydroxymethyltransferase, *panB*
PA4730	2.43 (0.21)	2.44 (0.35)		pantoate-beta-alanine ligase, *panC*
PA4731	2.74 (0.24)	2.66 (0.39)		aspartate 1-decarboxylase precursor, *panD*
PA5445	3.41 (0.65)	4.81 (1.29)		probable coenzyme A transferase
Electron transport and oxidative phosphorylation				
PA1317	34.43 (10.84)	38.84 (8.75)	12.14 (2.68)	cytochrome o ubiquinol oxidase, *cyoA*
PA1318	17.79 (1.27)	27.69 (4.91)	19.61 (14.31)	cytochrome o ubiquinol oxidase, *cyoB*
PA1319	21.75 (6.04)	25.49 (5.09)	8.02 (0.64)	cytochrome o ubiquinol oxidase, *cyoC*
PA1320	30.10 (7.72)	37.61 (14.53)	11.91 (6.77)	cytochrome o ubiquinol oxidase, *cyoD*
PA1321	10.72 (2.17)	7.07 (1.43)	3.72 (0.72)	cytochrome o ubiquinol oxidase protein, *cyoE*
PA1552	3.50 (0.44)	2.70 (0.31)		probable cytochrome c
PA1553	3.31 (0.12)	2.57 (0.37)		probable cytochrome c oxidase subunit
PA1554	2.47 (0.14)			probable cytochrome oxidase subunit
PA2266			2.48 (0.31)	probable cytochrome c precursor
PA4133	4.44 (0.18)			cytochrome c oxidase subunit (cbb3-type)
PA4429			2.40 (0.39)	probable cytochrome c1 precursor
PA5553	2.99 (0.31)	2.40(0.35)		ATP synthase epsilon chain, *atpC*
PA5554	2.72 (0.49)	2.36(0.37)		ATP synthase beta chain, *atpD*
PA5555	2.39 (0.21)			ATP synthase gamma chain, *atpG*
PA5556	2.15 (0.12)			ATP synthase alpha chain, *atpA*
PA5560	2.00 (0.00)			ATP synthase A chain, *atpB*
Fatty acid and phospholipid metabolism				
PA1288	2.39 (0.21)	2.51(0.16)		probable outer membrane protein precursor
PA1806	2.56 (0.23)		3.37 (0.30)	NADH-dependent enoyl-ACP reductase, *fabI*
PA1846	2.15 (0.22)			cis/trans isomerase, *cti*
PA2015			2.90 (0.41)	probable acyl-CoA dehydrogenase
PA2352	4.01 (0.38)	6.34(2.37)		probable glycerophosphoryl diester phosphodiesterase
PA2965	2.61 (0.32)			beta-ketoacyl-acyl carrier protein, *fabF1*
PA2968	2.89 (0.26)	2.75(035)		malonyl-CoA-[acyl-carrier-protein] transacylase, *fabD*
PA2969	2.26 (0.15)			fatty acid biosynthesis protein, *plsX*
PA3651	3.67 (0.25)			phosphatidate cytidylyltransferase, *cdsA*
PA4693	2.15 (0.22)			Phosphatidylserine synthase, *pssA*
PA4847		3.09 (0.20)		biotin carboxyl carrier protein (BCCP), *accB*
PA4848		3.42 (0.12)		biotin carboxylase, *accC*
PA5174	2.84 (0.32)			probable beta-ketoacyl synthase

