Genetic traits of *P. aeruginosa* morphotypes affecting virulence *in vivo*

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Abstract

The metabolically versatile and ubiquitous *Pseudomonas aeruginosa* is a major opportunistic pathogen for plants, animals and men. It is a leading cause for nosocomial infections, particularly for bronchopneumonia of ventilated patients at intensive care units. *P. aeruginosa* also causes chronic lung infections in individuals with cystic fibrosis (CF), bronchiectasis and chronic obstructive pulmonary disease.

During lung infections the colonizing *P. aeruginosa* clone diversifies into niche specialists and morphotypes, a phenomenon called ‚dissociative behaviour'. In the cystic fibrosis lungs, aerobic planctonic bacteria, microaerophilic mucoid morphotypes, biofilm forming bacteria, autoaggregative bacteria, small colony variants and other morphotypes were found.

In our study we investigated the genomic capacity of *P. aeruginosa* to diversify in morphotype by single-step gene inactivation. The screening of a signature-tagged mini-Tn*5* plasposon library of the cystic fibrosis airway isolate TBCF10839 under different culture and temperature conditions *in vitro* revealed that the transposon insertion in about 0,5 % of the genome led to a change of morphology into eight discernable morphotypes. Half of the 57 targets encode features of primary or secondary metabolism whereby quinolone production was frequently affected. In the other half the transposon had inserted into genes of the functional categories transport, regulation or motility/chemotaxis. Only three of the 57 targets identified in the screen were known from previous studies on genetic reference strain PAO1 to be involved in the variation of morphotype.

To mimic dissociative behaviour of isogenic strains in lungs, pools of 25 colony morphology variants were tested for competitive fitness in an acute murine airway infection model. Seventeen of the 57 mutants either grew better or worse *in vivo* than *in vitro*, respectively. Some of the variants were characterized in more depth by separate infection experiments and bioassays. Formal proof of reversion to wild type phenotype was performed for a significant proportion of targets by complementation *in trans*.

The most common morphotype of self-destructive autolysis did unexpectedly not impair fitness. Metabolic proficiency to utilize the substrates that are abundant in bronchial secretions and to synthesize the major secondary metabolites that exert bactericidal or host immunomodulatory functions, were identified as key determinants of better survival.

Key words: *Pseudomonas aeruginosa*, morphotypes, murine infection

Kurzfassung

Die metabolisch vielseitige und ubiquitär verbreitete bakterielle Spezies *Pseudomonas aeruginosa* gehört zu den bedeutendsten Pathogenen für Pflanzen, Tiere und Mensch. *Pseudomonas aeruginosa* ist einer der Hauptauslöser nosokomialer Infektionen, insbesondere von Infektionen der unteren Atemwege bei beatmeten Intensiv-Patienten. Zudem verursacht diese Spezies chronische Atemwegsinfektionen bei Patienten mit Cystischer Fibrose (CF), Bronchiektasen und chronisch obstruktiven Lungenerkrankungen.

Im Verlauf der Lungeninfektionen spezialisiert sich der kolonisierende Klon auf die verschiedenen sich bietenden (besiedelbaren) „Nischen“ und bildet verschiedene Morphotypen aus (sog. dissoziatives Verhalten). In CF-Lungen findet man so aerobisch planktonisch lebende Bakterien, mikroaerophile mukoide Morphotypen, Biofilm-bildende Bakterien, sog. *small colony variants* und noch andere Morphotypen.

Im Rahmen dieser Arbeit wurde analysiert, inwieweit *Pseudomonas aeruginosa* infolge der Inaktivierung einzelner Gene verschiedene Morphotypen ausbildet. Untersucht wurden dazu die Mutanten einer Mini-Tn*5*-Plasposon-Bibliothek des Stammes TBCF10839. Bei Wachstum bei verschiedenen Temperaturen und Kulturbedingungen wiesen mehrere Mutanten eine veränderte Morphologie auf, wobei insgesamt acht Arten von Morphotypen unterscheidbar waren. Die veränderten Morphotypen ließen sich auf Transposon-Insertionen in 57 verschiedenen Genen zurückführen. Die Hälfte dieser Gene codiert Proteine des Primär- oder Sekundärmetabolismus, darunter häufig Proteine zur Synthese von Chinolonen. Die durch andere Hälfte der mutierten Gene codierten Proteine gehörten in die funktionellen Kategorien Transport, Regulation, Motilität oder Chemotaxis. Lediglich drei dieser 57 Gene waren dabei in früheren Analysen mit dem Referenzstamm PAO1 in Bezug auf Änderung des Morphotyps aufgefallen.

Um das dissiozative Verhalten in der Lunge zu simulieren, wurden jeweils Gruppen von 25 isogenen Mutanten mit veränderter Kolonie-Morphologie mit Hilfe eines Modells zur akuten Infektion von Mäuselungen untersucht. Das Wachstumsverhalten in Konkurrenz zu den übrigen Mutanten (Fitness) unterschied sich in diesem *in vivo* – Experiment bei insgesamt 17 der 57 Mutanten gegenüber vergleichbaren *in vitro* – Analysen. Einige dieser 17 Mutanten wurden u. a. durch separate Infektionsexperimente noch genauer charakterisiert, die Wiederherstellung des Wildtyp-Phänotyps jeweils durch *in trans* - Komplementation des mutierten Gens überprüft.

Unerwarteterweise ergab sich kein Zusammenhang zwischen dem am häufigsten auftretenden veränderten Morphotyp, der Präsenz von autolytischen Bereichen in der Bakterienkolonie, und der beobachteten Fitness.

Als entscheidende Faktoren für erfolgreiches kompetitives Wachstum im selben Habitat (Wachstum in der Lunge) wurden metabolische Fähigkeiten identifiziert, die die effektive Verstoffwechselung von Substraten, die in Bronchialsekreten vorkommen, ermöglichen oder die die Synthese von Sekundärmetaboliten mit bakteriozider oder immunmodulierender Wirkung erlauben.

Schlüsselwörter: *Pseudomonas aeruginosa*, Morphotypen, Maus-Infektion

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I INTRODUCTION

1. Phenotype of the metabolically versatile *P. aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic and polarly flagellated rod which belongs to the bacterial family *Pseudomonadaceae* of the group of γ-proteobacteria (Palleroni, 1986). It is a metabolically versatile bacterium which inhabits terrestrial, aquatic, animal-, human-, and plant-host-associated environments (Hardalo and Edberg, 1997; Ramos, 2004).

The exceptional competence of *P. aeruginosa* to colonize a wide variety of ecological niches is based on its ability to utilize a broad spectrum of organic compounds as food sources and its capability to survive for a long time under extremely harsh conditions where nutrients are limited (Ramos, 2004; Madigan and Martinko, 2005).

With plants, *P. aeruginosa* induce symptoms of soft rot with *Arabidopsis thaliana* and *Letuca sativa* (Rahme *et al*, 1995, 1997). It is a pathogen for *Caenorhabditis elegans* (Mahajan-Miklos *et al*., 1999; Martínez *et al*., 2004), *Drosophila (*D'Argenio *et al*., 2001) and *Galleria mellonella (*Miyata *et al*., 2003).

This pathogen causes a wide range of infections in humans including localized infections such as urinary tract infections, acute ulcerative keratitis, malignant otitis media, peritonitis, acute ventilator associated pneumonia in endotracheal intubated patients, and burn wound infections, as well as chronic localized infections such as chronic destructive lung infections in cystic fibrosis (CF) patients, whose abnormal airway epithelia allows long-term colonization by *P. aeruginosa* (Tümmler *et al*., 1991; Lyczak *et al*., 2002; Pollack, 2000; Bush *et al*., 2006). In addition, patients with severe underlying diseases reducing physical (burned patients, mechanically ventilated patients) and/or immune defence mechanisms (neutropenias, AIDS patients) are at serious risk for evolution of localised infections toward systemic disease, which is associated with dramatically elevated mortality (Ramos, 2004)
Such a high metabolic versatility of *P. aeruginosa* is reflected in its core genome (Stover *et al.,* 2000). The genome of *P. aeruginosa* spans over approximately 6.3 Mb

and comprises 5,570 predicted open reading frames (ORFs) about 90 % of which are highly conserved (available on http://pseudomonas.com) (Wolfgang *et al.,* 2003). Analyses of this genome revealed a high proportion of two-component systems (2.1 % of genomic ORFs) and regulatory genes (7.2 %) to modulate the diverse genetic and biochemical properties of this bacterium to adapt to changing environmental conditions. Many of the genes being responsible for growth and metabolism of various organic compounds enhance the ability of the bacterium to survive under harsh environmental conditions.

Except of the core genome that is common to all strains of a taxon, the genome of *P. aeruginosa* consists of an accessory part that varies within and among clones (Schmidt *et al.,* 1996). The accessory genome represents the flexible gene pool that frequently undergoes acquisition and loss of genetic information and hence plays an important role for the adaptive evolution of this bacterium. The flexible gene pool is made up of elements such as bacteriophages, plasmids, insertion elements, transposons, conjugative transposons, integrons and genomic islands. Genomic islands may also increase the metabolic versatility or adaptability of the bacterium, or promote bacterium-host interaction in terms of symbiosis, commensalism or virulence (Dobrindt *et al*., 2004).
Additionally, genes encoding membrane transporters for nutrients uptake and antibiotic efflux play essential roles during the course of the infection including various iron-uptake systems and a series of resistance nodulation-cell division (RND) efflux transporters (Stover *et al.,* 2000).

Thus, 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.

2. The virulence factors produced by *P. aeruginosa*

There have been many discussions about what exactly constitutes a bacterial virulence factor (Domingue *et al*., 1997). Finlay and Falkow, in their 1997 review (Finlay and Falkow, 1997) discuss the various definitions of microbial pathogenicity and the idea that pathogens can be distinguished from their non-virulent counterparts by the presence of such virulence genes. Many novel virulence factors have been discovered through the use of homology searches like BLAST and FASTA with bacterial genomic sequence data. However, around a third of all ORFs in each genome published so far have unknown function (Weinstock, 2000).

P. aeruginosa possesses and produces a large variety of both cell-associated and excreted virulence factors (Van Delden and Iglewski, 1998). Mainly, these factors suppress host immune responses as well as being involved in the establishment of persistent infections (Cryz *et al*., 1984).

Cell-associated virulence factors include pili, flagella, lipopolysaccharide, a type III secretion system and alginate. The first step in *P. aeruginosa* infections is the adherence to and colonization of host epithelial surfaces. The primary *P. aeruginosa* adhesins for respiratory mucins are the flagella cap protein (Arora *et al*., 1998) and flagellin (Feldman *et al*., 1998; Lillehoj *et al*., 2002). Flagellum driven motility has been reported to enhance the efficiency of surface *P. aeruginosa* colonization by *P. aeruginosa* (O´Toole and Kolter, 1998). However, many *P. aeruginosa* strains isolated from chronically infected CF airways do not produce flagella, that indicates that flagella plays a role only in the early step of infection and longer term maintenance of the organism in this milieu involves other factors (Mahenthiralingam *et al*., 1994).

P. aeruginosa produces several extracellular products that, after the initial step of colonization, can cause extensive tissue damage, bloodstream invasion, and dissemination (Rahme *et al*., 1997; Tang *et al*., 1996).
Two hemolysins, phospholipase C and rhamnolipid, produced by *P. aeruginosa,* may act synergistically to break down lipids and lecithin. Phospholipase C has been shown to induce vascular permeability, organ damage and death in animal models as

well as to contribute to the release of inflammatory mediators (Berk *et al.,* 1987). *P. aeruginosa* produces two lipases, LipA and LipC, which were shown to enhance the induction of inflammatory mediators by phospholipase C (Konig *et al.,* 1996).

Rhamnolipid is a rhamnose-containing glycolipid biosurfactant with detergent-like structure believed to be responsible for dissolving of the lung surfactant phospholipids, making them more accessible to cleavage by phospholipase C (Liu, 1973). The resulting loss of lung surfactant may be responsible for the atelectasis associated with chronic and acute *P. aeruginosa* lung infection. Rhamnolipid also inhibits the mucociliary transport and ciliary function of human respiratory epithelium (Read *et al*., 1992). The rhamnolipids have been suggested to play an important role during the development of microbial communities inside biofilm (Davey *et al*., 2003). Recently, the rhamnolipids have been shown to induce a rapid necrosis of the polymorphonuclear leukocytes (PMNs) in mouse lungs infected with *P. aeruginosa* (Jensen *et al*., 2007).

Proteases are also thought to play a role in the pathogenesis of some *P. aeruginosa* infections (Sokol *et al*., 1979; Morihara and Homma, 1985). Alkaline protease is an important extracellular virulence factor implicated in corneal infections (Howe and Iglewski, 1984), degradation of components of the complement (Hong and Ghebrehiwet, 1992) and hydrolysis of fibrin and fibrinogen (Shibuya *et al*., 1991).
Elastases (LasA and LasB) are metalloproteases that degrade elastin which accounts for a significant part of human lung tissue and is an important component of blood vessels (Galloway, 1991), collagen and inactivate human immunoglobulin G, serum alpha-1, proteinase inhibitor and several complement components (Hamood *et al*., 1996). Both elastases, LasA and LasB, have been found in the sputum of CF patients (Storey *et al.,* 1992, Jaffar-Bandjee *et al.,* 1995). Protease IV is a serine protease implicated in the degradation of complement components as well as fibrinogen, plasmin and plasminogen (Engel *et al.,* 1998a) and plays an important role during corneal infections (Engel *et al.,* 1997; Engel *et al.,* 1998b).
The virulence factor exotoxin A is used by *P. aeruginosa* to ADP-ribosylate eukaryotic elongation factor 2 in the host cells, much as the diphtheria toxin does. Without elongation factor 2, eukaryotic cells cannot synthesize proteins and become necrotic (Plotkowski *et al*., 2002).

Other exoproteins, including exoenzyme S (Sokurenko *et al.,* 2001), exoenzyme T (Nicas and Iglewski, 1984; Barbieri, 2000), exoenzyme Y and exotoxin U (Finck-Barbancon *et al.,* 1998; Hauser *et al.,* 1998; Dacheux *et al.,* 2000; Sato *et al.,* 2003) require close contact with host cells and are secreted via the type III secretion pathway, using a complex secretion and translocation machinery to inject the effector proteins directly into the cytoplasm of target cells (Frank, 1997; Yahr *et al.,* 1997).

One of the extracellular compounds, hydrogen cyanide, has been found at relatively high concentrations in patients with freshly infected burns (Pessi and Haas, 2000). Cyanide is a potent inhibitor of cytochrome c oxidase, the terminal component of the aerobic respiratory chain in many organisms. This secondary metabolite is responsible for rapid paralytic killing of the nematode *Caenorhabditis elegans*, but its role during human infections is unclear (Gallagher and Manoil, 2001).

The blue-green pigmented pyocanin is by far the most extensively studied phenazine produced by *P. aeruginosa* (Britigan *et al.,* 1999; Muller, 2002). It induces apoptosis and inhibits generation of superoxide by neutrophils and inhibits proliferation of lymphocytes (Usher *et al.,* 2002).

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

The ability of *P. aeruginosa* to attach to biotic surfaces and the subsequent differentiation of the microorganisms into biofilm can be considered a major virulence trait in a variety of infections (Tang *et al*., 1996; Watnick and Kolter, 2000). Exopolysaccharides and alginate play an essential role in the formation of biofilms. Alginate producing *P. aeruginosa* strains were proven to be more resistant to antibiotics and disinfectants (Govan and Deretic, 1996). Due to its major role in the control of extracellular virulence factor production, the quorum-sensing circuit of *P. aeruginosa* could be also considered a virulence determinant (Van Delden, 2001).

3. Biofilm formation by *P. aeruginosa*

Bacterial biofilms consist of surface-attached organisms that live in highly differentiated communities inside an extracellular matrix composed of secreted polysaccharides, nucleic acids and proteins (Sutherland, 2001; Costerton *et al*., 1995; Kolter and Greenberg, 2006). Bacteria growing in bioflms possess characteristics distinct from their free-floating or swimming (planktonic) counterparts. Bacterial biofilms are resistant to antimicrobial treatments (Costerton *et al*., 1999; O'Toole and Kolter, 1998; Sutherland, 2001) and to the immune defence of hosts (Costerton *et al*., 1995, 1999). Biofilm formation by *P. aeruginosa* occurs in discrete steps: surface attachment and multiplication; microcolony formation; and differentiation into mature, structured, antibiotic-resistant communities (Figure I-1).

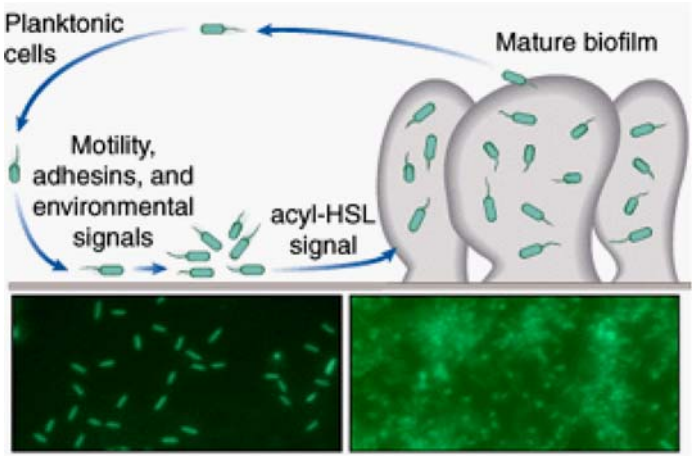

Figure I-1. Biofilm formation by *P. aeruginosa*. “One for all and all for one” (Kolter and Losick, 1998).

The biofilms are not simply random clusters of cells. It has been suggested that there may be some specialization within biofilms, analogous to how caste development in social insects allows individuals to specialize in certain behaviors. In *P. aeruginosa* biofilms, several phenotypically different cell variants that exhibited different behaviors have been isolated (Boles *et al*., 2004), in particular, a wrinkly variant that showed faster biofilm development, and greater resistance to stress, and a mini variant that showed greater dispersal from the biofilm (West *et al*., 2007).