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
I) Hydrogen peroxide induced genes				
B) Amino acid biosynthesis and metabolism				
Arginine				
PA0662	2.43 (0.21)			N-acetyl-gamma-glutamyl-phosphate reductase, *argC*
PA0895	2.84 (0.23)	2.43(0.21)	3.27 (0.41)	N-succinylglutamate 5-semialdehyde dehydrogenase, *aruC*
PA0896			2.56 (0.23)	arginine/ornithine succinyltransferase, *aruF*
PA0897			2.69 (0.18)	arginine/ornithine succinyltransferase, *aruG*
PA0898			2.07 (0.15)	Succinylglutamate 5-semialdehyde dehydrogenase, *aruD*
PA3537	2.38 (0.17)			ornithine carbamoyltransferase, *argF*
PA4756	2.40 (0.39)			Carbamoylphosphate synthetase, *carB*
Glycine and serine				
PA0008	2.31 (0.29)	2.27(0.23)		glycyl-tRNA synthetase beta chain, *glyS*
PA0316	2.80 (0.40)			D-3-phosphoglycerate dehydrogenase, *serA*
PA0904	4.47 (0.57)	3.76(0.59)	4.23 (0.43)	aspartate kinase alpha and beta chain, *lysC*
PA2445	2.59 (0.56)	3.88(0.35)		glycine cleavage system protein P2, *gcvP*2
PA2446	5.07 (0.86)	7.42(1.31)		glycine cleavage system protein H2, *gcvH*2
PA3736	3.31 (0.22)	3.57(0.56)	3.93 (1.15)	homoserine dehydrogenase, *hom*
Threonine				
PA0331	2.31 (0.29)			threonine dehydratase. Biosynthetic, *ilvA*1
PA1757	4.45 (0.40)	4.58(0.88)		homoserine kinase, *thrH*
PA3735	3.03 (0.00)	3.20 (0.22)	3.09 (0.28)	threonine synthase, *thrC*
Glutamate and glutamine				
PA2202	5.68 (3.71)	5.67(2.40)		probable amino acid permease
PA2203	3.46 (0.61)	4.42 (1.25)		probable amino acid permease
PA2204	17.81 (1.59)	15.85 (2.27)	4.60 (2.47)	probable binding protein component of ABC transporter
PA3068			2.39 (0.29)	NAD-dependent glutamate dehydrogenase, *gdhB*
PA3134	3.37 (0.30)	3.05 (0.34)		glutamyl-tRNA synthetase, *gltX*
PA5074	3.67 (0.25)	3.59 (1.03)		probable ATP-binding component of ABC transporter
PA5075	3.74 (0.21)	2.94(0.67)		probable permease of ABC transporter
PA5076	3.32 (0.38)			probable binding protein component of ABC transporter
PA5203	2.44 (0.37)	2.62 (0.46)		glutamate-cysteine ligase, *gshA*
PA5429		3.70 (0.71)		aspartate ammonia-lyase, *aspA*
PA5479	2.94 (0.26)			proton-glutamate symporter, *gltP*
Alanine and aspartate				
PA0903	4.01 (0.32)	3.22 (0.48)	3.02 (0.57)	alanyl-tRNA synthetase, *alaS*
PA2252	4.95 (0.63)	4.47 (0.57)	2.56 (0.23)	probable AGCS sodium/alanine/glycine symporter
PA2253	3.62 (0.32)	2.57 (0.37)		L-asparaginase I, *ansA*

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
I) Hydrogen peroxide induced genes				
B) Amino acid biosynthesis and metabolism				
Lysine				
PA0904	4.47 (0.57)	3.76 (0.59)	4.23 (0.43)	aspartate kinase alpha and beta chain, *lysC*
PA3700	3.40 (0.56)	2.67 (0.48)	2.70 (0.28)	lysyl-tRNA synthetase, *lysS*
PA4628	4.76 (1.04)	3.58 (1.24)	3.41 (1.30)	lysine-specific permease, *lysP*
PA5278			2.04 (0.07)	diaminopimelate epimerase, *dapF*
Leucine, isoleucine and valine				
PA1587	2.11 (0.14)			lipoamide dehydrogenase-glc, *lpdG*
PA1971	2.50 (0.51)			branched chain amino acid transporter, *praZ*
PA3834	3.05 (0.34)	2.52 (0.35)	2.72 (0.51)	valyl-tRNA synthetase, *valS*
PA3987			2.55 (0.17)	leucyl-tRNA synthetase, *leuS*
PA4560	2.30 (0.18)		2.80 (0.40)	isoleucyl-tRNA synthetase, *ileS*
PA4694			2.19 (0.20)	ketol-acid reductoisomerase, *ilvC*
PA4695			2.30 (0.13)	acetolactate synthase isozyme , *ilvH*
PA4696			2.19 (0.23)	acetolactate synthase, *ilvI*
PA5308	3.13 (0.64)	3.04 (0.71)		leucine-responsive regulatory protein, *lrp*
Proline				
PA0782	5.55 (1.11)	5.66 (0.00)	3.70 (0.53)	proline dehydrogenase, *putA*
PA0783	5.81 (0.90)	5.77 (0.52)	3.57 (0.50)	sodium/proline symporter, *putP*
PA0789	4.70 (0.54)	3.70 (0.53)		probable amino acid permease
PA0956	2.31 (0.29)		2.63 (0.47)	prolyl-tRNA synthetase, *proS*
Histidine				
PA3151			2.26 (0.19)	imidazoleglycerol-phosphate synthase, cyclase subunit, *hisF2*
PA4448	2.43 (0.21)	2.27 (0.23)		histidinol dehydrogenase, *hisD*
PA4449	2.83 (0.16)		2.75 (0.37)	ATP-phosphoribosyltransferase, *hisG*
PA5140	2.22 (0.09)	2.26 (0.19)		imidazoleglycerol-phosphate synthase. cyclase subunit, *hisF1*
PA5141	2.42 (0.08)	2.70 (0.28)		phosphoribosylformimino-5-aminoimidazole carboxamide, *hisA*
Tryptophan and phenylalanine				
PA0037	2.72 (0.49)	2.14 (0.00)		transcriptional regulator, *trpI*
PA0649	2.23 (0.20)	2.35 (0.27)		anthranilate synthase component II, *trpG*
PA1750	4.72 (0.74)	4.10 (0.58)	3.35 (0.61)	phospho-2-dehydro-3-deoxyheptonate aldolase
PA2739	2.39 (0.29)			phenylalanyl-tRNA synthetase, *pheT*
PA2740	2.28 (0.33)		2.07 (0.08)	phenylalanyl-tRNA synthetase, *pheS*
PA3164	3.70 (0.56)	2.78 (0.60)		still frameshift 3-phosphoshikimate 1-carboxyvinyltransferase prephenate dehydrogenase
PA3165	3.56 (0.41)	2.80 (0.14)		histidinol-phosphate aminotransferase, *hisC*2
PA4439	2.18 (0.14)	2.61 (0.32)	2.44 (0.37)	tryptophanyl-tRNA synthetase, *trpS*
PA4447	2.64 (0.00)	2.75 (0.37)		histidinol-phosphate aminotransferase, *hisC*1

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
I) Hydrogen peroxide induced genes				
C) Biosynthesis of cofactors, prosthetic groups and carriers				
Folic acid biosynthesis				
PA0342	2.34 (0.08)			thymidylate synthase, *thyA*
PA0350	2.99 (0.25)	2.56 (0.23)		dihydrofolate reductase, *folA*
PA0582	3.50 (0.35)			dihydroneopterin aldolase, *folB*
PA1674	2.95 (0.43)	2.81 (0.50)		GTP cyclohydrolase I precursor, *folE*2
PA1796	2.65 (0.30)		2.11 (0.14)	5.10-methylene-tetrahydrofolate dehydrogenase / cyclohydrolase, *fol*D
PA2444	6.00 (2.38)	18.29 (8.92)		serine hydroxymethyltransferase, *glyA*2
PA2964	2.43 (0.25)			4-amino-4-deoxychorismate lyase, *pabC*
PA4314	2.42 (0.08)	2.51 (0.22)	2.38 (0.10)	formyltetrahydrofolate deformylase, *purU*1
PA4602	2.95 (0.43)	2.62 (0.65)		serine hydroxymethyltransferase, *glyA*3
PA4664	3.42 (0.12)	2.98 (0.64)	4.40 (0.68)	probable methyl transferase, hemK
PA5415		2.57 (0.33)		serine hydroxymethyltransferase, *glyA*1
Cobalamin biosynthesis				
PA1271	2.49 (0.44)	2.86 (0.51)		probable tonB-dependent receptor
PA1272	2.18 (0.14)	2.34 (0.08)		cob(I)alamin adenosyltransferase, *cobO*
PA1273	2.26 (0.15)			cobyrinic acid a.c-diamide synthase, *cobB*
PA1274	3.44 (0.35)	3.33 (0.47)		conserved hypothetical protein
PA1275	2.18 (0.14)	2.30(0.18)		cobalamin biosynthetic protein, *cobD*
PA1276	5.11 (0.46)	3.80 (0.25)		cobalamin biosynthetic protein, *cobC*
PA1277		2.76 (0.45)		cobyric acid synthase, *cobQ*
PA1278	4.15 (0.95)	3.08 (0.17)		cobinamide kinase, *copP*
PA1279	9.14 (2.51)	3.51 (0.55)		nicotinate-nucleotide—dimethylbenzimidazole phosphoribosyltransferase, *cobU*
PA1281		3.03 (0.69)		cobalamin (5'-phosphate) synthase, *cobV*
PA2903	8.89 (0.59)	5.16 (2.16)		precorrin-3 methylase, *cobJ*
PA2904	2.43 (0.25)			precorrin-2 methyltransferase, *cobI*
PA2948		4.13 (0.77)		precorrin-3 methylase, *cobM*
Vitamin B6 biosynthesis				
PA0593	2.89 (0.30)			pyridoxal phosphate biosynthetic protein, *pdxA*
PA1375	2.54 (0.47)			erythronate-4-phosphate dehydrogenase, *pdxB*
PA2062	2.23 (0.20)			probable pyridoxal-phosphate dependent enzyme
D) Polyamine biosynthesis and transport				
PA0293	2.85 (0.36)			N-carbamoylputrescine amidohydrolase, *aguB*
PA0301		2.45 (0.43)		polyamine transport protein, *spuE*
PA0303			2.23 (0.20)	polyamine transport protein, *spuG*
PA0304			2.82 (0.61)	polyamine transport protein, *spuH*
PA0654	10.25 (1.23)	5.55 (1.59)		S-adenosylmethionine decarboxylase proenzyme, *speD*
PA1687	3.94 (0.26)	3.77 (0.64)		spermidine synthase, *speE*
PA3607	5.58 (1.26)			polyamine transport protein, *potA*