About 85 % of *P. aeruginosa* strains isolated from the lungs of patients, especially with advanced stages of disease have mucoid colony morphology. In contrast, only 1 % of strains isolated from other sites of infection are mucoid (Doggett *et al*., 1966; Fick *et al*., 1992; Govan and Deretic, 1996; Hoiby *et al*., 2001). This mucoid

phenotype is indicative of the overproduction of the extracellular polysaccharides (EPS) alginate, an *O*-acetylated linear polymer of D-mannuronate and L-guluronate residues (Evans and Linker, 1973). Infection with alginate-producing *P. aeruginosa* in CF patients has been associated with an overactive immune response and a poor clinical condition, suggesting that alginate production is a virulence factor (Hoiby, 1974; Baltimore *et al*., 1989; Pedersen *et al*., 1992). Animal studies support the view that alginate production impedes host immune clearance, contributes to tissue damage and favours survival in the lung (Boucher *et al*., 1997; Yu *et al*., 1998; Song *et al*., 2003).

The cell-to-cell communication is very important in the bacterial world. Bacteria make a continuous exchange by various molecules which help to coordinate their behavior. The biological fitness in this content will be dependent from other cells and their exudates altering the phenotype and as consequence the virulence of the pathogen. The change in behavior of bacteria to form the biofilm is triggered by many factors, including quorum-sensing. The following chapters will describe the mechanism of quorum sensing and its importance for *P. aeruginosa*.

4. Cell-to-cell communication by *P. aeruginosa*

4.1. Role of quorum-sensing in *P. aeruginosa*

During growth the bacteria secrete the specific signalling molecules, which accumulate in the surrounding environment as the population density increases until a critical threshold concentration is reached, which then triggers expression of certain sets of genes. This type of cell-to-cell communication was termed “quorum-sensing” in order to emphasize the fact that a sufficient number of bacteria, the bacterial “quorum”, is needed to induce or repress expression of target genes (Fuqua *et al.*, 1994; Van Delden and Iglewski, 1998; Williams *et al*., 2007). In the case of Gram-negative bacteria the signalling molecules utilized by quorum-sensing systems are often acylated homoserine lactones (AHL) (Winans, 2002). One of the most extensively studied AHL-dependent cell-to-cell communication systems is *P. aeruginosa* (de Kievit and Iglewski, 2000; Greenberg, 2003).

The multiple extracellular virulence factors including elastases, alkaline protease, exoenzyme S, neuraminidase, hemolysin, lectins, pyocyanin, rhamnolipids and hydrogen cyanide are produced and regulated in a cell density dependent manner under the control of quorum-sensing circuits composed of the LasRI and the RhlI quorum-sensing systems. The *las* system utilizes N-(3-oxododecanoyl)-L-homoserinelactone (3-oxo-C12 HSL) whereas the *rhl* system functions by means of N-butanoylhomoserine lactone as signal molecules (Latifi *et al*., 1996; Pesci and Iglewski, 1997; Pesci *et al*., 1997). The two regulatory circuits act in tandem to control the expression of a number of *P. aeruginosa* virulence factors (Van Delden and Iglewski, 1998). All these extracellular virulence factors are crucial for the competence of *P. aeruginosa* to establish and maintain the infection (Passador *et al.,* 1993; Van Delden and Iglewski, 1998; Hasset *et al.,* 1999; Pessi and Haas, 2000; Winzer *et al.,* 2000). Mutants defective in quorum-sensing were substantially less pathogenic than their parental strains in the burned mouse model, the mouse agar bead model or in the neonatal mouse model of pneumonia (Tang *et al.,* 1996; Rumbaugh *et al.,* 1999; Wu *et al.,* 2000).

4.2. PQS as an important signaling molecule for the cell-to-cell communicaion

An additional autoinducer has been demonstrated to be involved in quorum-sensing in *P. aeruginosa*. This signal is noteworthy because it is not of the homoserine lactone class and belongs to the family of 4-hydroxy-2-alkylquinolines (HAQs) (Deziel *et al*., 2004). Rather, it is 2-heptyl-3-hydroxy-4-quinolone (denoted PQS for *P*seudomonas quinolone signal) released into the extracellular milieu, the synthesis and bioactivity of which has been reported to be mediated via the *las* and *rhl* systems respectively (Pesci *et al.*, 1999). The direct precursor of PQS, 4-hydroxy-2-heptylquinoline (HHQ), is first released from and then taken up again by the cells, before it is eventually converted into PQS by the action of PqsH, whose expression is controlled by the *las* system (Gallagher *et al.,* 2002; Déziel *et al.,* 2004). While the production of PQS seems to be enhanced by the *las* system – although it can be produced in the absence of *lasR* (Diggle *et al*., 2003) – exogenous PQS induces the expression of *lasB* encoding for the major virulence factor LasB elastase and acts by upregulating the *rhl* QS system. On the contrary, the loss of PQS biosynthesis has been shown to result in the abolition of primarily *rhl*-dependent QS phenotypes despite continued C4-homoserine lactone biosynthesis (Diggle *et al*., 2003)

The global transcriptional profile of *P. aeruginosa* in response to PQS revealed a marked upregulation of genes belonging to the tightly interdependent functional groups of the iron acquisition and the oxidative stress response. The most of the differentially regulated genes, as well as the induction of *rhlR*, could be traced back to an iron-chelating effect of PQS (Bredenbruch *et al*., 2006).

PQS is produced in the lungs of cystic fibrosis patients infected with *P. aeruginosa* (Collier *et al*., 2002) and is required for virulence in nematodes, plants, and mice (Cao *et al*., 2001; Gallagher *et al*., 2002). PQS also induces apoptosis and decreases viability in eukaryotic cells (Calfee *et al*., 2005).

The PQS acts as a coinducer for a LysR-type transcriptional activator called PqsR (also referred to as MvfR) (Cao *et al*., 2001; Wade *et al*., 2005). In the presence of PQS, PqsR interacts with the promoter region of the *pqsABCDE* operon, which is part

of the PQS synthetic gene cluster (D'Argenio *et al*., 2002; Gallagher *et al*., 2002), thereby creating a positive feedback loop for PQS production (Wade *et al*., 2005).

Along with PQS, *P. aeruginosa* produces at least 55 other quinolone compounds, many of which were identified because of their antibiotic activities (Lepine *et al*., 2004). Studies of the synthesis of these compounds have shown that 4-quinolones are derived via the condensation of anthranilate and a fatty acid. By Farrow and Pesci (2007) was shown that anthranilate is a precursor for PQS and that the addition of an anthranilate analog to a *P. aeruginosa* culture would disrupt PQS production (Calfee *et al*., 2001). One of the anthranilate synthases happens to be encoded by genes within the PQS synthetic region. Adjacent to the *pqsABCDE* operon is the *phnAB* operon, which is positively controlled by PqsR and encodes the large and small subunits, respectively, of an anthranilate synthase. In addition, the three genes (*kynA*, *kynB* and *kynU*) of the anthranilate branch of the kynurenine (*kyn*) pathway, which converts tryptophan to anthranilate, are present in *P. aeruginosa* (Farrow and Pesci, 2007).

5. Colony morphology variations of *P. aeruginosa*

Adaptation to novel environments usually entails morphological changes. Morphological variation is the most visible component of biodiversity, and morphological changes of organisms in a novel environment are the most visible indicator of organismal adaptation (Smith, 1998).

P. aeruginosa isolates from acute and chronic airway infections of the human host display high phenotypic diversity (Zierdt and Schmidt, 1964; Tümmler, 2006). Once CF patients become colonized by *P. aeruginosa*, there is a subsequent gradual deterioration in lung function, which determines the course and prognosis in most CF patients. Despite the fact that chronically infected CF patients harbour only one or few *P. aeruginosa* genotypes (Breitenstein *et al*., 1997), there is a significant phenotypic variation in *P. aeruginosa* isolates from the CF lung, known as dissociative behaviour (Zierdt and Schmidt, 1964). Spatial compartmentalization in the supply of oxygen and nutrients and in the exposure to host inflammatory responses is associated with the diversification of *P. aeruginosa* into morphotypes and the establishment of niche specialists (Häussler, 2004; Tümmler, 2006; Oliver *et al*., 2000 Palmer *et al*., 2005; Worlitzsch *et al*., 2002; Yoon *et al*., 2002; Alvarez-Ortega and Harwood, 2007).

The appearance of multiple morphotypes may be facilitated by “hypermutable” strains of *P. aeruginosa*, which have been isolated at high frequency from CF patients (Oliver *et al*., 2000).

The morphological diversity of *P. aeruginosa* recovered from the respiratory tract material of CF patients is well known (Zierdt and Schmidt, 1964) and has been suggested to be almost pathognomonic for the chronically infected CF lung (Govan and Deretic, 1996). Those phenotypic variants that can face the challenges of the heterogeneous and changing habitat imposed by antibiotic therapies and the host immune system dominate in clonal bacterial populations (Oliver *et al*., 2000).

In the context of chronic *P. aeruginosa* CF lung infection, attention has long focused on the appearance of the most common alginate-overproducing mucoid *P. aeruginosa* phenotype (Govan and Deretic, 1996; Mathee *et al*., 1999; Merighi *et al*., 2007; Worlitzsch *et al*., 2002; Yoon *et al*., 2002). Other phenotypes, however, including small colony variants, (SCVs) (Häussler, 2004; Häussler *et al*., 1999, 2003;

Drenkard *et al*., 2002), colourless variants (De Vos *et al*., 2001) or colonies with visible autolysis (D'Argenio *et al*., 2002) or autoaggregative behaviour (Häussler *et al*., 2003; D'Argenio *et al*., 2002 ; von Götz *et al*., 2004) were described (Figure I-2).

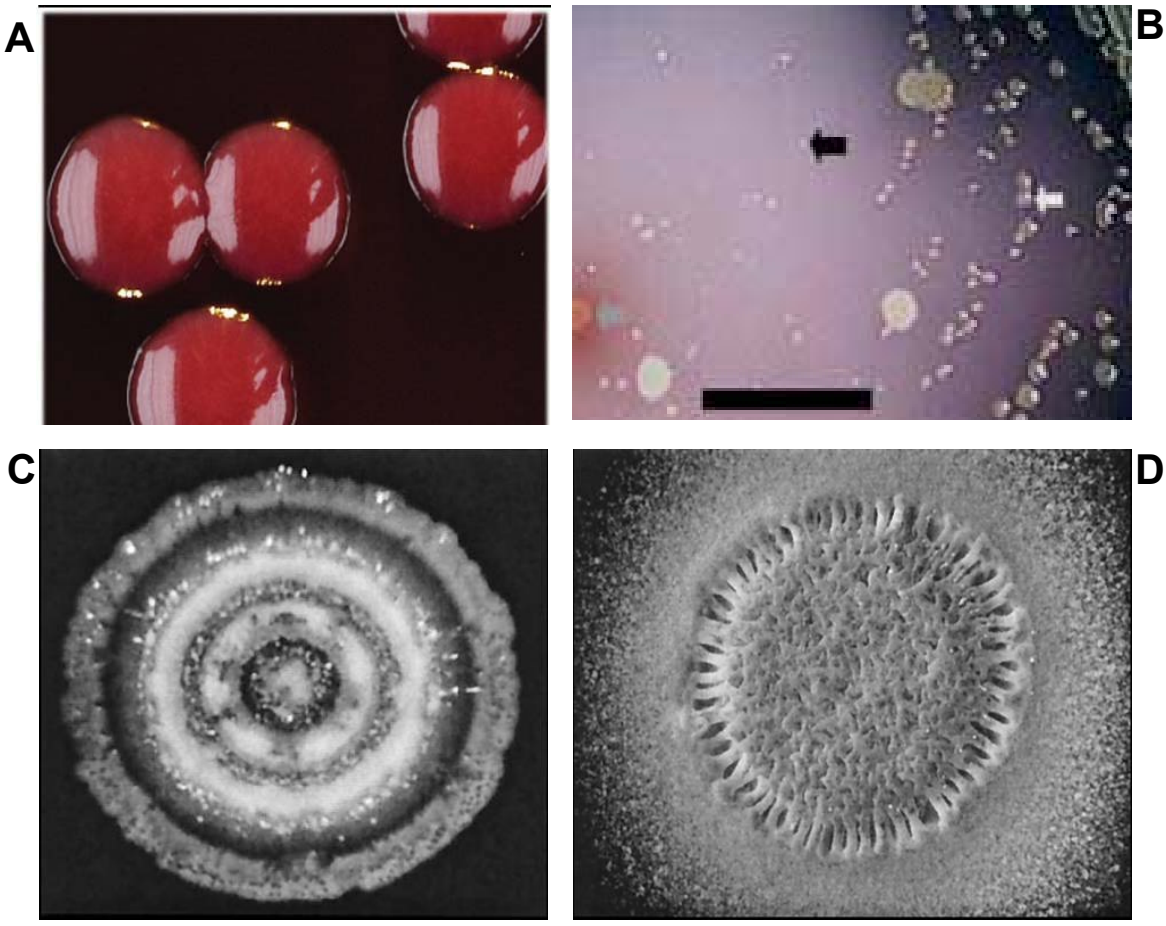

Figure I-2. Colony morphology variations of *P.aeruginosa:* mucoid colony morphology, A; SCVs, B; colony with visible autolysis, C; autoagregative colony morphology, D.

5.1. Fitness of colony morphology variants of *P. aeruginosa*

Growth and morphology are easy-to-follow phenotypic traits of organismal adaptation that may or may not be genetically fixed by mutation and selection. The biological fitness of biological organisms is not necessarily equal, but depends on the environment in which the organisms live (Smith, 1998; Seifert and Dirita, 2006).

Mucoid *P. aeruginosa* variants preferentially grow in biofilms under microaerophilic or anaerobic conditions (Worlitzsch *et al*., 2002; Yoon *et al*., 2002) and as shown above

the expression of polysaccharides confers increased resistance to the host immune response and results in chronic pulmonary infection and poor prognosis for the patient. Apart from the mucoid *P. aeruginosa* phenotype, it has been recognized for many years that dwarf colonies can be isolated from the chronically infected respiratory tract of CF patients (Zierdt and Schmidt, 1964). The recovery of dwarf small-colony variants (SCVs) of *P. aeruginosa* could be correlated with parameters revealing poor lung function and the use of inhaled antibiotics (Häussler *et al*., 1999). Among the heterogeneous group of clinical SCV isolates from different patients, a subgroup was identified exhibiting hyperpiliation and increased twitching motility as well as increased fitness under stationary growth conditions, better biofilm-forming capabilities, and a high adherence to a pneumocytic cell line in comparison with the clonal fast-growing wild types (Häussler *et al.*, 2003), better biofilm-forming capabilities and high adherence to airway epithelial cells (Dacheux *et al*., 1999; Häussler *et al*., 1999).

Even autolysis, which might seem unambiguously detrimental to an unicellular organism, is an adaptive behaviour of *P. aeruginosa* mediated by overproduction of the quinolone PQS being an extracellular signal increasing the stringent response and the formation of protective biofilm by released DNA after the cells lysis (Häussler, 2004; D'Argenio *et al*., 2002; Yang *et al*., 2007; Allesen-Holm *et al*., 2006). Moreover, c-di-GMP levels regulate the differentiation of *P. aeruginosa* populations into macroscopic cell aggregates and planctonic cells (Klebensberger *et al*., 2007).

5.2. Autolysis and autoagregation colony morphology of *P. aeruginosa*

The plaque-like clearings and the metallic, iridescent sheen associated with the lysed areas are both properties noted in early descriptions for *P. aeruginosa* isolates (Berk, 1963, 1965; Hadley, 1924; Holloway, 1969). In many cases, visible autolysis of *P. aeruginosa* is induced by the bacteriophage lysogenic process. The genome of *P. aeruginosa* strain PAO1 contains a filamentous bacteriophage (Stover *et al.*, 2000) whose genes are highly upregulated during biofilm growth (Whiteley *et al*., 2001). In addition, inserted between the two anthranilate synthase subunit genes, *trpE* and *trpG*, is a gene cluster encoding bacteriocins believed to be evolved phage tails (Nakayama *et al.*, 2000) and whose induction shares elements with the SOS

response (Matsui *et al*., 1993). Mutation in gene *vfr* was also associated with visible autolysis. It could be an analogous to the situation in *E. coli* where mutation of *crp* shifts the balance from lysogeny to lysis (Hong *et al*., 1971), given that *vfr* and *crp* are homologs (Beatson *et al*., 2002, West *et al*., 1994), and suggests an alternate pathway for autolysis in *P. aeruginosa.*

One gene with visible autolysis was described after the screening of PAO1 mutant's library, where out of 6,000 transposon insertion mutants the *pqsL* mutant formed colonies that lysed at their centers (D'Argenio *et al.*, 2002). The phenotype was linked with phenazine and quinolone pathways. Authors demonstrated the positive correlation between autolysis and increased production of PQS by *pqsL* mutant. The PQS overproduction by autolysed mutants can be activated by starvation either physiologically (Van Delden *et al*., 2001) or by mutation (Van Delden *et al*., 1998). This situation may underlie the selection pressure for compensatory mutations in certain quorum-sensing mutants (D'Argenio *et al.*, 2002).

A key feature of mature *P. aeruginosa* biofilms is the presence of an extracellular matrix. Matrix production occurs at a late stage in biofilm development, when cells display a high degree of autoaggregation (D'Argenio *et al*., 2002; Sauer *et al.*, 2002). The ability to autoaggregate leads to several macroscopic phenotypes; among them are the production of pellicles at the air-liquid interface of standing liquid cultures and the production of highly structured colonies on agar plates. Different strains of *P. aeruginosa* display variability in the abilities to form pellicles under different culture conditions and to show different colony morphologies. This phenotypic diversity most likely results from genetic differences among isolates (Deziel *et al*., 2001).

D'Argenio *et al.* (2002) showed that the autoaggregative mutant phenotype of *P. aeruginosa* strain PAO1 can result from mutations in *wspF* (aCheB-like methylesterase; PA3703), PA2933 (an efflux proteinof the major facilitator superfamily), or PA0171 and PA1121 (two genes of unknown function). Similar mutations in *Pseudomonas fluorescens* and *Salmonella enterica* serovar *Typhimurium* resulted in the identification of a cellulose-like polymer component of the matrix in those species (Romling *et al*., 2000; Spiers *et al*., 2002; Zogaj *et al*., 2001). A mutation of one of the EPS loci, the *psl* locus (PA2231 to PA2245), was contributed with formation of the autoaggregative colonies of *P. aeruginosa* (Kirisits

et al., 2005). The polysaccharide synthesis locus (*psl*) was predicted to encode an exopolysaccharide, which is critical for biofilm formation. The *psl* gene products synthesize an EPS contained mainly of galactose and mannose, as well as glucose and trace amount of rhamnose, xylose and N-Acetylglucosamine (GlcNAc) (Ma *et al*., 2007).