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
I) Hydrogen peroxide induced genes				
D) Polyamine biosynthesis and transport				
PA3609	3.26 (0.26)			polyamine transport protein, *potC*
PA3610	5.80 (0.82)	3.02 (0.90)		polyamine transport protein, *potD*
PA4839	2.81 (0.50)			biosynthetic arginine decarboxylase, *speA*
E) Iron-sulfur metabolism				
Iron acquisition				
PA0929			2.23 (0.20)	two-component response regulator
PA3812	2.15 (0.17)			probable iron-binding protein, *iscA*
PA3813	2.26 (0.19)			probable iron-binding protein, *iscU*
PA3901	6.09 (2.57)		30.74 (29.69)	Fe(III) dicitrate transport protein, *fecA*
PA4221	3.62 (0.32)	13.66 (2.71)	20.22 (12.39)	Fe(III)-pyochelin receptor precursor, *fptA*
PA4222	3.98 (1.09)			probable ATP-binding component of ABC transporter
PA4223	4.23 (0.36)	8.31 (0.74)	2.85 (0.36)	ATP-binding component of ABC transporter
PA4224	5.81 (1.98)	5.29 (0.42)	4.83 (0.96)	pyochelin biosynthetic protein, *pchG*
PA4225	4.08 (0.92)	5.11 (0.46)	14.71 (9.30)	pyochelin synthetase, *pchF*
PA4226	4.25 (0.61)	11.49 (7.51)	26.00 (13.98)	dihydroaeruginoic acid synthetase, *pchE*
PA4227	2.43 (0.21)	2.98 (0.63)	2.26 (0.15)	transcriptional regulator, *pchR*
PA4228	3.44 (0.35)	10.22 (2.35)	7.93 (3.48)	pyochelin biosynthesis protein, *pchD*
PA4229	3.62 (0.41)	10.58 (0.85)	6.52 (0.66)	pyochelin biosynthetic protein, *pchC*
PA4230	2.49 (0.40)	10.23 (0.91)	7.48 (0.16)	salicylate biosynthesis protein, *pchB*
PA4231	3.03 (0.67)	9.49 (3.29)	14.72 (9.30)	salicylate biosynthesis isochorismate synthase, *pchA*
PA4358			3.31 (0.12)	probable ferrous iron transport protein
PA4514			3.08 (0.62)	probable outer membrane receptor for iron transport
PA4687		5.22 (0.67)	7.61 (0.50)	ferric iron-binding periplasmic protein, *hitA*
PA4688	2.62 (0.46)	4.29 (0.24)	3.57 (1.38)	iron (III)-transport system permease, *hitB*
PA4710	3.45 (0.49)	3.09 (0.28)	5.17 (3.33)	haem/haemoglobin uptake outer membrane receptor PhuR precursor, *phuR*
PA5217		2.64 (0.15)		probable binding protein component of ABC iron transporter
PA5531		2.79 (0.24)	5.05 (0.67)	TonB protein, *tonB*
Sulfur metabolism				
PA0280	4.64 (0.74)	5.90 (0.85)		sulfate transport protein, *cysA*
PA0281	17.17 (0.97)	24.31 (16.0)		sulfate transport protein, *cysW*
PA0282	4.27 (0.77)	4.36 (0.15)		sulfate transport protein, *cysT*
PA0283	5.77 (1.34)	6.83 (2.40)		sulfate-binding protein precursor, *spb*
PA0390	3.15 (0.28)	3.32 (0.38)		homoserine O-acetyltransferase, *metX*
PA1493	3.22 (0.48)	2.43 (0.29)	2.74 (0.24)	sulfate-binding protein of ABC transporter, *cysP*
PA1795			2.28 (0.33)	cysteinyl-tRNA synthetase, *cysS*
PA1838	6.73 (0.27)	7.69 (2.14)		sulfite reductase, cysI

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
I) Hydrogen peroxide induced genes				
E) Iron-sulfur metabolism				
Sulfur metabolism				
PA3809	3.04 (0.24)			ferredoxin [2Fe-2S], *fdx2*
PA3814	2.15 (0.17)			L-cysteine desulfurase, *iscS*
PA4442	12.49 (2.27)	15.77 (3.55)	4.46 (0.56)	ATP sulfurylase GTP-binding subunit/APS kinase, *cysN*
PA4443	12.01 (1.67)	12.32 (3.69)	4.68 (0.31)	ATP sulfurylase small subunit, *cysD*
F) Transcription, RNA processing and degradation				
PA1161	3.10 (0.36)			rRNA methyltransferase, *rrmA*
PA3246	3.36 (0.71)			pseudouridine synthase, *rluA*
PA3308	2.76 (0.45)			RNA helicase, *hepA*
PA3743	2.65 (0.34)			tRNA (guanine-N1)-methyltransferase, *trmD*
PA3861	2.35 (0.25)		2.85 (0.41)	ATP-dependent RNA helicase, *rhlB*
PA4269	3.80 (0.13)	2.40 (0.39)		DNA-directed RNA polymerase beta* chain, *rpoC*
PA4270	4.71 (0.67)			DNA-directed RNA polymerase beta chain, *rpoB*
PA4275	2.22 (0.09)			transcription antitermination protein, *nusG*
PA4742	2.73 (0.11)			tRNA pseudouridine 55 synthase, *truB*
PA4755	2.47 (0.14)			transcription elongation factor, *greA*
G) Translation and post-translational modification				
PA0018	2.07 (0.15)			methionyl-tRNA formyltransferase, *fmt*
PA0067			2.43 (0.21)	oligopeptidase A, *prlC*
PA0538	2.23 (0.20)	2.40 (0.39)		disulfide bond formation protein, *dsbB*
PA0579	2.57 (0.37)			30S ribosomal protein S21, *rpsU*
PA0594	3.00 (0.36)			peptidyl-prolyl cis-trans isomerase, *surA*
PA0767	2.71 (0.38)			GTP-binding protein, *lepA*
PA0768	2.64 (0.15)			signal peptidase I, *lepB*
PA1793		2.56 (0.23)	2.51 (0.22)	peptidyl-prolyl cis-trans isomerase B, *ppiB*
PA1805	2.83 (0.00)	3.20 (0.22)	2.62 (0.39)	peptidyl-prolyl cis-trans isomerase D, *ppiD*
PA2748	3.26 (0.76)			probable methionine aminopeptidase
PA2851	2.55 (0.10)		2.44 (0.35)	translation elongation factor P, *efp*
PA2970	2.47 (0.20)		2,.40 (0.35)	50S ribosomal protein L32, *rpmF*
PA3262	3.17 (0.52)	3.52 (0.56)	2.63 (0.51)	probable peptidyl-prolyl cis-trans isomerase
PA3653	2.18 (0.08)			ribosome recycling factor, *frr*
PA3655	3.80 (0.25)	3.08 (0.66)	3.47 (0.67)	elongation factor Ts, *tsf*
PA3656	2.94 (0.26)		3.24 (0.62)	30S ribosomal protein S2, *rpsB*
PA3717	2.11 (0.14)			probable peptidyl-prolyl cis-trans isomerase
PA3737	2.28 (0.33)		2.28 (0.33)	thiol:disulfide interchange protein, *dsbC*
PA3742	2.43 (0.21)			50S ribosomal protein L19, *rplS*
PA3831			2.36 (0.33)	leucine aminopeptidase, *pepA*
PA3903	3.34 (0.56)			peptide chain release factor 3, *prfC*
PA4237	3.49 (0.20)	2.58 (0.43)	2.71 (0.38)	50S ribosomal protein L17, *rplQ*
PA4239	2.72 (0.51)		2.40 (0.39)	30S ribosomal protein S4, *rpsD*