Recently, the formation of cell aggregates in *P. aeruginosa* and other bacteria has been shown to involve cyclic-di-guanosine monophosphate (c-di-GMP) signalling (Romling *et al*., 2005; Jenal and Malone, 2006). This intracellular second messenger molecule was originally found in *Gluconacetobacter xylinus* where it acts as an allosteric regulator of cellulose synthase (Ross *et al*., 1991). C-di-GMP biosynthesis from 2 GTP is catalysed by diguanylate cyclases containing a characteristic GGDEF-domain as the active center (Chan *et al*., 2004; Ryjenkov *et al*., 2005). The hydrolysis of c-di-GMP is catalysed by specific phosphodiesterases containing either an EAL or a HD-GYP domain (Christen *et al*., 2005; Ryan *et al*., 2006). *P. aeruginosa* harbours 17 genes with a GGDEF-domain, 6 genes with an EAL domain, and 14 genes containing both domains. A function in cell aggregation or biofilm formation has been demonstrated for several of them (D'Argenio *et al*., 2002; Drenkard and Ausubel, 2002; Hickman *et al*., 2005; Hoffman *et al*., 2005).

By Klebensberger *et al.* (2007) was demonstrated that during growth of *P. aeruginosa* strain PAO1 with the toxic detergent SDS, a part of the population actively formed macroscopic cell aggregates. The SDS-induced aggregation involved c-di-GMP signalling with the *psl* operon as a possible target. Cell aggregation could in this case serve as a pre-adaptive strategy ensuring survival and growth of *P. aeruginosa* populations in environments with multiple toxic chemicals (Klebensberger *et al.,* 2007).

Recently, the PelD protein of *P. aeruginosa* was shown as a novel c-di-GMP receptor that mediates c-di-GMP regulation of PEL polysaccharide biosynthesis (Lee *et al*., 2007). Analysis of PelD orthologues identified a number of conserved residues that are required for c-di-GMP binding as well as synthesis of the PEL polysaccharide. The combination of a c-di-GMP binding site with a variety of output signalling motifs within one protein domain provides an explanation for the specificity for different cellular responses to this regulatory dinucleotide.

6. Studying host-pathogen interaction of *P. aeruginosa*

In vitro assays contribute greatly to our understanding of bacterial pathogenesis, but they frequently cannot replicate the complex environment encountered by pathogens during infection. The information gained from such studies is therefore limited. *In vivo* models, on the other hand, can be difficult to use, and this has to some extent diminished the incentive to perform studies in living animals.

6.1. Pathogenicity of *P. aeruginosa* in murine infection model

Understanding the complex interplay between pathogen and host will help us determine the biological foundation of pathogenicity. *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). Since the establishment of the first animal model of chronic *P. aeruginosa* lung infection in rats by Cash *et al.* in 1979, several animal models of acute and chronic lung infection have been described including, 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). Many of these models required that *P. aeruginosa* was embedded in an artificial biofilm (e.g., agar, agarose, or seaweed alginate) to prevent mechanical clearing (Johansen, 1993). Furthermore, these models led to chronic infections of the conducting airways in mice, due to the size of the beads mechanically blocking the bronchi (Wu *et al*., 2000).

In the reported animal experiments 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; Coleman *et al*., 2003; Hoffmann *et al*., 2005).

Since the first CF mouse model was generated by Snouwaert *et al.* (1992) and Clarke *et al.* (1992), several groups have tried to use different variants of transgenic CF mice for the study of *P. aeruginosa* lung infection (Davidson *et al*., 1995; Coleman *et al*., 2003).

6.2. Identification of novel virulence associated genes

Several recently developed techniques permit *in vivo* examination of many genes simultaneously. Most of these methods fall into two broad classes: *in vivo* expression technology (IVIT) and signature-tagged mutagenesis (STM) (Hensel *et al.*, 1995). *In vivo* expression technology is a promoter-trap strategy designed to identify genes whose expression is induced in a specific environment, typically that encountered in a host (Mahan *et al.*, 1993; Mahan *et al.*, 1995).

Signature-tagged mutagenesis uses comparative hybridization to isolate mutants unable to survive specified environmental conditions and has been used to identify genes critical for survival in the host. It 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 the infection (Hensel *et al.*, 1995). STM is also used successfully in identification of factors involved in virulence/colonization in many pathogenic bacteria, for example *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), and *Mycobacterium tuberculosis* (Camacho *et al.*, 1999). 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.

7. The phenotype of *P. aeruginosa* TBCF10839

P. aeruginosa TBCF10839 isolate is a highly virulent strain of *P. aeruginosa,* which belongs to a major clone in the *P. aeruginosa* population (Wiehlmann *et al*., 2007). TBCF10839 was isolated in 1983 from the sputum of a CF patient who had suffered from an acute and chronic infection with *P. aeruginosa.* The patient was severely ill and this *P. aeruginosa* strain had been eradicated from his airways only by high-dose antipseudomonal chemotherapy (Tümmler, 1987).

TBCF10839 was the most virulent isolate from clone TB isolates that were frequently identified in CF patients and burn patients at the Medizinische Hochschule Hannover in the 1980s (Tümmler *et al*., 1991; Kiewitz and Tümmler, 2000).

Phagocytosis assays with freshly isolated polymorphonuclear neutrophils (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). Figure I-3 shows the survival of TBCF10839 in PMNs after 30 and 60 min incubation by electron microscopic analyzes. Proliferation of TB inside the phagolysosomes starts after 60 min incubation with PMNs.

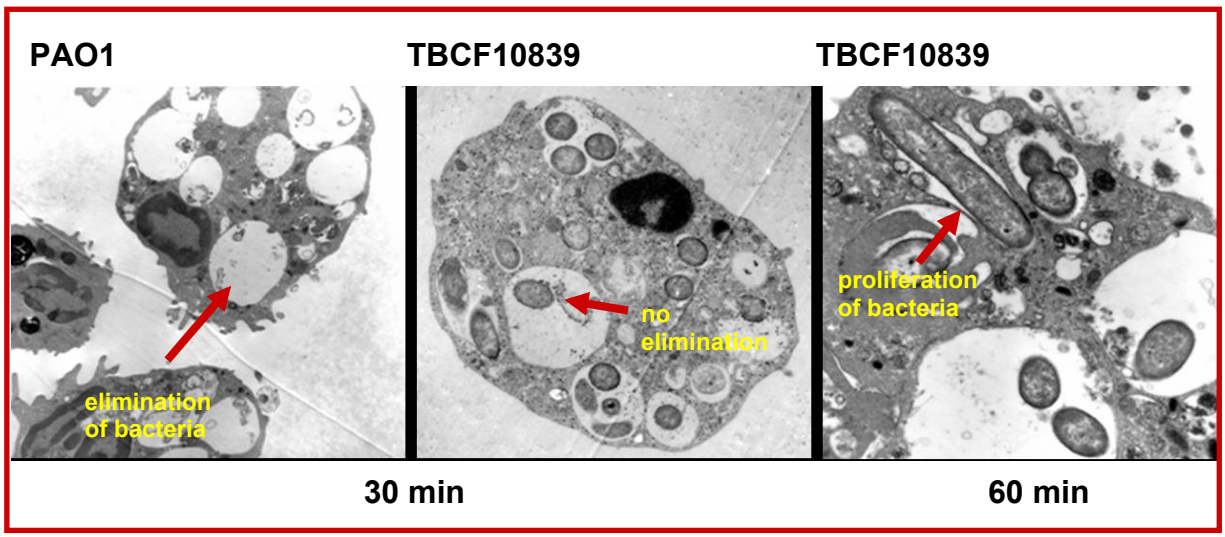

Figure I-3. Electron micrograph of *P. aeruginosa* PAO1 and TBCF10839 incubated with PMNs isolated from human peripheral blood. Bacteria were added at a multiplicity of infection (MOI) 20 and incubated at 37 °C for 30 and 60 min.

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

Being a strong producer of virulence effector proteins and of siderophores, quinolones and phenazines, it is pathogenic for *Drosophila melanogaster* and *Caenorhabditis elegans* and causes substantial airway pathology in mice and rats after intratracheal instillation. TBCF10839 is more virulent than the genetic reference strain PAO1 in infection models and can colonize naive murine airways (Wiehlmann *et al*., 2007).

It is part of a contagious cluster that had caused numerous outbreaks at ICUs and the ward for burn wounds at the authors' institution and had spread among patients of the local CF clinic by nosocomial acquisition (Tümmler *et al*., 1991). Transcriptome (Salunkhe *et al*., 2005) and proteome analyses (Arevalo-Ferro *et al*., 2004) indicated that TBCF10839 orchestrates many more metabolic and signalling pathways upon exposure to inanimate and animate stressors than the sequenced reference strain PAO1 (Stover *et al*., 2000).

8. Objectives of the present work

Airway infections with *P. aeruginosa* are major determinants of morbidity and mortality for ventilated patients at ICU (Diaz *et al*., 2007) and individuals with CF (Lyczak *et al*., 2002; Bush *et al*., 2006), but the time scales of adaptation are different. *P. aeruginosa* rapidly diversifies within a few days in the airways of intubated patients in traits of virulence and antimicrobial resistance. During the infection, *P. aeruginosa* modifies its cell envelope to produce a different spectrum of exopolysaccharides and exo-products and initiates mechanisms to evade the immune response of the host. All the virulence factors presumably make unique and overlapping contribution to bacterial fitness and adaptation during an infection.

In the CF lung, the colonizing *P. aeruginosa* clone will diversify in morphotype and lifestyle concurrently with airway remodelling and dedifferentiation and sequentially accumulates mutations over a period of months to decades (Tummler, 2006). High frequency of phenotype switching is often the result of adaptive genetic diversification resulting in increased chances of bacterial survival in their niche.
Based on our knowledge that *P. aeruginosa* modifies its own morphotype during the lung infection, the objectives for this work were: first, to identify genes that are responsible for divergent morphotypes of *P. aeruginosa* and second, to answer how the changes in colony morphology affect fitness and virulence of the pathogen *in vivo*.

An existing signature tagged transposon mutant library (Wiehlmann *et al.*, 2002) of a clinical *P. aeruginosa* TBCF10839 isolate was screened under different culture and temperature conditions to reveal the mutants with unusual morphotypes distinct from the wild type. The transposon inactivated genes were identified either by Y-linker methodology or plasmid rescue. As a model for the differentiation and evolution in the CF lung, pools of the isogenic transposon colony morphology variants were tested in acute murine airway infections. Mutants with higher or lower fitness were complemented for verification of their phenotypic characteristics.
Finally, the phenotype of the most striking isogenic colony morphology variants was described after testing with the various bioassays.

II Materials and methods

1. Materials

1.1. Equipment and consumables

1.1.1. Equipment

Digital Camera 7.1 Megapixel	Olympus
Centrifuge 5415D / 5417R	Eppendorf
Sorvall - Centrifuge RC5B Plus	DuPont
Rotors: GS-3 (SLA3000)	
GSA (SLA1500)	
SS34	
Tabletop centrifuge Hettich universal	Hettich
Thermomixer Comfort	Eppendorf
Ultracentrifuge (Rotor: SW 40 Ti)	Beckman
Glass plates (200 x 250 mm & 1.5 mm spacer)	Amersham Biosciences
DALT vertical electrophoresis system	Amersham Biosciences
Bio-Rad GenePulser	BioRad
Hybridization oven 400 HY	Bachofer
Incubator	Heraeus
Minifold I Vacuum blotter	Schleicher and Schuell
pH-Meter 761	Calimatic Knich
Spectrophotometer U3000	Hitachi
Thermocycler	Landgraf
Thermomixer	Eppendorf
UV- Transilluminator	Bachofer
UV Stratalinker 1800	Stratagene
Vacuum concentrator	Bachofer
Voltage supply power pack 300	Bio-Rad
ImageScanner™	Amersham Biosciences
Labofuge I / Megafuge	Heraeus

Sonifier	250 Branson
Water bath Model G76	New Brunswick Scientific
X-ray processor XP 505	M3

1.1.2. Consumables

Electroporation cuvettes 1 mm, 2 mm	BioRad
Eppendorf tubes (0.5 mL, 1.5 mL, 2 mL)	Sarstedt
Filter Celluloseester HA 0.45 µM pore size	Millipore
Filter paper GB003	Schleicher and Schuell
Hybond N+ Nylon Membrane	Amersham Biosciences
Glass Pasteur pipettes	Sarstedt
Petri dishes 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 X-Omat AR	Kodak, AGFA

1.2. Chemicals and enzymes

Agar and agarose	Gibco BRL
Anti-digoxigenin AP Blocking Reagent Klenow Polymerase DNA DIG Labeling and Detection Kit	Roche
CDP Star	Tropix
Ethidium bromide Gentamicin Ampicillin Tetracycline Carbenicilin Chelex-100 (iminodiacetic acid)	Sigma

Congo red	
2,6 - dichlorophenol indophenol (DCPIP)	
Phenazine methosulfate	
Ethylamine	
KCN	
FAD	
L-malate	
Phenol TE buffered (pH 7.5 and pH 5.5) aqueous (pH 8)	Fluka
Formaldehyde 35 %	Merck
Hydrogen peroxide (30 %)	
Oligodeoxynucleotides, Primers	MWG-Biotech
DNA-ladder standards	New England Biolabs
Restriction enzymes + buffer system	
T4-DNA-Ligase	
Antarctic phosphatase + buffer system	
Qiaprep Spin kit	Qiagen
Mini Prep plasmid kit	
QIAquick Gel Extraction Kit	
RNeasy Kit	
RNase A	
Acrylamide solution (Rotiphorese-Gel 40)	Roth
Phenol (Rotiphenol)	
Chelex-100 (iminodiacetic acid)	
HEPES	
Gentamicin	Serva
Bromophenol blue, MOPS buffer	Invitrogen
Nitrocellulose membrane	Schleicher & Schuell
FailSafeTM PCR System + buffer system	Epicentre® Biotechnologies
Fast-link ligase kit	
GoldStar polymerase + buffer system	Eurogentec
InviTaq polymerase + buffer system	InViTek
Revert aidTM H Minus cDNA Synthesis Kit	Fermentas

1.3. Media and solutions

1.3.1. Media

LB Medium:	Peptone	15 g/L
	Yeast Extract	5 g/L
	NaCl	10g/L (0.17 M)
LB-Gm:	LB medium with 25 µg/mL Gentamicin	
LB-Tet:	LB medium with 50 µg/mL or 100 g/mL Tetracycline	
LB-Ap:	LB medium with 100 or 200 µg/mL Ampicillin	
LB-Cr:	LB medium with 200 µg/mL Carbenicillin	
LB- Agar:	LB medium was solidified by adding 15 g/L agar and autoclaved.	
M9- Medium (10X):	Na_2HPO_4	68.14 g/L (0.48 M)
	KH_2PO_4	30 g/L (0.22 M)
	NaCl	5 g/L (85 mM)
	NH_4Cl	10 g/L (0.18 M)
M9- Agar:	The M9 medium (10x) and water agar (15 g/L water) were autoclaved separately. Different carbon sources: casein (0.75 % w/v), ethanol (25 mM, 86 mM), glucose (25 mM) were dissolved in a 50 mL of M9 (10x) medium and subsequently added to the 450 mL of melted water agar.	
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)
SOC:	SOB + Glucose, 3.6 g/L (20 mM)	
ABC minimal medium:		
A:	$(NH_4)_2SO_4$	20 g/L (0.15 M)
	$Na_2HPO_4 \cdot 2H_2O$	60 g/L (0.33 M)

	KH_2PO_4	30 g/L (0.22 M)
	NaCl	29 g/L (0.5 M)
B:	$MgCl_2 \cdot 6H_2O$	0.4 g/L (2 mM)
	$CaCl_2 \cdot 2H_2O$	0.014 g/L (0.1 mM)
	$FeCl_3 \cdot 6H_2O$	0.008 g/L (0.003 mM)
C:	Sodium citrate	10 mM
	pH	5.5

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

Columbia blood agar (Becton Dickinson): This complex agar, which is used frequently in microbiology, was exploited for the analysis of hemolytic activity of the investigated *P. aeruginosa* strains.

King's A medium:	Peptone	20 g/L
	KOH	20 g/L (0.3 M)
	H_2SO_4	5.5 mL
	$MgCl_2$ x $6H_2O$	3.3 g/L (16mM)
	Glycerol	1% (w/V)
	pH	7.2
GYT medium:	Glycerol	10% (v/v)
	Yeast extract	0.125% (v/v)
	Tryptone	0.25% (v/v)
	Storage condition:	4 °C

1.3.2. Solutions

TBE-Buffer (10X):	Tris	108 g/L (0.9 M)
	Boric Acid	55 g/L (0.9 M)
	EDTA	7.7 g/L (0.02 M)
	pH	8.3-8.5
Loading Buffer (6X):	Ficoll 400	15 % (v/v)
	Bromophenol Blue	0.25 % (w/v)
	Xylene cyanol	0.25 % (w/v)
	EDTA	146 g/L (0.5 M)
	pH	8.0

PBS (10X):	NaCl	80 g/L (1.37 M)
	KCl	2 g/L (27 mM)
	$Na_2HPO_4x7H_2O$	11.5 g/L (4.3 mM)
	KH_2PO_4	2 g/L (1.4 mM)
	pH	7.3
TB Buffer:	PIPES	3 g/L (10 mM)
	$CaCl_2$	1.6 g/L (15 mM)
	KCl	18.6 g/L (250 mM)
	Sterilized by filtration and stored at 4 ^{o}C.	
TE Buffer:	Tris-HCl	1.2 g/L (10 mM)
	EDTA	0.38 g/L (1 mM)
	pH	8.0
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)
Lysis Buffer:	Tris-acetate	4.84 g/L (40 mM)
	Sodium acetate	2.72 g/L (20 mM)
	EDTA	0.38 g/L (1 mM)
	SDS	1 % (w/v)
	pH	7.8
Blot buffer:	NaOH	16 g/L (0.4 M)
Blot wash buffer:	NaH_2PO_4 x $2H_2O$	7.8 g/L (50 mM)
	pH	6.5
Pre-hybridization buffer:	SDS	7 % (w/v)
	NaH_2PO_4 x·$2H_2O$	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 °C.	
Hybridization wash buffer:	NaH_2PO_4 x $2H_2O$	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
Wash solution:	NaOH	8 g/L (0.2 M)
	SDS	0.1 % (w/v)
Stop solution:	Tris-Cl	24 g/L (200 mM)
	EDTA	5.84 g/L (20 mM)
	NaN_3	1.3 (20mM)
	aurin tricarboxylate	20mM
	pH	8.0
	Storage condition: -21 °C in the dark	
STET-solution:	Sucrose	8 %
	Triton-X100	5 %
	EDTA	14.6 g/L (50 mM)

	Tris-Cl	6 g/L (50 mM)
	pH	7.0
	The solution was prepared in DEPES treated water and supplemented with 2 µL of Super RNasin (20 U/µL)	
MOPS buffer (10X):	MOPS	41.8 g/L (200 mM)
	Sodium acetate	8.2 g/L (100 mM)
	EDTA	2.9 g/L (10 mM)
	pH	7.0
RNA loading buffer:	Glycerol	50 % (v/v)
	EDTA	0.29 g/L (1 mM)
	Bromophenol blue	0.25 % (w/v)
	pH	6.0
Buffer A (enzymatic assay):	Tris-Cl	12g/L (0.1 M)
	$MgSO_4$	1.2 g/L (10 mM)
	Glycerol	10% (w/v)
	2-mercaptoethanol	0.4mL (5 mM)
	pH	7.4
Buffer B (enzymatic assay):	HEPES	11.9 g/L (50mM)
	potassium acetate	0.99 g/L (10 mM)
	$CaCl_2$	1.1 g/L (10 mM)
	$MgCl_2$	0.42 g/L (5 mM)
	pH	7.5

1.4. Biological materials

Table II-1. Strains, plasmids, oligonucleotide primers and culture conditions.

Strains	**Genotype and/or source**	**Reference**
E. coli DH5α	F^-, φ80m80*1ac*ZΔM15, Δ(*lacYZA-arg*F), U169*, rec*A1*, end*A1*, hsd*R17*, (r_{k-}; m_{k+}), pho*A, *sup*E44*, λ*$^-$*, thi*-1*, gyr*A96, *rel*A1	Ausubel *et al*., 1987
E. coli XL10 - Gold	Δ(*mcr*A)183, Δ(*mcrCB-hsdSMR-mrr*)173, *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1*, lac*Hte, [F´, *pro*AB, *lacI*^{q}Z*ΔM15*, Tn*10* (Tet^r) Amy Cam^r]	Stratagene
P. aeruginosa TBCF10839	CF airways, serotype 4; pyocin type: 1h, phage lysotype: F8, M4, PS2, PS24, PS31, 352, 46b/2, 1214, Col21, F7, F10, PS21, PS73, no plasmids. Hexadecimal SNP genotype: 3C52.	Wiehlmann *et al*., 2007 Tümmler *et al*., 1991
Plasmids	**Genotype and/or source**	
pME6010	Shuttle vector for Gram-negative bacteria; Tc^r	Heeb *et al*., 1988
pUCP20	*Escherichia-Pseudomonas* shuttle vector; Ap^r	Garrity-Ryan *et al*., 2000
pME6010::TB*pilY1*	pME6010 containing the *Bgl*II/*Eco*RI PCR product bearing the *pilY1* gene	This study
pME6010::TB*pilW*	pME6010 carrying the *Kpn*I/*Eco*RI PCR product bearing the *pilW* gene	This study
pUCP20::TB*mvfR*	pUCP20 carrying the *Hin*dIII/*Sac*I PCR product bearing the *mvfR* gene	This study
pUCP20::TB*pqsD*	pUCP20 carrying the *Hin*dIII/*Sac*I PCR product bearing *pqsABCD* operon	This study
pUCP20::TB*edd*	pUCP20 carrying the *Hin*dIII/*Sac*I PCR product bearing the *edd* gene	This study
pUCP20::TB*mqoB*	pUCP20 carrying the *Hin*dIII/*Sac*I PCR product bearing the *mqoB* gene	This study
pUCP20::TBPA4131	pUCP20 carrying the *Hin*dIII/*Sac*I PCR product bearing the PA4131 gene	This study
pUCP20::TBPA0785	pUCP20 carrying the *Hin*dIII/*Sac*I PCR product bearing the PA0785-PA0787 genes	This study
pUCP20::TBPA4916	pUCP20 carrying the *Hin*dIII/*Sac*I PCR product bearing the PAPA4916-PA4917 genes	This study

Primers	Sequence	Reference
5'pilW_*Kpn*I	GCCGGTACCCGACTTCTTCAAGGCCAAGG	This study
3'pilW_*Eco*RI	GCGAATTCCGCGCTGTTGTGCAGGGAAGT	
5'pilY1_*Bgl*	CGGAGATCTGGAACAACCTGCCCATTCCC	This study
3'pilY1_*Eco*RI	GCCGAATTCGAAGGTCTGGGGATCTTCGG	
5'mvfR_*Hin*dIII	GGATAAGCTTACACCTGAAGGCGCAACAGC	This study
3'mvfR_*Sac*I	CTAGAGCTCCGGAAGGTTTCGACTGCCTG	
5'pqsD_*Hin*dIII	GGATAAGCTTGAAGCCTGCAAATGGCAGGC	This study
3'pqsD_*Sac*I	CTAGAGCTCGACGCCAGGACCTGTACGTT	
5'edd_*Hin*dIII	GGATAAGCTTGCGTTCGAGACGATCCGATG	This study
3'edd_*Sac*I	CTAGAGCTCCCGGCGCTTCTCTTGTTGTCG	
5'mqoB_*Hin*dIII	GGATAAGCTTCACTGAGCAACAGGCGATGCAGC	This study
3'mqoB_*Sac*I	CTAGAGCTCCCTGTTTCGGTACCCTGGTGG	
5'PA4131_*Hin*dIII	GGATAAGCTTCAGGTAAAGGTACAGGCCGATG	This study
3'PA4131_*Sac*I	CTAGAGCTCTCTCGTAGCGCTTCATCTTG	
5'PA0785_*Hin*dIII	GGATAAGCTTCAGTGGTGGAGACCGTCAGGTTG	
3'PA0785_*Sac*I	CTAGAGCTCCTGCCAGTGCAGGTACTCAAG	
5'PA4916_*Hin*dIII	GCCTAAGCTTGTTCCGCCAGATCGTGGTAG	This study
3'PA4916_*Sac*I	GCAGAGCTCGTGAAGACCTCCACCTCCAG	
3'PA4640qRT-PCR	CGGTACCGGGTTGATGAAGG	This study
5'PA4640qRT-PCR	GTCGACATGCTGCTGGTAGG	
3'PA3452qRT-PCR	GTTCTCGTTGATCGCCACCG	This study
5'-PA3452qRT-PCR	GCTTTTGTGCGTCAGCGTGC	
3'-PA4916qRT-PCR	ACGAAGTCGAGGTCGTCGTC	This study
5'-PA4916qRT-PCR	CAGCGGAAGTATTGGCCAGC	
5'P1	GTACCCCACTAGTCCCAAGC	This study
5'P2	GTACCTCCACTCACCCAAGC	
5' Y-linker	CTGCTCGAGCTCAAGCTTCG	Kwon and Ricke, 2000
5' Tn*5*MOD	TGCGTTCGGTCAAGGTTCTGG	This study
5'Y1	TTTCTGCTCGAGCTCAAGCTTCGAACGATGTACGGGGACAC ATG	Kwon and Ricke, 2000
5'Y2	TGTCCCCGTACATCGTTCGAACTACTCGTACCATCCACAT	

2. Methods

2.1. Microbiological methods

2.1.1. Bacterial growth conditions

The cultures of *P. aeruginosa* or *E. coli* were grown in LB medium at 37 ^{o}C (230 rpm) overnight (12 – 16 hrs) unless noted otherwise. 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.

E. coli strains transfected with pME6010 (DH5α) or pUCP20 constructs (XL10-Gold) were growing in the presence of 50 µg/mL tetracycline or 100 µg/mL ampicillin, respectively, and recombinant *P. aeruginosa* were cultured in the presence of 100 µg/mL tetracycline (pME6010) or 200 µg/mL carbenicillin (pUCP20).

2.1.2. Determination of bacterial cell density

The optical density (OD) of *P. aeruginosa* or *E. coli* cultures was measured spectrophotometrically at 578 nm (OD_{578nm}) or 600 nm (OD_{600nm}) (Wiehlmann, 2001):

P. aeruginosa	OD(578nm) 0.6 ~ 1 x 10^9 cfu / mL
E. coli	OD(600nm) 1.0 ~ 0.8 x 10^8 cfu / mL

2.1.3. Growth of transposon mutants

A library of *P. aeruginosa* TB signature tagged mini Tn*5* OGm transposon mutants was constructed by Wiehlmann (Wiehlmann *et al*., 2002). The STM library was maintained as glycerol stocks in 96 well plates at -80 ^{o}C.

2.1.4. Assessment of colony morphology

The transposon mutants of the STM library stored at -80^{o}C were inoculated in 96 microtiter plates containing 200 µl LB medium and incubated at 37 ^{o}C for 16 h.
For the assessment of colony morphology for each single mutant, the bacteria were inoculated from frozen glycerol stock and grown in 5 mL glass tube at 37 ^{o}C for 16 h. After incubation time, mutants were plated on different media and incubated (Table II-2). Colony morphology was documented after 48 hrs of incubation.

Table II-2. Media and culture conditions used in the present work.

Media	**Culture conditions**
Luria broth (LB)	4^{0}C, ambient temperature, 37^{0}C, 42^{0}C
LB supplemented with 4mM $FeSO_4$	37^{0}C
LB depleted from iron by Chelex-100 beads (Sigma)	
Minimal medium (M9)	
Blood-agar (Columbia agar containing 5% sheep blood)	
LB supplemented with Congo red (40µg/mL)	

2.1.5. Maintenance of bacterial cultures

Bacterial cultures were maintained in LB medium containing 15% glycerol and stored at – 80 ^{o}C. For a limited period of time, LB agar plates were used to store cultures at 4^{o}C.

2.1.6. Generation of transformation competent cells

2.1.6.1. Generation of chemically competent cells

Competent cells from *E. coli* DH5α and XL-10 Gold were prepared according to Inoue *et al.* (1990). 10-12 colonies from LB agar plate were inoculated in a 1L Erlenmeyer flask containing 250 mL of SOB medium and grown at 18-20 ^{o}C on a

rotary shaker (300 rpm) up to an optical density of 0.6-0.8. At the appropriate cell density, flasks were chilled on ice and the cells were harvested by centrifugation at 2500 x *g* for 10 min at 4 ^{o}C. The pellet was resuspended in 80 mL of ice-cold TB buffer. After 10 min incubation on ice, the cells were centrifuged again at 2500 x *g*, for 10 min at 4 ^{o}C and the pellet completely resuspended in 20 mL of ice cold TB buffer and 1.4 ml of DMSO (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 ^{o}C.

2.1.6.2. Generation of electrocompetent cells

The single colony of *E. coli* DH5α and XL-10 Gold from a fresh agar plate was inoculated into a flask containing 25 mL of LB medium and incubated at 37^{o}C overnight with vigorous aeration (250 rpm). 250 mL of prewarmed LB medium was inoculated by 12.5 ml of overnight culture and the cells were incubated at 37^{o}C with (300 rpm). The growing bacterial culture was measured every 20 min. The optimum OD (OD_{600nm} = 0.35-0.4) was achieved after 2.5-3 hrs of incubation. Then, the flasks were transferred on ice for 15-30 min and swirled occasionally to ensure that cooling occurred evenly.

For maximum efficiency of transformation, it is crucial that the temperature of the bacteria do not rise above 4^{o}C at any stage of the protocol. After cooling, the cultures were transferred to ice-cold centrifuge bottles. The cells were harvested by centrifugation at 1000 x *g* for 15 min at 4^{o}C. The supernatant was decanted and the cell pellet were resuspended in 250 mL ice-cold H_2O. The cells were centrifuged at 1000 x *g* for 20 min at 4^{o}C and the cell pellet was resuspended in 125 mL of ice-cold water containing 10 % glycerol. The centrifugation step was repeated at 1000 x *g* for 20 min and the pellet was carefully resuspended in 5 mL of ice-cold pure water containing 10 % glycerol. Finally, the cells were harvested by centrifugation at 1000 x *g* for 20 min at 4^{o}C. The supernatant was carefully decanted to remove any remaining buffer. The pellet was resuspended in 0.5 mL of ice-cold GYT medium. Afterwards, the cell suspension was diluted to a concentration of 2 x 10^{10} cell/mL with ice-cold GYT medium. 40 µL of the suspension were aliquoted into ice-cold 0.5 mL eppendorf tubes, dropped into a bath of liquid nitrogen and transferred to a -80 ^{o}C freezer.

2.1.7. Introduction of foreign DNA into bacteria

2.1.7.1. 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 ^{o}C for 40 sec 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 ^{o}C for 1 h. After the incubation period, the whole mixture was plated on LB agar containing an appropriate antibiotic and incubated at 37 ^{o}C. The colonies harboring plasmids were visible after 16-24 h of incubation.

2.1.7.2. Electrotransformation of *E. coli*

Electrocompetent *E. coli* cells (Dh5α and XL10-Gold) were prepared as described above. 1-3 µl of the ligation product were added to 40 µl of *E. coli* cells. The mixture was poured in the electroporation 1 mm cuvette and the *P. aeruginosa* cells were electroporated with the Bio-Rad pulser (400 W, 25 µF, 1.25 kV, time constant 5 ms). 0.9 mL of SOC medium was added in the cuvette and the whole volume was transferred into a fresh eppendorf tube. Bacteria were incubated at 300 rpm at 37 ^{o}C for 1 h. Afterwards, the bacterial culture was centrifuged at 6000 x *g* for 10 min, the pellet was resuspended in 100 µl SOC medium and spread on LB plates containing an appropriate antibiotic. The plates were inverted and incubated at 37 ^{o}C overnight. Positive transformants were analysed for the presence of the insert by plasmid isolation and digestion.

2.1.7.3. Electrotransformation of *P. aeruginosa*

Introduction of DNA into *P. aeruginosa* was carried out by an electroporation protoicol modified from Enderle and Farwell (1998). 2 mL overnight cultures were sedimented by centrifugation at 6000 x *g* at 4 °C for 10 min. To achieve successful electroporation, the amount of exopolysaccharides in the culture needs to be as low as possible so they were removed from the pellet with repeated washing with ddH_2O and centrifugation at 13000 x *g* for 3 min. After the last washing step, the bacteria pellet was resuspended in 100–200 µL, ice-cold ddH_2O and mixed with ~ 50 ng plasmid. 100 µL of this mixture were transferred into pre-cooled electroporation cuvettes (1 mm, BioRad) and *P. aeruginosa* cells were electroporated with the Bio-Rad Pulser (400 W, 25 µF, 1.25 kV, time constant 5 ms) to induce DNA uptake. 900 µL prewarmed LB broth was added to the cell suspension followed by incubation at 37 °C with constant shaking (350 rpm) for 3 h. Positive revertants were selected by plating 100 µL aliquots on LB agar containing an appropriate antibiotic and incubated overnight at 37 °C. Recombinant plasmids were analyzed from selected colonies by plasmid isolation and restriction digestion.

2.1.8. Genetic complementation

After STM competition experiments *in vivo*, the mutants with enhanced or impaired fitness in the lung were selected for the future experiments. Subsequently these selected genes were complemented *in trans* to ensure that the observed striking phenotypes were caused by the transposon inactivation and not by any other secondary genetic event.

Complementation *in trans* was performed by using plasmids pME6010 or pUCP20. The genes with their own promoter regions were amplified from TBCF10839 by PCR with GoldStar polymerase system (Eurogentec) (see chapter 2.2.4). The PCR product was purified by Qiaquick GelExtraction Kit (Qiagen) according to the manufacturer's protocol to remove polymerase enzyme that might block the restriction sites and 20-25 µL were digested by 2x2.5 µL (5 U/µL) of restriction nucleases, 5 µL of the recommended buffer was added and 50 µL of the total volume was adjusted by adding of ddH_2O. Digestion was preformed overnight at 37 ^{o}C. 5-10 µg of the plasmid were subjected to restriction digestion. The digested products were

again purified using Qiaquick GelExtraction Kit (Qiagen) according to the manufacturer's protocol.

To avoid the self-ligation of the vector, 5´ phosphate groups were removed before ligation from the plasmid DNA. This property can be used to decrease the vector background in cloning strategies (Sambrook *et al*., 1989). For this, the plasmid (3-5 µg) was dephosphorylated by addition of 4 µL of antarctic phosphatase, 5 µl of Antarctic Phosphatase Reaction Buffer was added and 50 µL of the total volume was adjusted by ddH_2O. The reaction was incubated at 37 ^oC for 1 h. Inactivation of enzyme was done at 65 ^oC for 5 min.

To obtain maximal ligation efficacy the vector and insert were mixed in proportion 1:3. The mixure was ligated (see chapter 2.2.6) and the plasmid was introduced into chemically competent or electrocompetent *E. coli* (DH5α or XL10-Gold) and subsequently into the respective *P. aeruginosa* transposon mutant by electroporation.

2.2. Molecular biological methods

2.2.1. Isolation of DNA

2.2.1.1. Isolation of genomic DNA from *P. aeruginosa*

The genomic DNA from *P. aeruginosa* was isolated according to the protocol by Chen and Kuo (1993). The bacteria were harvested from 5 mL of overnight grown culture in LB medium by centrifugation at 14000 x *g* for 3 min. The pellet of harvested bacterial cells was resuspended in 900 µl of Lysis buffer. 300 µl of 5 M NaCl were added to the suspension and the whole volume was mixed thoroughly. Cell debris were separated by centrifugation at 14000 x *g* for 60 min at 4 ^{o}C and the supernatant was transferred into a fresh eppendorf tube. RNA was removed by incubation of the supernatant with 5 µl of RNase (10 mg/mL) at 37 ^{o}C for 30 min. Proteins were removed by mixing of the supernatant with equal volumes of phenol, phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) and separation of the phases by centrifugation at 14000 x *g* for 15 min. DNA was precipitated by addition of an equal volume of isopropanol and subsequent centrifugation at 14000 x *g* for 15 min. The pellet of the genomic DNA was washed with 70 % v/v ethanol, dried and resuspended in 25 – 50 µl in ddH_2O or TE buffer.
DNA isolated by this method was used as template in PCR reactions, Y – linker method or Southern blotting.