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
I) Hydrogen peroxide induced genes				
G) Translation and post-translational modification				
PA4244	2.31 (0.26)		2.65 (0.21)	50S ribosomal protein L15, *rplO*
PA4245			2.27 (0.23)	50S ribosomal protein L30, *rpmD*
PA4250	2.26 (0.19)		2.40 (0.35)	30S ribosomal protein S14, *rpsN*
PA4252	2.45 (0.47)		2.82 (0.78)	50S ribosomal protein L24, *rplX*
PA4254			2.47 (0.28)	30S ribosomal protein S17, *rpsQ*
PA4255	3.83 (0.54)		2.69 (0.61)	50S ribosomal protein L29, *rpmC*
PA4256	2.14 (0.00)			50S ribosomal protein L16, *rplP*
PA4257			2.40 (0.35)	30S ribosomal protein S3, *rpsC*
PA4266	2.19 (0.23)			elongation factor G, *fusA*1
PA4268	2.23 (0.20)			30S ribosomal protein S12, *rpsL*
PA4271	3.20 (0.22)	2.23 (0.27)	3.70 (0.53)	50S ribosomal protein L7 / L12, *rplL*
PA4272	2.94 (0.26)		3.72 (0.72)	50S ribosomal protein L10, *rplJ*
PA4273	2.89 (0.30)			50S ribosomal protein L1, *rplA*
PA4432	2.88 (0.10)		2.40 (0.39)	30S ribosomal protein S9, *ssb*
PA4483			2.44 (0.34)	Glu-tRNA(Gln) amidotransferase, *gatA*
PA4484			2.43 (0.25)	Glu-tRNA(Gln) amidotransferase, *gatB*
PA4558	2.53 (0.39)		2.93 (0.10)	probable peptidyl-prolyl cis-trans isomerase
PA4567	2.64 (0.15)		2.34 (0.15)	50S ribosomal protein L27, *rpmA*
PA4568			2.04 (0.07)	50S ribosomal protein L21, *rplU*
PA4665	3.20 (0.22)	2.74 (0.63)	3.50 (0.44)	peptide chain release factor 1, *prfA*
PA4671	3.21 (0.39)	2.31 (0.29)	3.30 (0.67)	probable ribosomal protein L25
PA4672	3.99 (0.77)			peptidyl-tRNA hydrolase
PA4741	3.04 (0.31)	2.49 (0.42)	3.67 (0.12)	30S ribosomal protein S15, *rpsO*
PA4743	3.34 (0.52)			ribosome-binding factor A, *rbfA*
PA4744	2.22 (0.16)			translation initiation factor IF-2, *infB*
PA4768	2.31 (0.29)		2.37 (0.43)	SmpB protein, *smpB*
PA4850	2.74 (0.63)			ribosomal protein L11 methyltransferase, *prmA*
PA4932	3.68 (0.38)	2.81 (0.50)		50S ribosomal protein L9, *rp1I*
PA4934	2.34 (0.15)		2.52 (0.29)	30S ribosomal protein S18, *rpsR*
PA4935	2.60 (0.17)		2.55 (0.54)	30S ribosomal protein S6, *rpsF*
PA5049	2.69 (0.09)	2.62 (0.46)	2.88 (0.10)	50S ribosomal protein L31, *rpmE*
PA5117	6.58 (1.17)	3.87(0.86)	4.02 (0.51)	regulatory protein, *typA*
PA5129	2.89 (0.33)	2.77 (0.55)	2.57 (0.37)	glutaredoxin, *rgx*
PA5134	2.15 (0.17)			probable carboxyl-terminal protease
PA5240		2.14 (0.00)	2.39 (0.21)	thioredoxin, *trxA*
PA5256	2.62 (0.39)			disulfide bond formation protein, *dsbH*
H) Cellular organisation and biogenesis				
PA0973	2.42 (0.08)			peptidoglycan associated lipoprotein, *oprL*
PA1812	2.27 (0.23)			membrane-bound lytic murein transglycosylase D precursor, *mltD*
PA1959	3.56 (0.37)			bacitracin resistance protein, *bacA*

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
I) Hydrogen peroxide induced genes				
H) Cellular organisation and biogenesis				
PA2272	3.02 (0.54)			penicillin-binding protein 3A, *pbpC*
PA3047		2.51 (0.16)	2.35 (0.32)	probable D-alanyl-D-alanine carboxypeptidase
PA3243	2.79 (0.34)	2.44 (0.35)		cell division inhibitor, *minC*
PA3245	2.55 (0.10)	2.31 (0.26)		cell division topological specificity factor, *minE*
PA3643	3.43 (0.29)	3.04 (0.71)		lipid A-disaccharide synthase, *lpxB*
PA3644	2.79 (0.24)			UDP-N-acetylglucosamine acyltransferase, *lpxA*
PA3646	2.71 (0.38)			UDP-3-O-[3-hydroxylauroyl] glucosamine N-acyltransferase, *lpxD*
PA3652	3.04 (0.17)			undecaprenyl pyrophosphate synthetase, *uppS*
PA3659	2.26 (0.15)	2.34 (0.15)		probable aminotransferase
PA3666	2.27 (0.23)	2.65 (0.21)	2.89 (0.26)	Tetrahydrodipicolinate succinylase, *dapD*
PA3984	3.62 (0.32)	2.79 (0.29)	2.07 (0.08)	apolipoprotein N-acyltransferase, *lnt*
PA3999	2.11 (0.14)			D-ala-D-ala-carboxypeptidase, *dacC*
PA4002	2.65 (0.27)			rod shape-determining protein, *rodA*
PA4003	2.53 (0.39)		2.74 (0.24)	penicillin-binding protein 2, *pbpA*
PA4450	2.15 (0.17)		3.00 (0.36)	UDP-N-acetylglucosamine 1-carboxyvinyltransferase, *murA*
PA4479	2.78 (0.19)			rod shape-determining protein, *mreD*
PA4480	2.86 (0.51)			rod shape-determining protein, *mreC*
PA4481	2.51 (0.09)			rod shape-determining protein, *mreB*
PA4512	3.25 (0.18)			lipopolysaccharide biosynthetic protein, *lpxO*1
PA4662	3.19 (0.11)			glutamate racemase, *murI*
PA4668	2.99 (0.25)	2.89 (0.33)	4.48 (0.74)	probable lipoprotein localization protein
PA4930	2.15 (0.12)			biosynthetic alanine racemase, *alr*
PA5009	2.56 (0.23)	2.31 (0.22)		lipopolysaccharide kinase, *waaP*
PA5549	4.22 (0.28)	3.21 (0.39)		glucosamine—fructose-6-phosphate aminotransferase, *glmS*
PA5562	2.26 (0.15)			chromosome partitioning protein, *spoOJ*
II) Hydrogen peroxide repressed genes				
A) Quorum sensing				
PA0996			5.81 (1.57)	probable coenzyme A ligase, *pqsA*
PA0999			5.06 (1.72)	3-oxoacyl-[acyl-carrier-protein] synthase III, *pqsD*
PA1003	4.88 (0.70)	6.03 (2.12)	6.54 (3.88)	transcriptional regulator, *mvfR*
PA1246	3.95 (0.95)	2.19 (0.20)		alkaline protease secretion protein, *aprD*
PA1247	3.42 (0.69)			alkaline protease secretion protein, *aprE*
PA1248	2.30 (0.13)			alkaline protease secretion protein, *aprF*
PA1249	7.75 (0.69)	5.66 (0.32)		alkaline metalloproteinase precursor, *aprA*
PA1250	3.19 (0.68)	3.16 (0.46)		alkaline proteinase inhibitor, *aprI*
PA1430			2.65 (0.27)	transcriptional regulator, *lasR*
PA1431	4.93 (1.09)	5.82 (2.16)		regulatory protein, *rsaL*