2.2.1.2. Isolation of plasmid DNA

For sequencing or cloning procedures, the extra pure plasmid DNA was isolated by using Qiagen Mini and Maxi Prep kit according to the manufacture's protocol. The plasmid DNA from the transformed *E. coli* or *P. aeruginosa* was isolated by the modified alkaline lysis method (Birnboim and Doly, 1979). For this, 5 mL of the overnight grown culture in LB medium was centrifuged at 5000 x *g* for 5 min and resuspended in 300 µl solution I. Then, 300 µl solution II were added and mixed properly. After 5 min incubation period, 300 µl solution III were added, thoroughly mixed and incubated on ice for 15 min. To precipitate cell debris, the centrifugation at 10000 x *g* for 10 min at 4 ^{o}C was done 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 at 14000 x *g* for 2 min at 4 °C for phase separation. The aqueous phase was mixed with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 10000 x *g* for 10 min at 4 °C.
After centrifugation, an equal volume of isopropanol was added to the supernatant for the precipitation of plasmid DNA and centrifuged at 14000 x *g* for 15 min at ambient temperature. Finally, the pellet was washed with 70 % (v/v) ethanol and dried at 37°C for 15 min. The dried pellet was resuspended in 25 - 50 µL TE buffer.

2.2.2. Separation of DNA

2.2.2.1. Agarose gel electrophoresis

The 0.8 - 3 % w/v agarose gel was prepared by solubilizing agarose in TBE buffer. 1x TBE buffer was used as a running buffer and 8.5 V/cm field strength was applied. The DNA was mixed with 1/5th volume of loading buffer and loaded on an agarose gel. Lambda phage DNA digested with *BstE*II restriction endonuclease was used as molecular size standard. After the run, the agrose gel was stained with 0.5 µg/mL ethidium bromide for 20 min and destained twice in water for 2 x 20 min. The DNA was visualized and photographed on a UV transilluminator at 312 nm.

2.2.2.2. Polyacrylamide gel electrophoresis

To separate the small DNA fragments (20–80bp), the polyacrylamide gel electrophoresis was applied. For this, the DNA fragments were loaded on a 10 % gel (19+1 acrylamide/bis-acrylamide) in TBE buffer and separated with field strength of 6.5 V/cm. The gel was stained with 0.5 g/mL ethidium bromide for 5 min and destained in water for 5 min and photographed on a UV transilluminator at 312 nm.

2.2.3. Quantification of DNA and RNA

The absorption was measured at 260 nm in a 1 cm thick quartz cuvette (Ausubel *et al.*, 1988; Sambrook *et al.*, 1989). Double distilled water was used as a control. The ratio of OD_{260} / OD_{280} provides for the estimation of DNA purity. For the ultra-pure, DNA or RNA has a ratio 1.8 and 2.0 respectively.

The concentration of the DNA and RNA can be easily calculated:

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

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

2.2.4. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was developed by Mullis and Faloona in 1986. In this work, PCR was used for the amplification of signal sequences from each Tn*5* inserted mutant, for probe preparation for Southern blotting and cloning procedures.

2.2.4.1. Construction of primers for PCR

The primers were designed according to the *Pseudomonas* genome database (Stover *et al.,* 2000, www.pseudomonas.com). Since a critical factor for yield and purity of the PCR product is the construction of correct primers, the following criteria were used:

- Length of the primers was kept to approximately 20-25 base pairs.
- G+C content of the primers was 60 – 66 %
- The primers were not self-complementary to avoid a formation of hair-pin structure and primer dimer formation
- The melting temperature (T_m) of the primers was above 60 ^oC to avoid the non specific amplification.
- The melting temperature was calculated as Tm = [Σ(A+T) x 2 + Σ(G+C) x 4] °C
- Two different restriction sites (one in each primer from the respective primer pair) were incorporated into the primers used for complementation.

2.2.4.2. PCR protocols

For quantitative PCR, probe preparation for Southern Blot detection and signal sequence amplification, InviTaq polymerase (InViTek) was used. For complementation and sequencing, amplifications were carried out either by using proofreading polymerase GoldStar polymerase system (Eurogentec) or FailSafeTM PCR system (Epicentre). PCR was performed according to the following protocols (Tables II-3. and II-4.) if not noted otherwise.

Table II-3.
The protocol of amplification of *P. aeruginosa* genomic sequences by InviTaq polymerase (InViTek) or GoldStar Taq polymerase (Eurogentec)

Standard reaction (50 µL)		**PCR program:**	**a)**	**b)**
(10x) reaction buffer	5 µl	Initial denaturation	94 °C, 10 min	96 °C, 10 min
Template DNA	5-100 ng			
5'-primer (5 pmol/µL)	5 µl	Annealing	variable, 45 sec	58 °C, 20 sec
3'-primer (5 pmol/µL)	5 µl	Elongation	72 °C, 60 sec/1kb	72 °C, 20sec
$MgCl_2$ (50 mM)	4 µl	Denaturation	94 °C, 30 sec	95 °C, 30 sec
dNTP (each 2 mM)	6 µl	35 cycles		
DMSO	2.5 µl			
Polymerase (5 U/ µL)	0.2 µl	Final cycle	72 °C, 120 sec/1 kb	72 °C, 40 sec
ddH2O	ad 50 µl			

a) 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.* For the cloning procedure 4M Betain as an enhancer of PCR reaction was added.

b) Amplification of specific signal sequences:

Primers P1 and P2 were used to amplify the signal sequence (80bp) of Tn*5* insert. This PCR required the appropriate amount of template since small inaccuracy leads to substantial loss of product.

Table II-4.
The protocol for amplification of *P. aeruginosa* genomic sequences by FailSafeTM PCR System (Epicentre)

Standard reaction (25 µL)		**PCR program:**	
Template DNA	5-100 ng	Initial denaturation	96 °C 10 min
5'-primer (5 pmol/µl)	2.5 µl		
3'-primer (5 pmol/ µl)	2.5 µl	Annealing	variable, 45 - 60 sec
FailSafe enzyme	0.5 µl	Elongation	72 °C, 60 sec/1kb
ddH2O	12.5 µl	Denaturation	96 °C, 30 sec
		35 cycles	
+ (2x) Premix buffer	12.5 µl		
		Final cycle	72 °C, 120 sec/1 kb

2.2.5. Restriction digestion of DNA

The restriction digestion was applied for the restriction of the genomic DNA, the PCR products of specific signal sequences or PCR products used for the cloning. The restriction enzymes together with the buffer system were applied according to the manufacturer's protocols (New England Biolabs). For the small sequences, the digestion to gain the specific signal sequence was done by *Hin*dIII. 80 µL of PCR product were digested with 250 U of restriction enzyme in a total volume of 250 µL and the reaction mixture was incubated overnight. For all other procedures, the unit definition of restriction enzyme was defined as the quantity of enzyme required for the digestion of 1 µg of DNA in 1 h in the assay buffer.

2.2.6. Ligation

The plasmids constructed in this study either for plasmid rescue or complementation were obtained by ligation. Ligation was performed according to the following protocols (Tables II-5. and II-6.) if not noted otherwise.

Table II-5. **Ligations of Insert DNA with Cohesive Ends by Fast-link ligase (a)**	
10X Fast-Link Ligation Buffer	1.5 µL
ATP (10 mM)	1.5 µL
Vector DNA	50 µg
Insert DNA	150 µg
Fast-Link DNA Ligase	1 µL
ddH_2O	x µL
Total reaction volume	15 µL

Table II-6. **Ligations of DNA with Cohesive Ends by T4 – ligase (b)**	
1 X Reaction Buffer	2 µL
T4 DNA Ligase (20 U)	1 µL
ATP (10mM)	2 µL
Digested DNA	50 - 500ng
ddH_2O	x µL
Total reaction volume	20 µL

a) For the construction of vectors for complementation, Fast-link ligase kit (Epicentre) was used. The protocol (Table II-5) was optimised for the construction of vectors in a short time (for as little as 5 min). Incubation of the reaction was done for 5 minutes at room temperature. After the indicated time, the reaction mixture was heated to 70^{o}C for 15 min for inactivation of the enzyme (failure to inactivate the ligase may decrease transformation efficiencies). This additional step could be done as to determine the extent of ligation. For this, the ligation products (5 µg) have to be separated on an agarose gel (3%).

b) For the plasmid rescue procedure T4 DNA ligase (New England Biolabs) was used (Table II-6). Incubation of the reaction was done for 16 hrs at 4^{o}C. Inactivation of the enzyme was performed for 10 min at 65^{o}C.

2.2.7. Sequencing of transposon flanking genes

2.2.7.1. 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 of the transposon mutant was digested with 40 U of *Pst*I at 37 ^{o}C overnight in 40 µl restriction buffer. Digested DNA was purified by phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and by chloroform:isoamyl alcohol (24:1, v/v) extractions and the DNA was precipitated by ethanol. The pellet was dried for 30 min at 37^{o}C and resuspended in 25 µl of TE buffer. 500 ng digested genomic DNA was incubated with

1000 cohesive end ligation units of T4-DNA ligase according to the protocol described above. 50 ng of ligated DNA were transformed into highly competent *E. coli* XL-10 Gold and plasmid-harbouring cells were selected on LB agar with getamycin (30 µg/mL). The plasmid was purified from these cells and sent for sequencing to QIAGEN.

2.2.7.2. The Y – linker method

This method based on the ligation of a linker of known sequence to the digested DNA and performing a PCR primed by an oligonucleotide specific for the non-complementary part of the linker and on the other side by a transposon-specific primer sequence. By this, a PCR product is generated that can subsequently be sequenced (Kwon and Ricke, 2000).

4 µL of linker strand 2 (3,5 µg/µl) were phosphorylated by adding 4 µL of 10 mM ATP, 4 µL of 10x concentrated polynucleotide kinase buffer (NEB), 1 µL of T4 polynucleotide kinase (NEB) and 27 µL ddH_2O to a final volume of 40 µL. The reaction was incubated at 37^{o}C for 15 min and then heated to 95^{o}C for 20min.

4 µL of linker strand 1 (3,5 µg/µL) and 36 µl ddH_2O were added to the mixture which was slowly cooled to room temperature. The linker strands annealed and formed the ready-to-use Y-linker. Genomic DNA of the mutants in aliquots of 1 µg was digested either with *Nla*III or *Sph*I in a total volume of 20 µL. The reaction was incubated at 37^{o}C for 3 h. 500 ng of digested genomic DNA was mixed with 5 µL of the Y-linker and ligated according to the protocol described above. The reaction was incubated at 25^{o}C for 2 h, 20-50 ng of Y-linker ligated product was used as a template for the PCR reaction with the Y- and pTnMod-specific primers. PCR products were purified and the transposon insertion site was determined by sequencing. The resulting PCR products were purified by agarose gel electrophoresis and extracted using a Qiagen Gel extraction Kit. Sequencing was done by QIAGEN using TnMOD-specific primer.

2.2.8. RNA working technique

2.2.8.1. RNA handling and storage

Due to the short life of bacterial mRNA, special precautions were taken when working with RNA. To avoid RNA degradation, all reagents and equipment 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 forceps were dipped in the 70% (v/v) ethanol.
- Glass wares were sterilised 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 to 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.
- Gel chambers for electrophoresis of RNA samples were cleaned with 3 % H_2O_2 and rinsed with 70 % EtOH
- Fresh stock of plastic wares was used every time.
- When working with RNA, all samples were placed on ice and RNase inhibitor was added.
- For long-term storage, RNA dissolved in RNase-free buffer or water was stored at -80 ^{o}C.

2.2.8.2. RNA extraction

Total RNA of *P. aeruginosa* mutants and strains was extracted by bacterial lysis in the late stationary phase. 50 - 100 mL overnight culture of bacterial cells in stationary phase (OD_{578nm} ~ 3.0) was cooled on ice and treated with 5 mL STOP-solution. Sedimentation of the cells was achieved by centrifugation at 5500 x g at 4°C for 10 minuts. Afterwards, the cell pellet was resuspended in 2 mL STET-solution. The samples were subsequently purified by phenol/chloroform extraction (aqueous phenol; pH 4.5-5.5). The upper aqueous phases of each lysate were pooled into one and the nucleic acid was precipitated with 1/10 sample volume 3 M sodium acetate

and 2 volumes of 95 % Ethanol (-21 °C). For complete precipitation, the sample was stored at -21 °C for 1 h and recovered by centrifugation at 10000 x g at 4 °C for 10 min. The pellet containing crude nucleic acid was air-dried at 37 °C and resuspended in 200 µL of DEPC-treated water. To get highly pure RNA, the resuspended pellet was purified by Qiagen RNeasy MiniKit according to the manufacturer's protocol. Resuspension of the RNA pellet in 200 µL DEPC-treated ddH_2O allowed long time storage of the RNA at – 80 °C for 6 months.

2.2.8.3. Formaldehyde agarose gel electrophoresis

In order to check the RNA preparations, formaldehyde agarose gel electrophoresis was performed. The formaldehyde gel electrophoresis was preformed in mini gel chambers: 5 x 7 cm gel (Forschungswerkstätten, MHH). 1.2 % agarose gel was prepared from 50 mL MOPS buffer with a gel volume of 50 mL and supplemented with 2.4 mL formaldehyde at a temperature lower than 50 °C.
The RNA extracts stored at -80 °C were denatured at 65 °C for 10 min and cooled down on ice.

The RNA samples preparation

RNA extract	2 µL
37 % Formaldehyde	2 µL
Formamide	5 µL
RNA loading buffer	2 µL
(10x) MOPS	1 µL
1 % Ethidium bromide	0.5 µL

The samples were loaded on the gel (5 min, 60 V). The constant applied field strength for mini gels and blot gels was 5 V/cm (running time approx. 1 h). The electrophoresis of the blot gels was stopped when the bromophenol blue dye has migrated two-thirds of the gel length.

2.2.9. Semi-quantitative RT- PCR (qRT-PCR)

cDNA first strand synthesis for the qRT-PCR was performed by using a modified protocol suggested by the Revert Aid^{TM} H Minus cDNA Synthesis Kit (Fermentas).
In the presence work, 3'-primers binding specifically to following genes were used: PA4640, PA3452 and PA4916.
For the cDNA synthesis, 2 µg of mRNA isolated from each mutant / strain was mixed with ~ 20 pmol of the 3'-primers. DEPC water was added to 12 µL and the mix was incubated for 5 min at 70 °C. After cooling down the sample on ice, 2 µL dNTP mix (10 mM), 1 µL Ribonuclease inhibitor (RiboblockTM, Fermentas) and 4 µL (5x) reaction buffer were added to the samples and incubated for 5 min at 37 °C. Afterwards, 1 µL Revert Aid^{TM} Reverse Transcriptase (Fermentas) was added to the sample to start the elongation reaction during incubation at 42 °C for 1 h followed by 70 °C for 10 min.
The cDNA first strands of each sample serve then as template for the following quantitative PCR carried out in a 50 µL reaction volume. 3 aliquots of 50 µL PCR reactions were prepared for each strain with the following 5'-primer using InviTaq polymerase (InViTek) according to the PCR protocol described above. During PCR, 8 µL aliquots were taken every second cycle from 16th cycle on until the final step after 32 cycles and subsequently applied to a 1.5 % agarose gel electrophoresis.

2.2.10. DNA fixation and hybridization

2.2.10.1. Dot-blot preparation

The dot blots were used during the selection experiments carried out for the screening of STM library in murine infection model.
80 µL of PCR products were 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 on ice, 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. 95 µL of the DNA solution was applied to a Minifold-Dot-vacuum-bloter. 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.

2.2.10.2. Probe generation

The PCR was performed with the use of primers P1 and P2 to amplify the signature tags from the mutants. The PCR products were digested for 16 h with *Hin*dIII and the specific 40 bp sequence tags were separated from the common flanking 20 bp sequences by polyacrylamide gel electrophoresis (10 % gel (19+1 acrylamide/bis-acrylamide) in TBE buffer). The 40 bp sequence tags were cut out from the gel and purified (QIAGEN). The 40 bp sequences were labelled with DIG-ddUTP using a terminal transferase (Roche). 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. For this, the cleaned specific signal sequences (40bp) 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 (Figure II-1.).

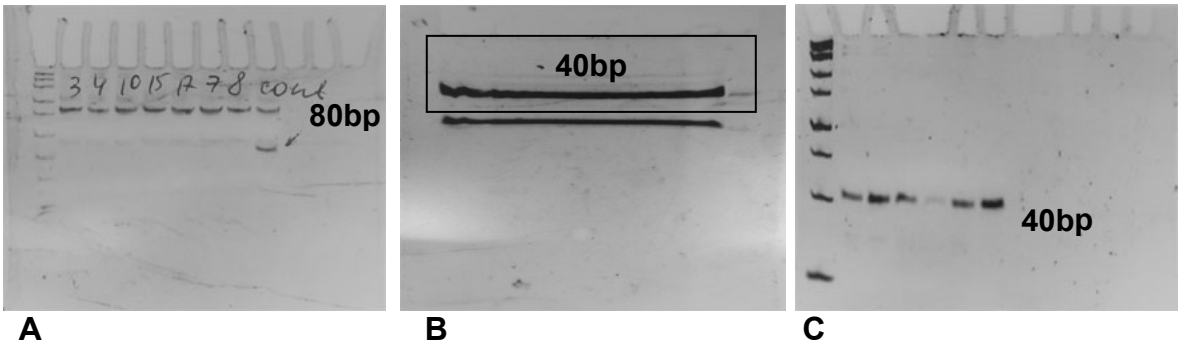

Figure II-1. Polyacrilamide gels for separation of small DNA sequences of signature tags after PCR amplification. (a) 80 bp of the Tn*5* region, (b) separation of specific 40bp sequences, (c) 40 bp sequences after purification are ready for labelling.