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
II) Hydrogen peroxide repressed genes				
A) Quorum sensing				
PA1871	19.31 (3.8)	7.77 (0.97)	6.54 (5.48)	LasA protease precursor, *lasA*
PA1901	16.50 (3.0)			phenazine biosynthesis protein, *PhzC*2
PA1902	4.83 (1.9)			phenazine biosynthesis protein, *PhzD*2
PA1904	11.13 (4.3)			probable phenazine biosynthesis protein, *phzF*2
PA1985	2.47 (0.23)	2.30 (0.13)	7.84 (5.05)	pyrroloquinoline quinone biosynthesis protein A, *pqqA*
PA1986			2.59 (0.52)	pyrroloquinoline quinone biosynthesis protein B, *pqqB*
PA1987			2.71 (0.40)	pyrroloquinoline quinone biosynthesis protein C, *pqqC*
PA1988		2.43 (0.21)		pyrroloquinoline quinone biosynthesis protein D, *pqqD*
PA2193	13.11 (4.4)	16.66 (5.38)		hydrogen cyanide synthase, *hcnA*
PA2194	17.02 (4.5)	14.41 (6.76)		hydrogen cyanide synthase, *hcnB*
PA2195	19.40 (5.9)	10.85 (1.69)		hydrogen cyanide synthase, *hcnC*
PA2586			2.15 (0.22)	response regulator, gacA
PA2587	2.51 (0.22)			probable FAD-dependent monooxygenase, *pqsH*
PA3006	2.83 (0.62)	6.08 (2.57)		transcriptional regulator, *psrA*
PA3476	2.07 (0.08)	4.09 (0.43)		autoinducer synthesis protein, *rhlI*
PA3477	5.48 (0.49)	8.19 (2.03)	4.01 (1.47)	transcriptional regulator, *rhlR*
PA3478	28.70 (5.2)	19.20 (5.01)		rhamnosyltransferase chain B, *rhlB*
PA3479	34.50 (4.3)	35.00 (8.13)		rhamnosyltransferase chain A, *rhlA*
PA3724	8.02 (3.39)			elastase, *lasB*
PA4209	4.76 (0.19)	3.11 (0.48)		probable phenazine specific methyltransferase, *phzM*
PA4211	5.73 (1.02)	6.43 (1.64)		probable phenazine biosynthesis protein, *phzC*1
PA4217	3.00 (0.42)			flavin-containing monooxygenase, *phzS*
PA5499	2.36 (0.37)			transcriptional regulator, *np*20
B) Flagella biogenesis and chemotaxis				
PA0173	23.79 (12.3)	31.55 (16.5)	9.11 (4.86)	probable methylesterase
PA0175	15.37 (5.2)	12.36 (2.79)	8.20 (2.04)	probable chemotaxis protein methyltransferase
PA0176	24.54 (4.3)	22.52 (6.24)	16.06 (1.52)	probable chemotaxis transducer
PA0177	22.76 (2.8)	25.60 (10.5)	12.33 (5.79)	probable purine-binding chemotaxis protein
PA0178	17.31 (2.7)	16.33 (3.79)	12.68 (1.92)	probable two-component sensor
PA0179	20.55 (2.9)	35.34 (9.97)	13.66 (2.73)	probable two-component response regulator
PA0180	8.35 (1.20)	8.47 (2.03)	7.43 (1.34)	probable chemotaxis transducer
PA1077	2.62 (0.39)	8.20 (1.16)		flagellar basal-body rod protein, *flgB*
PA1078	2.72 (0.49)	8.33 (1.00)		flagellar basal-body rod protein, *flgC*
PA1079		5.97 (0.40)		flagellar basal-body rod modification protein, *flgD*
PA1080	2.00 (0.00)	6.19 (0.65)		flagellar hook protein, *flgE*
PA1081		5.90 (1.35)		flagellar basal-body rod protein, *flgF*

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
II) Hydrogen peroxide repressed genes				
B) Flagella biogenesis and chemotaxis				
PA1082	2.59 (0.09)	5.42 (1.47)		flagellar basal-body rod protein, *flgG*
PA1083	2.51 (0.09)	5.72 (0.97)		flagellar L-ring protein precursor, *flgH*
PA1084	2.52 (0.26)	4.72 (0.74)		flagellar P-ring protein precursor, *flgI*
PA1085	2.26 (0.15)	3.45 (0.49)		flagellar protein, *flgJ*
PA1086	2.30 (0.00)	5.55 (1.11)		flagellar hook-associated protein 1, *flgK*
PA1087	2.47 (0.14)	6.78 (0.98)	2.39 (0.21)	flagellar hook-associated protein type 3, *flgL*
PA1088	3.05 (0.38)	5.57 (0.37)		hypothetical protein
PA1089	2.27 (0.27)	5.48 (0.49)		conserved hypothetical protein
PA1092	3.09 (0.11)	5.72 (0.97)	2.93 (0.12)	flagellin type B, *fliC*
PA1093	5.31 (0.67)	10.23 (0.9)	4.38 (0.51)	hypothetical protein
PA1094	4.40 (0.62)	10.66 (2.8)	3.75 (0.47)	flagellar capping protein, *fliD*
PA1095	3.00 (0.36)	8.47 (1.02)	2.65 (0.21)	hypothetical protein
PA1098	2.34 (0.21)	3.28 (0.93)		two-component sensor, *fleS*
PA1099	2.74 (0.24)	4.87 (1.63)		two-component response regulator, *fleR*
PA1100	2.67 (0.48)	4.62 (1.74)		flagellar hook-basal body complex protein, *fliE*
PA1101	2.56 (0.23)	4.77 (1.10)		flagellar M-ring protein, *fliF*
PA1102		2.19 (0.23)		flagellar motor switch protein, *fliG*
PA1105		2.27 (0.27)		flagellar protein, *fliJ*
PA1423			4.61 (0.37)	probable chemotaxis transducer
PA1452		3.70 (0.56)		flagellar biosynthesis protein, *flhA*
PA1453	2.43 (0.29)	3.78 (1.10)		flagellar biosynthesis protein, *flhF*
PA1455	2.18 (0.14)	2.90 (0.45)		sigma factor, *fliA*
PA1456	2.31 (0.29)	3.02 (0.54)		two-component response regulator, *cheY*
PA1457	2.65 (0.21)	3.05 (0.38)		chemotaxis protein, *cheZ*
PA1458	2.51 (0.16)	3.75 (0.47)	2.26 (0.15)	probable two-component sensor
PA1459	3.25 (0.00)	4.40 (0.69)	2.51 (0.16)	probable methyltransferase
PA1460	2.55 (0.17)	3.61 (0.14)		probable chemotaxis transmembrane proton channel
PA1461	2.44 (0.37)	3.74 (0.21)		probable chemotaxis protein
PA1464	2.47 (0.14)	2.85 (0.45)		probable purine-binding chemotaxis protein
PA1561	5.58 (0.47)	12.28 (6.1)	2.98 (0.20)	aerotaxis receptor, *aer*
PA1608		3.70 (0.51)	3.06 (0.49)	probable chemotaxis transducer
PA1930	26.09 (2.6)	19.06 (8.01)	16.67 (2.15)	probable chemotaxis transducer
PA2561			2.63 (0.47)	probable chemotaxis transducer
PA2573	28.24 (8.2)	19.11 (5.99)	9.75 (1.36)	probable chemotaxis transducer
PA2652		2.90 (0.45)	2.59 (0.51)	probable chemotaxis transducer
PA2654	2.47 (0.20)	4.53 (0.39)	2.53 (0.43)	probable chemotaxis transducer
PA2788	13.70 (0.4)	23.03 (4.8)	4.10 (1.03)	probable chemotaxis transducer
PA2920	9.01 (1.80)	9.75 (1.40)	5.77 (0.52)	probable chemotaxis transducer
PA3348	2.90 (0.42)	3.57 (0.50)		probable chemotaxis protein methyltransferase
PA3349	3.37 (0.30)	5.08 (0.95)	2.15 (0.12)	probable chemotaxis protein

PAO No.	Average fold change[a]			Protein name[b]
	TB	892	PAO1	
II) Hydrogen peroxide repressed genes				
B) Flagella biogenesis and chemotaxis				
PA3708	2.39 (0.21)	2.86 (0.48)		probable chemotaxis transducer, *wspA*
PA4117	4.29 (0.24)	3.02 (0.54)		probable bacteriophytochrome
PA4307		2.53 (0.39)		chemotactic transducer, *pctC*
PA4309	5.38 (0.35)	10.84 (1.6)	5.33 (1.42)	chemotactic transducer, *pctA*
PA4310	2.78 (0.19)	10.04 (0.6)	2.96 (0.52)	chemotactic transducer, *pctB*
PA4520		2.59 (0.09)	3.05 (0.38)	probable chemotaxis transducer
PA4633	5.77 (0.52)	9.66 (1.93)	4.96 (0.64)	probable chemotaxis transducer
PA4915	13.72 (4.2)	14.82 (5.0)	6.41 (0.66)	probable chemotaxis transducer

[a]: Values are means (SD) of four independent RNA preparations.

[b]: Gene names are indicated in Italics.

Table 7.2. Genes down-regulated from arginine metabolism in 25A12 mutant when grown in LB medium up to late exponential phase[1,2].

PAO No.	Average fold change	Protein name[3]
PA0888	1.60	arginine/ornithine binding protein, *aotJ*
PA0889	1.81	arginine/ornithine transport protein, *aotQ*
PA0890	1.52	arginine/ornithine transport protein, *aotM*
PA0892	1.52	arginine/ornithine transport protein, *aotP*
PA0893	1.45	transcriptional regulator, *argR*
PA0895	1.02	N-succinylglutamate 5-semialdehyde dehydrogenase, *aruC*
PA0896	2.07	arginine/ornithine succinyltransferase, *aruF*
PA0897	1.94	arginine/ornithine succinyltransferase, *aruG*
PA0898	2.34	succinylglutamate 5-semialdehyde dehydrogenase, *aruD*
PA0899	1.59	succinylarginine dihydrolase, *aruB*
PA1544	1.50	transcriptional regulator, *anr*
PA5171	1.42	arginine deiminase, *arcA*
PA5172	1.52	ornithine carbamoyltransferase, *arcB*
PA5173	1.70	carbamate kinase, *arcC*

[1]: No down-regulated genes observed in mutant 45A7 under the same condition.

[2]: No down-regulation of genes from this category were observed when both the mutants were grown in presence of hydrogen peroxide and PMNs.

[3]: Gene names are indicated in italics.

Curriculum vitae

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Publication list:

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