2.2.10.3. 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 and Gilbert, 1984). Southern hybridization (Southern, 1975) involves a reaction between denatured DNA immobilized on the nylon membrane and single stranded DNA probe. It is depending on temperature, time, salt concentration, G+C content, probe length and concentration. 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 required volume of prehybridization buffer was preheated to 58 ^{o}C and the blots were kept in large glass tubes (Biometra) or in 50 mL 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 58 ^{o}C for at least 2-3 hours with constant shaking. The DIG labeled probe was denatured by heating for 5-10 min at 95 ^{o}C and added in 10 mL total volume of prehybridization buffer to the tube containing blot. The blots were hybridized for 16-24 hours at 58 ^{o}C in the rotary hybridization oven.

2.2.10.4. Immunological detection of the hybridized blot

To remove non-specific bound DNA probe, the blots were washed twice with 20 mL of hybridization washing buffer and transferred into a plastic container and equilibrated in buffer I on a rotary shaker for 5 min. To block unspecific binding sites, the blot membrane was incubated in buffer II for 30 min. Antibody solution was prepared in buffer II (1:5000) and added to the blot for another 30 min. For detection, the membrane was rinsed three times in buffer I and equilibrated in freshly filtered buffer III for 5 min before addition of CDP-Star^{TM} diluted in buffer III (1:1000). The maximal chemiluminescence is reached after 5 - 30 min and can be used for exposure to X-ray film.

2.2.10.5. Washing and stripping of hybridized membranes

The labeled and hybridized membranes can be used several times after washing (stripping) under alkaline conditions. For washing, the membranes were 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.

2.2.10.6. Signals strength quantification

Signals were quantified by PCBAS, version 2.09f. The signal strength of each dot was compared to the corresponding signal of a probe prepared from pooled bacteria grown on LB agar without *in vivo* selection. The mutants were retested and transposon mutants with consistently strong differences in their ability to survive were selected for further examinations.

2.3. The infection experiments *in vivo*

2.3.1. Mice infection experiment

The animal experiments were performed in collaboration with Dr. Antje Munder of the Clinical Research Group at the Medical School Hannover.

Prior to animal experiments, bacteria were grown in LB broth overnight at 37 ^{o}C (230rpm) to stationary-phase. The bacteria were pelleted by centrifugation at 5000 x *g* for 10 min, washed twice with sterile phosphate buffered saline (PBS) and the optical density of the bacterial suspension was adjusted by spectrophotometry at 578 nm. The intended number of cfu was extrapolated from a standard growth curve and appropriate dilutions with sterile PBS were made to prepare the inoculum for the mice. To verify the correct dilution, an aliquot was plated on LB agar plates. Ten to 12 week old female mice of the inbred strain C3H/HeN (Charles River, Sulzfeld, Germany) were inoculated with 30 µl of this bacterial suspension containing 7.5 x 10^6 CFU of the different *P. aeruginosa* mutants via view controlled intratracheal instillation. This noninvasive application technique (Munder *et al*., 2002) via catheter allows controlled delivery of the bacteria to the lungs. During the experiments mice

were maintained in microisolator cages with filter top lids at 21 ± 2°C, 50±5 % humidity and 12 h light-dark-cycle. They were supplied with autoclaved, acidulated water and fed ad libitum with autoclaved standard diet. Prior to the start of the experiments animals were acclimatized for at least seven days. All animal procedures were approved by the local District Governments and carried out according to the guidelines of the German law for the protection of animal life.

In case of 14-day infection experiments, the weight and rectal temperature of the mice were measured daily and their body condition was determined using a self-developed score (Munder *et al*., 2005). Murine behaviour was scored for the parameters vocalisation, piloerection, attitude, locomotion, breathing, curiosity, nasal secretion, grooming and dehydration. Two mice were sacrificed by 48 h for the evaluation of lung histology or the determination of cfu in homogenized organs (lungs, liver, spleen and brain).

2.3.2. Screening of the STM mutants for survival in vivo

Transposon mutants with different signature tags were separately grown in LB at 37^{o}C overnight and pooled directly before mice infection. From this pool, 100 µL of bacterial suspension was cultivated on LB agar or liquid for 48 h at 37^{o}C (control) and 30 µL (7.5 x 10^{6} cfu) was used for the intratracheal mice infection (experiment). After 48 h of infection, mice were sacrificed and organs (lungs, liver and spleen) were homogenised. Bacteria from the homogenised organs were recovered in LB and on LB agar at 37^{o}C overnight. In parallel, bacteria from the control plates were collected and incubated on LB and LB agar at 37^{o}C overnight in the same incubator. Genomic DNA was prepared from both control and experiment pools and PCR was preformed to amplify the signature tags from the mutants. The PCR products were digested, labelled and hybridized onto dot blots.

2.4. Bioassays

2.4.1. Measurement of malate-quinone oxidoreductase (MQO) enzymatic activity

2.4.1.1. Preparation of cell-free extracts and membrane fractions

All operations were done at 4 °C by the use of a modified protocol of Kretzschmar *et al.* (2002). Bacteria were grown to late exponential phase, harvested, washed twice with ice-cold buffer A and resuspended in the same buffer. After cell disruption by sonication, cell debris were removed by centrifugation for 30 min at 6000 x *g*. Part of the supernatant was used as the cell free extract; the other part was used to obtain the membrane fraction.

The supernatant was centrifuged for 1h at 100000 x *g*; the resulting membrane-rich pellet was washed with ice-cold buffer B before being resuspended in a small volume of the same buffer buffer. Cell-free extracts and membrane fractions were split into aliquots and either assayed immediately for enzymic activity or stored at -20 °C. The protein content of the cell-free extracts was measured by adding the Bradford reagent (Bradford *et al*., 1976).

2.4.1.2. Enzyme assay

The measurement of MQO activity was carried out at 25 °C in a total volume of 1 ml. The MQO activity was assayed with the membrane fraction. The activity was determined by measuring the decrease of the absorbance of 2,6 - dichlorophenol indophenol (DCPIP) at 600nm, assuming an absorption coefficient of 22 cm^{-1} mM^{-1} (Armstrong, 1964). The test of O'Brien and Taylor (1977) was modified and contained, in a total volume of 1 ml, 50 mM Tris/HCl, 0.05 mM DCPIP, 0.1 mM phenazinemethosulfate, 5 mM ethylamine, 5 mM KCN, 100 hl FAD and 1 mM L-malate.

2.4.2. Phenotype MicroArrays (PMs) of *P. aeruginosa* (BIOLOG)

PMs for the mutants of interest were performed as described elsewhere (Bochner *et al*., 2001) by BiOLOG Inc., Hayward, CA according to standard protocols (http://www.biolog.com).

5 micro plates used in this study contain compounds related to the main catabolic pathways for carbon (C-source, PM1, PM2A), nitrogen (N-source, PM3B), phosphorous and sulphur (P-source and S-source, PM4A). One plate was used for stimulation of bacterial growth by nutrients (nutrient supplements, PM5).

Different chemicals were tested in 96-well microtiter plates with each well containing a cell culture medium that is designed to test a unique phenotype or cell function. The components of the culture media were dried onto the bottom of each well. After inoculation the cells were incubated for 24 to 48 hours, and their phenotypic behavior was observed and recorded. The response of the cells in each well was monitored colorimetrically using a patented redox chemistry that monitors the respiration of the cells over the time course of the incubation.

2.4.3. HAQ detection and quantification

The assessment of *Pseudomonas* quinolone molecules production was performed in collaboration with Dr. Bredenbruch and Dr. Häußler of the Research Group Chronic Pseudomonas Diseases at the Helmholtz Centre for Infection Research in Braunschweig, Germany, as described elsewhere (Bredenbruch *et al.,* 2005).

For this, bacterial cultures were grown in 50 mL-flask in 10 mL BHI medium (Brain Heart-Infusion) and incubated with constant shaking at 37 °C for 18 h. 5 mL of this culture was extracted with 5 mL dichlormethane by shaking and the two phases were separated by centrifugation at 5000 x *g* for 10 min. 2 mL of the organic phase which contains PQS and pyocyanin was dried by evaporation, and the pellet was subsequently resuspended in 50 µL methanol. 2 - 4 µL of these quinolone fractions was applied onto a TLC plate and separated by 5 % methanol / 95 % dichlormethane.

Fluorescent spots were visualized under UV light and photographed. Starting from 4-hydroxy-2-heptylquinoline, PQS was synthesized by the procedures described by Pesci *et al* (1999) and used as standard.

2.4.4. Cytotoxicity assay

The cytotoxicity assays were performed in collaboration with Stephanie Tamm of the Clinical Research Group at the Medical School Hannover.

Chinese hamster ovary (CHO) cells were routinely grown in RPMI medium supplemented with 5% fetal calf serum (FCS) and 1% Penicillin and Streptomycin. For the LDH assay nearly confluent CHO cells were trypsinised and seeded in 6well plates to a density of $6.0x10^5$ cells per well and again cultivated overnight. Prior to infection, cells were washed and covered with colourless CD-CHO-medium without any supplements. *P. aeruginosa* was grown overnight in LB, subcultured into fresh LB, and grown to mid-log phase. CHO cells were infected with mid-log-phase *P. aeruginosa* at an initial multiplicity of infection (MOI) of 10. Culture supernatants were collected at the times indicated in the figure legends and centrifuged for 10 min at 3220 x *g* to sediment bacteria and CHO cells. Lactate dehydrogenase (LDH) in the supernatant was measured with a Roche LDH kit in accordance with the manufacturer's instructions. Percent LDH release was calculated relative to that of the uninfected control, which was set at 0% LDH release, and that of cells lysed with Triton X-100, which was set at 100% LDH release.

2.4.5. Assessment of pyocyanin secretion

Pyocyanin is a blue redox-active secondary metabolite that is produced by *P. aeruginosa* and being one of the virulence factors of the bacterium (Lau *et al.,* 2004).

For the assessment of pyocyanin production, King A medium (King *at al.,* 1954) was inoculated with 0.2 OD (578nm) and incubated at 300 rpm at 37°C. Two mL samples were taken every 2 hrs starting from first hour for further 36 hrs: 1 mL for the optical density measurement of the cells at 578nm and 1 mL for the assessment of pyocyanin production by measuring the optical density of the supernatant at 695 nm after sedimentation of the bacteria culture at 8000 rpm for 10 min. In all optical density measurements, pure King A medium served as negative control.

2.4.6. Assessment of protease secretion

P. aeruginosa produces an arsenal of proteases such as LasB, alkaline protease and protease IV which can degrade casein (Cowell *et al.,* 2003). All of them are virulence factors which are under the control of quorum sensing in *P. aeruginosa* (Passador *et al.,* 1993). Hence, growth of *P. aeruginosa* on minimal medium containing casein as a carbon source shall indicate functional quorum sensing in the strain to be tested.
For this assay, 2 µL of overnight culture were inoculated onto plates of M9 minimal medium containing 0.8 % (w/v) casein solidified with 1 % agar and incubated overnight at 37 °C. Protease secretion was indicated by a white halo around the colony.

2.5. Internet databases and software

The sequenced DNA was aligned with the *P. aeruginosa* PAO1 genome (http://www.pseudomonas.com) or at the National Center for Biotechnology Information (http://www.ncbi.nlm.hiv.gov/) using the BLAST algorithms (Benson *et al*., 2002).
To get information about the gene function, operon structure, domains, motifs, predicted biological function and others, the databases of the Institute for Genomic Research TIGR (http://www.tigr.org) or the integrated database retrieval system for major biological databases DBGET (http://www.genome.ad.jp/dbget/) were used.
Signals on dot blots after southern blotting were quantified by PCBAS, version 2.09f.
For the multiple sequences alignment the Jalview alignment editor was used (http://www.jalview.org).

III Results and discussion

The STM mini-Tn*5* transposon library was generated in the *P. aeruginosa* strain TBCF10839 (Wiehlmann *et al.*, 2002). Strain TBCF10839 was isolated in 1983 from an exocrine pancreas-insufficient F508del homozygous CF patient who was chronically infected by this strain (Tummler, 1987).

By studies of TBCF10839 in our laboratory, the strain was shown as a strong producer of the quinolone PQS and N-acylhomoserinelactones, the major signal molecules involved in quorum sensing (Juhas *et al*., 2005). The strain can persist in polymorphonuclear leukocytes (PMNs) (Tummler, 1987), is proficient in the expression of type I, type II and type III secretion dependent virulence effector molecules and secretes large amounts of secondary metabolites such as phenazine and pyocyanin during stationary phase.

Due to indicated properties, TBCF10839 strain was shown to be more virulent than the genetic reference strain PAO1 in infection models and can colonize naive murine airways (Wiehlmann *et al.,* 2007). Transcriptome (Salunkhe *et al*., 2005) and proteome analyses (Arevalo-Ferro *et al*., 2004) indicated that TBCF10839 manages many more metabolic and signalling pathways than the sequenced reference strain PAO1 (Stover *et al*., 2000).

For the present work, the strain TBCF10839 was chosen for studies on morphotype variation and airway adaptation between the sequenced burn wound isolates PAO1 and PA14, because of several reasons.

First, the infection model could not be established with the strain PA14 (Lee *et al*., 2006), the dose was either cleared or lethal within a day (A. Bragonzi, personal communication).

Second, TBCF10839 strain was isolated from a chronic CF lung infection and compared to PAO1 strain has a set of adaptations supporting its survival *in vivo*.

Third, being a mucoid strain, TBCF10839 had already acquired the morphological signature of chronic airway colonizers, and hence we expected that single hits in the genome should generate more informative morphological variants than the same approach in strains PAO1 or PA14.

1. Screening of the STM library

Different stress conditions were simulated in the STM library of strain TBCF10839 by culturing under different conditions. The 3,500 transposon mutants of the STM library were inoculated in 96 microtiter plates, incubated overnight and plated on square plates with LB agar at 4°C, 22-25°C, 37°C or 42°C and to grow at 37°C with blood agar or agar with minimal medium, iron-depleted LB, iron supplemented Lb or with LB supplemented with the dye Congo red.

After 48 hours of incubation, morphology was documented by shape, size, margin, colour and texture of the colonies. After three rounds of repeated screenings, there remained 57 mutants with a morphotype other than wild type.

The insertion site of the plasposon was identified for all 57 mutants by sequencing of PCR products generated either by the Y-linker method (Kwon and Ricke, 2000) or by plasmid rescue (Dennis and Zylstra, 1998).

The Y-linker method is a readily applicable easy technique (Figure III-1).

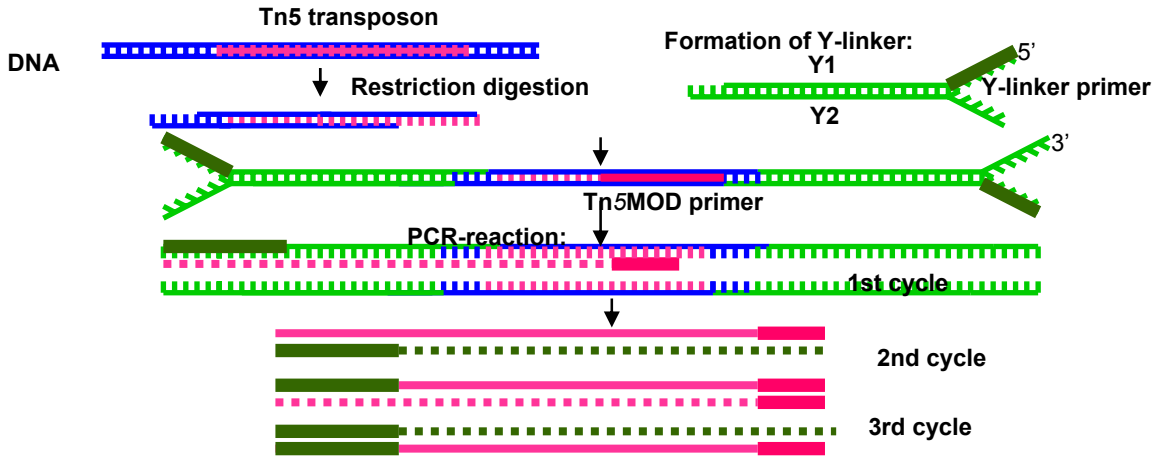

Figure III.1. The scheme of the Y-linker procedure. The Y-linker was prepared by the annealing of two oligonucleotides, Y1 and Y2. For this, the 3' end of the double stranded linker forms a "Y" upon annealing of both strands. A primer for the Y-linker is designed to bind to the non-complementary region of the Y-linker (Y-linker primer) which prevents annealing to the linker itself. This way a second primer (Tn*5*MOD*)* is necessary for logarithmic amplification of the targeted sequence. DNA fragments that were not ligated to the Y-linker will not give any product.

The principle is based on the ligation of a linker of known sequence to the digested DNA and performing a PCR primed by an oligonucleotide specific for the non-complementary part of the linker and on the other side by a transposon-specific primer sequence. A PCR product is hence generated that can be subsequently be sequenced.

Sequencing revealed that the transposon had inactivated genes encoding for elements of energy metabolism, chemotaxis, motility, attachment, secretion or transcription regulators (Figure III-2).

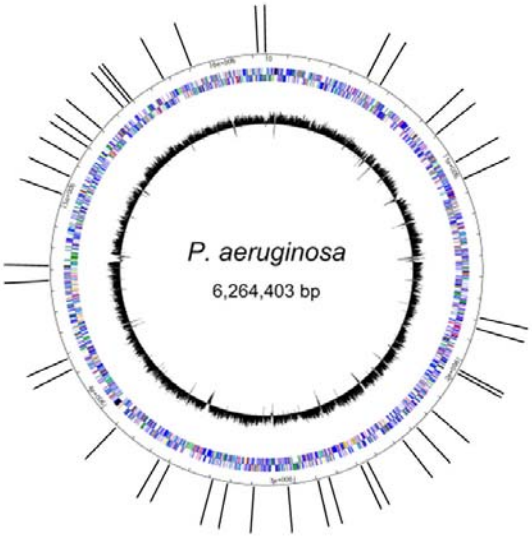

Figure III-2. Map position of sequenced knock-out genes in the PAO1 genome.

Out of 57 sequenced genes, the plasposon had inactivated a PAO1-homologous sequence in 52 TBCF10839 mutants, whereas sequences of five mutants had not been found in the PAO1 genome database (Table III-1).

Table III-1. *P. aeruginosa* TBCF10839 Tn*5* minitransposon colony morphology mutants.

Map position of Tn5: gene number in PAO1 genome	Mutant	Type of Morphology#	Gene name	Gene annotation
Stable morphotypes distinct from wild type				
PA0424	8dC3	A	*mexR*	Multidrug resistance operon repressor MexR
PA2028	8dB10	A	-	Probable transcriptional regulator
PA2122	8dB6	A	-	Hypothetical protein
PA3462	10dB7	A	-	Probable sensor/response regulator hybrid, transcriptional regulatory protein
PA3748	10dA1	A	-	Conserved hypothetical protein; putative magnesium and cobalt transporter (CorB)
PA4190	22D2	A	*pqsL*	Probable FAD-dependent monooxygenase
PA4489	2D1	A	-	Conserved hypothetical protein
PA5524	2bH1	A	-	Probable short-chain dehydrogenase
A1	9dA5	A	*topA*	Topoisomerase 1A, *P. aeruginosa* 2192: genomic island PAGI-2, *P. aeruginosa* strain C.
A2	8dA5	A	*fpvA*	Siderophore receptor for type III ferripyoverdine, *P. aeruginosa* strain 59.20
A3	9dH5	A	*phiCTXp40*	*Pseudomonas* phage phiCTX, hypothetical protein, ORF37
PA0999	3D9	B	*pqsD*	3-oxoacyl-[acyl-carrier-protein] synthase III
PA1003	46A8	B	*mvfR*	Transcription regulator
PA2361	8cD6	B	-	Hypothetical protein
PA2588	2D4	B	-	Probable transcriptional regulator
PA4915	9dC6	B	-	Probable chemotaxis transducer
PA2537	3D2	C	-	Probable acyltransferase
PA2579	9bA10	C	*kynA*	Tryptophan 2,3-dioxygenase
PA2838	8A9	C	-	Probable transcriptional regulator

Map position of Tn5: gene number in PAO1 genome	**Mutant**	**Type of Morphology#**	**Gene name**	**Gene annotation**
PA4734	9D2	C	-	Hypothetical protein
PA4552	14D1	D	*pilW*	Type IV fimbrial biogenesis protein
PA4554	10cB5	D	*pilY1*	Type IV fimbrial biogenesis protein
PA0413	39B4	E	*chpA*	Chemotaxis protein
PA0415	8bB12	E	*chpC*	Chemotaxis protein
PA1846	46D12	E	*cti*	Cis/trans isomerase
PA4916	15D9	E	-	Hypothetical protein; predicted ADP-ribose pyrophosphatase
PA4954	17A12	E	*motC*	membrane protein, part of the torque generator of the flagellar motor
PA2388	39A12	F	*fpvR*	Transcriptional regulator
PA2391	25D7	F	*opmQ*	Probable outer membrane protein precursor
PA3194	24C8	G	*edd*	Phosphogluconate dehydratase
PA4640	14D12	G	*mqoB*	Malate:quinone oxidoreductase
H1	6eB10	H	-	No homologies in PAO1 genome, GC low region at pos. 3291786 – 3291972 in a genomic island of the *P. aeruginosa* 2192 genome
Unstable mutant morphotypes with rapid reversion to wild type morphotype				
PA0482	8aC12	-	*glcB*	Malate synthase G
PA0728	7bE4	-	-	Probable bacteriophage integrase
PA0785	8dH9	-	-	Probable acyl carrier protein phosphodiesterase
PA0920	18D12	-	-	Hypothetical membrane protein
PA1589	31D7	-	*sucD*	Succinyl-CoA synthetase alpha chain
PA1633	10cB10	-	*kdpA*	Potassium-transporting ATPase
PA1823	8dE1	-	*nudC*	NADH pyrophosphatase
PA2706	30A10	-	-	Hypothetical protein

Map position of Tn5: gene number in PAO1 genome	Mutant	Type of Morphology#	Gene name	Gene annotation
PA2946	5cB6	-	-	Hypothetical protein; predicted integral membrane protein
PA3012	48C4	-	-	Hypothetical protein
PA3238	19B12	-	-	Hypothetical protein
PA3239	10dB4	-	-	Conserved hypothetical protein, predicted surface lipoprotein (VacJ)
PA3804	16D9	-	-	Hypothetical protein
PA4131	18A11	-	-	Probable iron-sulfur protein
PA4703	8dE7	-	-	Hypothetical protein; predicted regulator of competence-specific genes (TfoX)
PA4797	44B7	-	-	Probable transposase.
PA4949	1C8	-	-	Conserved hypothetical protein; predicted sugar kinase
PA4951	1C2	-	*orn*	Transcription, RNA processing and degradation, oligoribonuclease
PA5121	6B12	-	-	Hypothetical membrane protein; predicted small-conductance mechanosensitive channel (MscS)
PA5231	36C4	-	-	Probable ATP-binding/permease fusion ABC transporter
PA5546	30A4	-	-	Conserved hypothetical protein; predicted cyclopropane fatty acid synthase (Cfa)
PA5563	21C1	-	*soj*	Chromosome partitioning protein
UK1	9dD7	-	-	No homologies in PAO1 genome
PR1	46C4	-	-	Promoter region of PA1266 gene
PR2	8dA12	-	-	Promoter region of PA3782 gene

Morphotype: **A** - shiny autolysis, **A$^+$** - shiny autolysis, but not white on iron supplemented medium; **B** – white colony on blood agar and iron supplemented media, on Congo red agar the colour is concentrated in the center of colony; **C** – light rose or colourless on Congo red agar, **D** – autolysis, but no shine, **E** - non mucoid at room temperature; **F** – orange pigment in the center of colony; **G** – small colony; **H** – highly mucoid.

Four of these sequences were ascribed to either *Pseudomonas* phage phiCTX (Nakayama *et al*., 1999), two genomic islands known from *P. aeruginosa* strain 2192 (GI 84328724) or to a non-PAO type of the pyoverdine receptor FpvA (Smith *et al*., 2005), respectively. One sequence (9dD7) was not homologous to any sequence in the database.

Of the 57 genes, properties of the encoded product have been characterized for 19 genes (Table III-1). A functional category could be ascribed *in silico* to 18 genes, whereas 20 ORFs encode hypothetical proteins of yet unknown function. All 57 sequenced genes exhibited an average of 99.8 % nucleotide homology using the BLAST algorithms (http://www.ncbi.nlm.hiv.gov).

1.2. Colony morphology variants of *P. aeruginosa*

The selection of the mutants on different media revealed 57 mutants (Table III-1). To confirm the indicated morphotype, an assessment of colony morphology was performed for each single mutant and repeated twice. Representative phenotypes of the eight robust morphotypes are shown in Figure III-3.

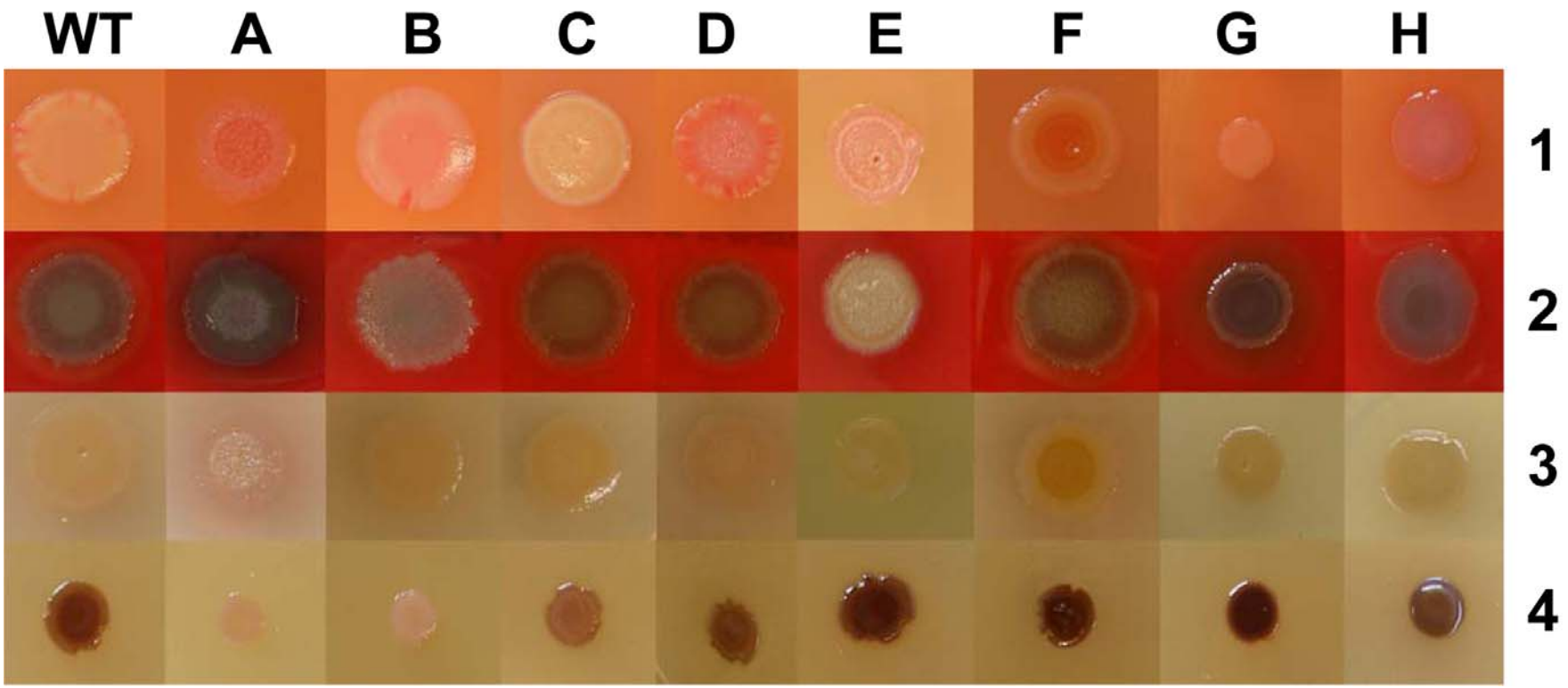

Figure III-3. Types of colony morphology of *P. aeruginosa* TBCF10839 mini-Tn*5* transposon mutants on LB agar with Congo red (1), blood agar (2), LB agar (3), LB agar supplemented with 4 mM $FeSO_4$ (4): WT, TBCF10839 strain; **A**, shiny autolysis; **B**, white colony on blood agar and iron supplemented media, on Congo red agar the colour is concentrated in the center of colony; **C**, light rose or colourless on Congo red agar; **D**, soft autolysis, no shine; **E**, non mucoid structure at room temperature; **F**, orange pigment in the center of colony; **G**, small colony size; **H**, highly mucoid. Transposon insertions into the TBCF10839 homolog of PA2028 (A), PA1003 (B), PA2579 (C), PA4554 (D), PA0415 (E), PA2388 (F), PA4640 (G), GC reach region at pos. 3291786 – 3291972 in a genomic island of the *P. aeruginosa* 2192 genome (H)

The metallic, iridescent sheen colonies with an inner circle of lysed cells (Figure III-3 A) represented the most frequent morphotype in our panel (Table III-1). The colonies with visible autolysis are noted in early descriptions for *P. aeruginosa* isolates (Berk, 1963, 1965; Holloway, 1969). In our experiments, the autolysis was visible after 24 hours of incubation at 37 ^{o}C on all tested media. The mechanism of autolysis for these mutants is not clear and has so far only been described for the *pqsL* mutant. By D'Argenio *et al.* (2002) PqsL was established as a negative regulator of HAQ biosynthesis and the autolysis of the *pqsL* mutant was attributed to the uncontrolled overproduction of bactericidal HAQs. Colonies of wild-type strains are typically brownish on high-iron medium (4 mM $FeSO_4$), but all mutants other than PqsL were colourless indicating that iron uptake was compromised. The fact that the pyoverdine receptor FpvA, the major uptake system for iron in *P. aeruginosa* (Smith *et al*., 2005; Ravel *et al*., 2003), is amongst these mutants, is consistent with this interpretation.

Type B colony morphology mutants (Figure III-3 B) appeared white on iron supplemented medium and blood agar, were impaired in hemolytic activity and accumulated the dye Congo red in the center of the colony. Congo red is known to bind extracellular matrix components and is taken as a surrogate marker for biofilm formation (Solano *et al*., 2002; Zogaj *et al*., 2001).
All type B mutants secreted proteases, being one of the most important determinants in the pathogenesis of *Pseudomonas* infections (Kessler *et al*., 1998) (Figure III-4), but lacked HAQ production including PQS (Bredenbruch *et al*., 2005) (Figure III-5).

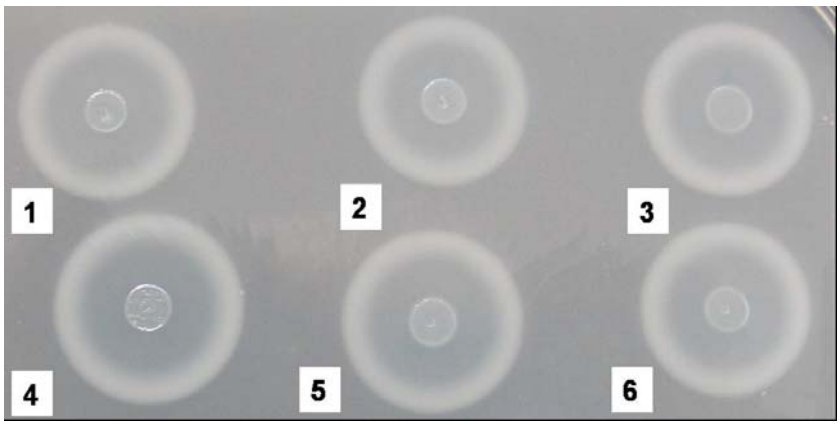

Figure III-4. Examination of inducible proteolytic activity on casein agar by: Tn*5*::PA0999 (1), Tn*5*::PA1003 (2), Tn*5*::PA2361 (3), Tn*5*::PA4915 (4), Tn*5*::PA2588 (5), TBCF10839 wild type (6).

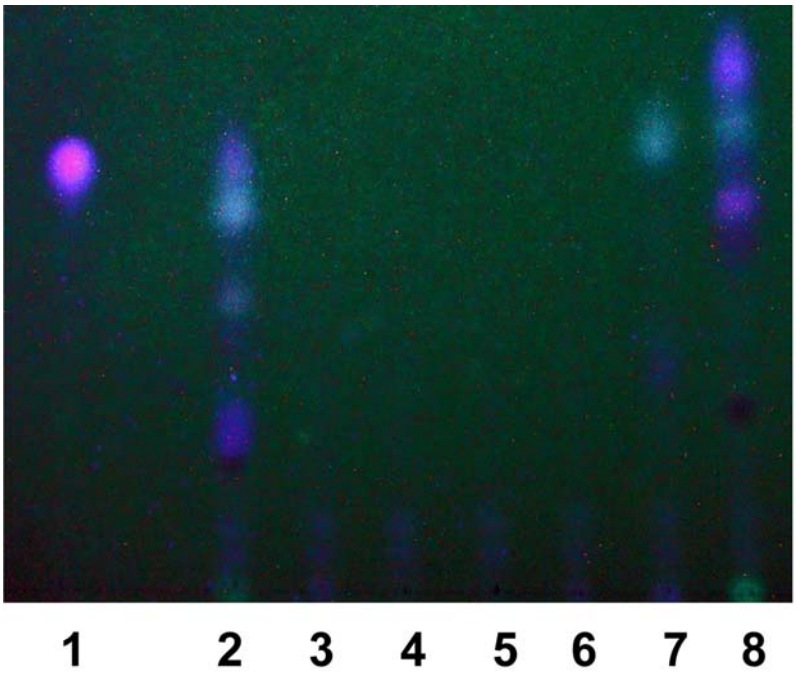

Figure III-5. Thin layer chromatogram of HHQ metabolites. Extracts isolated from TBCF10839 (lane 2) and its isogenic transposon mutants Tn*5*::PA0999 (lane 3), Tn*5:* PA1003 (lane 4), Tn*5*::PA2361 (lane 5), Tn*5*::PA4915 (lane 6), Tn*5*::PA2838 (lane 7), Tn*5*::PA4190 (lane 8), synthesized PQS (standard, lane 1).

The pleiotropic B phenotype was not only caused by the inactivation of members of the HAQ biosynthesis operon (*pqsD* and *mvfR*) (Xiao *et al*., 2006; Wade *et al*., 2005; Farrow *et al*., 2007), but also by that of three yet uncharacterized genes (PA2361, PA2588, PA4915) (Table III-1). The phenotype of these mutants will be described in the following chapters in detail.

Colony type C mutants were light rose or colourless on Congo red agar (Figure III-3 C) suggesting that biofilm formation was affected. The phenotype was shared by mutants of metabolic genes (PA2537, PA2579), a LysR transcriptional regulator (PA2838) and a conserved hypothetical (PA4734) that has numerous orthologs in 'honorary pseudomonads' that are the metabolically related *Burkholderia*, *Ralstonia* and *Xanthomonas* bacteria.

Mutants of the non-piliated TBCF10839 strain (Chang *et al*., 2007) in the pilin biosynthesis genes *pilY1* and *pilW* showed colonies with soft autolysis on LB agar, but not on blood agar or on plates supplemented with or depleted of iron (Figure III-3

D). Autolysis became visible by 9 hours of incubation and was maximal by 48 hours (Figure III-6).

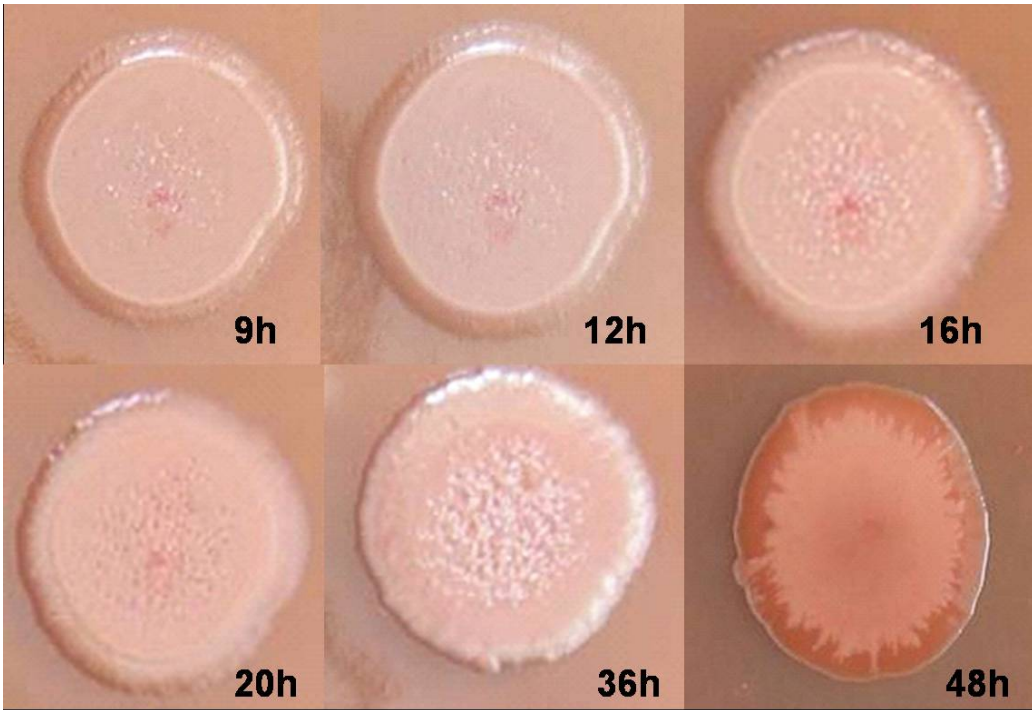

Figure III-6. The autolysis formation by Tn*5*::*pilY1* mutant on LB agar with 40 µg/mL Congo red dye during 48 hours of incubation at 37^{o}C.

This medium-dependent phenotype of cell lysis points to pili-unrelated roles of PilY1 and PilW for the *P. aeruginosa* cell. By Chang (2006) it was shown that the *pilY1* gene plays a role in the biosynthesis of phenazines. PilY1, previously only known as a retraction regulating tip-associated adhesin, was shown also to be involved in the intracellular packaging, handling and/or controlling the release of quinolones to protect the cell against the accumulation of these metabolites (Chang, 2006).

In five mutants the alginate-overproducing TBCF10839 strain had reverted to a non-mucoid phenotype (Figure III-3 E). Two mutants PA0413 and PA0415 are members of one operon of the genes involved in chemotaxis, motility and attachment. Another one (PA4954) is also a membrane protein, a part of the torque generator of the flagellar motor involved in chemotaxis. The last gene from the group encodes a cis/trans isomerase.

On the contrary, the transposon mutagenesis in the 6eB10 mutant induced an even stronger mucoid morphotype (Figure III-3 H). Because no homologies in the PAO1

genome were found after the BLAST search, the region surrounding the Tn*5* insert was obtained by plasmid rescue method. The 8.2 kbp plasmid was multiplied in *E. coli* XL-10 Gold and sequenced. The sequencing revealed that the Tn*5* is inserted in a GC low region of a 38461 kbp genomic island of the *P. aeruginosa* 2192 genome at position 3291786 – 3291972 (Figure III-7 A, B).

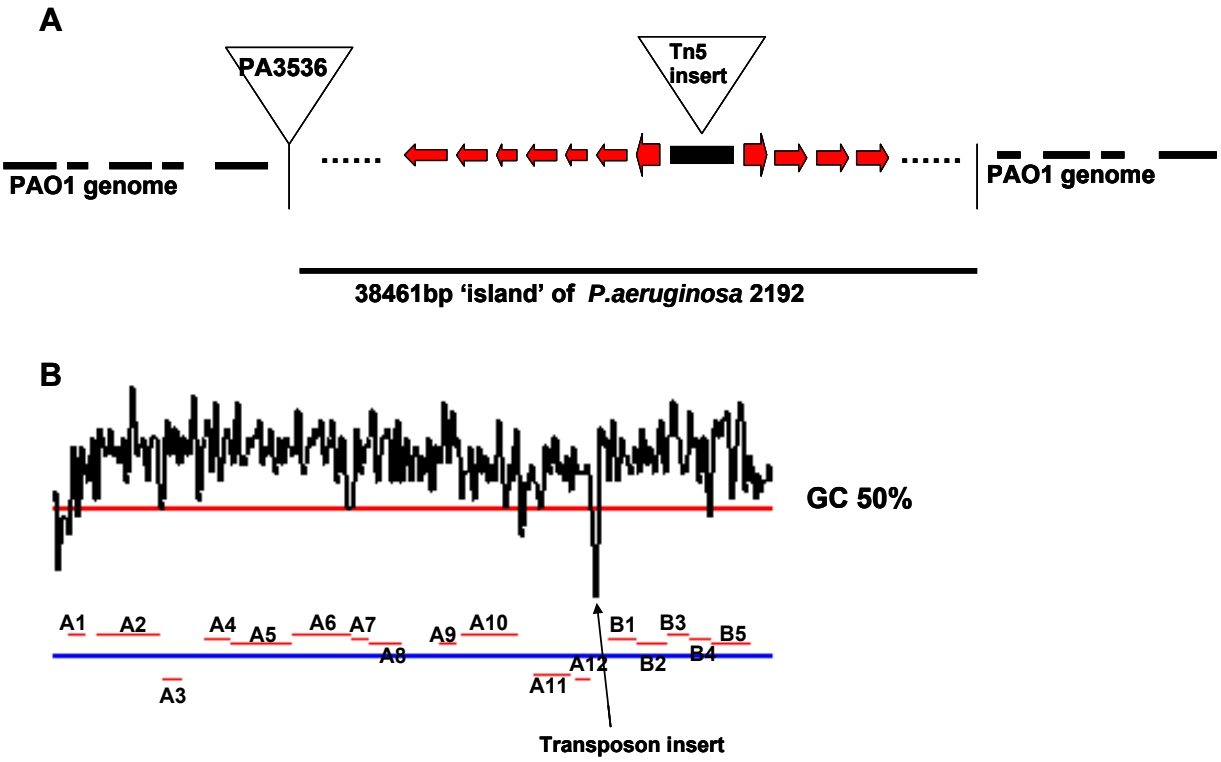

Figure III-7. (**A**) The position of the Tn*5* insert in the PAO1 genome. (**B**) The genes surrounding the Tn5 insert (see Appendix 1).

Two mutants turned orange on Congo red agar due to the knock-out of genes (*fpvR, opmQ*) of the pyoverdine locus (Smith *et al*., 2005; Ravel and Cornelis, 2003) (Figure III-3 F). The antisigma factor FpvR is involved in signal transduction that itself negatively regulates the activity of the extracytoplasmic family sigma factor protein PvdS. It results in the production of the virulence factors: pyoverdine, exotoxin A and others. OpmQ has an over 30% identity with OMF proteins of RND/MFP/OMF-type efflux systems (Poole, 2001; Zgurskaya and Nikaido, 2000).

Furthermore, colonies were smaller when transposon mutagenesis had inactivated one of two core genes of energy- and carbohydrate- metabolism, the

phosphogluconate dehydrogenase Edd that converts 6-phosphate-gluconate to 2-keto-3-deoxy-6- phosphate -gluconate in the Entner-Douderoff pathway (Cuskey and Phibbs, 1985) and the malate:quinone oxidoreductase MqoB that catalyzes the conversion of malate to oxaloacetate in the citric acid / glyoxylate cycles (Kretzschmar *et al*., 2002).

The morphology of TBCF10839 mutants was compared with the transposon mutant collections of the sequenced strain PAO1. TBCF10839 homologues are 99.8 % or more identical in sequence with the respective PAO1 genes. In contrast, the complex trait of a colony morphology variant found in a TBCF10839 transposon mutant was not reproduced by the insertion of a transposon into the homologous gene of the PAO1 strain in 16 of 19 loci indicating that the genetic background is essential for shaping complex phenotypes (Table III-2).

Table III-2. Comparison of colony morphology of TBCF10839 and PAO1 transposon mutants that are inserted into homologous genes.

PAO1 gene number of transposon insertion	**Morphotype***	
	TBCF10839 transposon mutants	**PAO1 transposon mutants****
PA2122+	A	not A; wild type PAO1
PA3462++	A	not A; wild type PAO1
PA3748++	A	not A; wild type PAO1
PA4190++	A	A
PA4489++	A	not A; wild type PAO1
PA0999++	B	B
PA1003++	B	B
PA2361++	B	not B; wild type PAO1
PA4915++	B	not B; wild type PAO1
PA4734++	C	not C; wild type PAO1
PA4552++	D	not D; wild type PAO1
PA4554++	D	not D; wild type PAO1
PA0413++	E	not E; wild type PAO1
PA0415++	E	not E; wild type PAO1
PA1846++	E	not E; wild type PAO1
PA4954++	E	not E; wild type PAO1
PA2388+	F	not F; wild type PAO1
PA2391++	F	not F; wild type PAO1
PA4640+	G	not G; wild type PAO1

*See Table III-1, footnote #

**PAO1 mutants were provided by the University of Washington Genome Center "*Pseudomonas aeruginosa* PAO1 mutant collection" (http://www.genome.washington.edu) (Jacobs *et al*., 2003).

+One PAO1 transposon mutant; ++Two PAO1 transposon mutants with an insert at the 5' end (mutant 1) or at the 3' end (mutant 2) of the gene.

All other 25 mutants listed in Table III-3 after several repeats did not show a robust, stable morphotype distinct from TBCF10839 wild type strain, but together with others were included in the STM competition experiments (Table III-3).

Table III-3. STM Tn*5* TBCF10839 colony morphology variants: media and culture conditions[1] used in the study that led to detectable changes in morphotype.

PAO1 gene number of transposon insertion	**1 LB agar**	**2 LB agar + Congo red dye**	**3 LB agar supplemented with iron (Fe^{2+})**	**4 LB agar depleted from iron**	**5 LB agar / *LB agar + Congo red dye**	**6 LB agar**	**7 LB agar[1]**	**8 Minimal medium (M9 agar)**	**9 Blood-agar**
	37°C				**22 – 25°C**	**42°C**	**4°C**	**37°C**	
PA0424	**x**	**x**	**x**	**x**	**x**	**x**		**x**	**x**
PA2028	**x**	**x**	**x**	**x**	**x**	**x**		**x**	**x**
PA2122	**x**	**x**	**x**	**x**	**x**	**x**		**x**	**x**
PA3462	**x**	**x**	**x**	**x**	**x**	**x**		**x**	**x**
PA3748	**x**	**x**	**x**	**x**	**x**	**x**		**x**	**x**
PA4190	**x**	**x**		**x**	**x**	**x**			**x**
PA4489	**x**	**x**	**x**	**x**	**x**	**x**		**x**	**x**
PA5524	**x**	**x**	**x**	**x**	**x**	**x**		**x**	**x**
A1	**x**	**x**	**x**	**x**	**x**	**x**		**x**	**x**
A2	**x**	**x**	**x**	**x**	**x**	**x**		**x**	**x**
A3	**x**	**x**	**x**	**x**	**x**	**x**		**x**	**x**
PA0999		**x**	**x**						**x**
PA1003		**x**	**x**						**x**
PA2361		**x**	**x**						**x**
PA4915		**x**	**x**						**x**
PA2537		**x**							
PA2579		**x**							
PA2588		**x**							

PAO1 gene number of transposon insertion	**1 LB agar**	**2 LB agar + Congo red dye**	**3 LB agar supplemented with iron (Fe^{2+})**	**4 LB agar depleted from iron**	**5 LB agar / *LB agar + Congo red dye**	**6 LB agar**	**7 LB agar[1]**	**8 Minimal medium (M9 agar)**	**9 Blood-agar**
PA2838		**x**							
PA4734		**x**							
PA4552	**x**	**x**							
PA4554	**x**	**x**							
PA0413					**x / x***				
PA0415					**x / x***				
PA1846					**x / x***				
PA4915					**x / x***				
PA4954					**x / x***				
PA2388	**x**	**x**							
PA2391	**x**	**x**							
PA3194	**x**	**x**				**x**			**x**
PA4640	**x**	**x**				**x**			**x**
H1	**x**	**x**	**x**						**x**
PA0482		**o**							
PA0728		**o**							
PA0785		**o**							
PA0920		**o**							**o**
PA1589		**o**							
PA1633		**o**							
PA1823		**o**							**o**
PA2706		**o**							
PA2946	**o**	**o**							

PAO1 gene number of transposon insertion	**1 LB agar**	**2 LB agar + Congo red dye**	**3 LB agar supplemented with iron (Fe^{2+})**	**4 LB agar depleted from iron**	**5 LB agar / *LB agar + Congo red dye**	**6 LB agar**	**7 LB agar[1]**	**8 Minimal medium (M9 agar)**	**9 Blood-agar**
PA3012		**o**							
PA3238		**o**							
PA3239		**o**							
PA3804		**o**							**o**
PA4131		**o**		**o**					
PA4703		**o**							
PA4797		**o**							
PA4949		**o**							
PA4951	**o**	**o**							
PA5121		**o**						**o**	
PA5231		**o**							
PA5546	**o**	**o**							
PA5563		**o**							
UK1		**o**							
PR1		**o**							
PR2		**o**							**o**

X Stable morphotype distinct from wild type; **O** Unstable morphotype with rapid reversion to wild type morphotype. [1] Cultures of conditions 1–6, 8, 9 were examined after 48 h cultures in LB agar at 4°C were examined every day for a period of 21 days.

1.3. Survival of colony morphology variants in a murine airway infection model

To mimic the dissociative behaviour of *P. aeruginosa* in lung infections, the 57 isogenic colony morphology variants were tested for their competitive fitness to survive in murine airways and to spread to other organs. For this, mutants were grown separately in LB agar and liquid by overnight incubation. Pools of 25 mutants each of which harbouring different signature tags were instilled into murine airways (three mice per experiment) and bacteria were recovered 48 hours later from lungs, spleen and liver and cultivated on LB. In parallel, bacteria from the control plates after 48 h hours of incubation were plated again on fresh LB and incubated in the same incubator. Genomic DNA was prepared from both input and output pools, and an amplification of the signature tags was performed by PCR (Figure III-8).

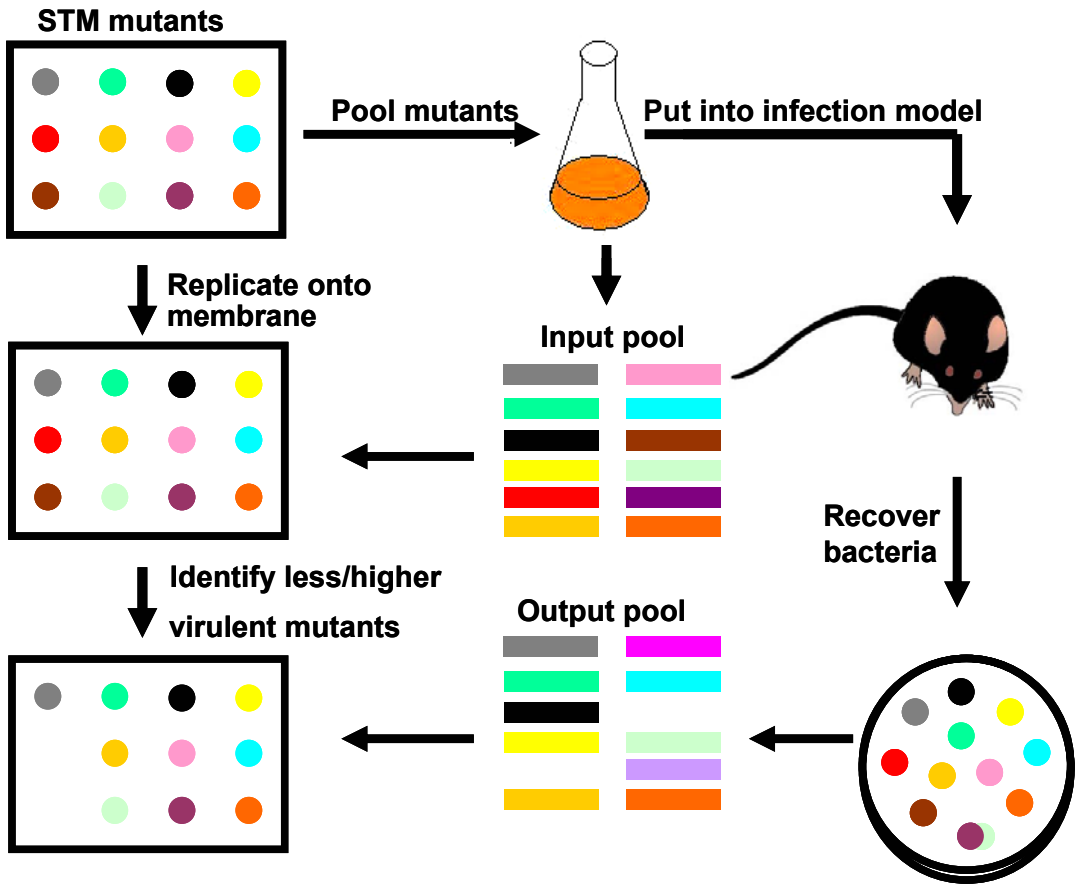

Figure III-8. The scheme of the STM competition experiment *in vivo*. Transposon mutants with different signature tags were separately grown and pooled together directly before mice infection. From this pool, one aliquot of bacterial suspension was cultivated on LB agar or liquid (input pool) and another one was used for the intratracheal mice infection (output pool).

The amplified signal sequences were hybridized on dot blots, which were previously prepared by PCR amplification of signal sequences from selected mutants. The signal intensities from the output pool were compared with the signal intensities from the input pool. The evaluation of the selection results was performed by determining the optical density on a X-ray film after hybridization. These X-ray films were scanned and densities of the respective dots were determined with the PCBAS program, version 2.09f. An example of the scanned blot for selection is shown in the Figure III-9. The dot blots show transposon mutants with higher (1) or lower survival (2) in vivo and mutants that were detected at similar rates (4) or not at all (3) from the pools of mutants grown in vitro or in vivo.

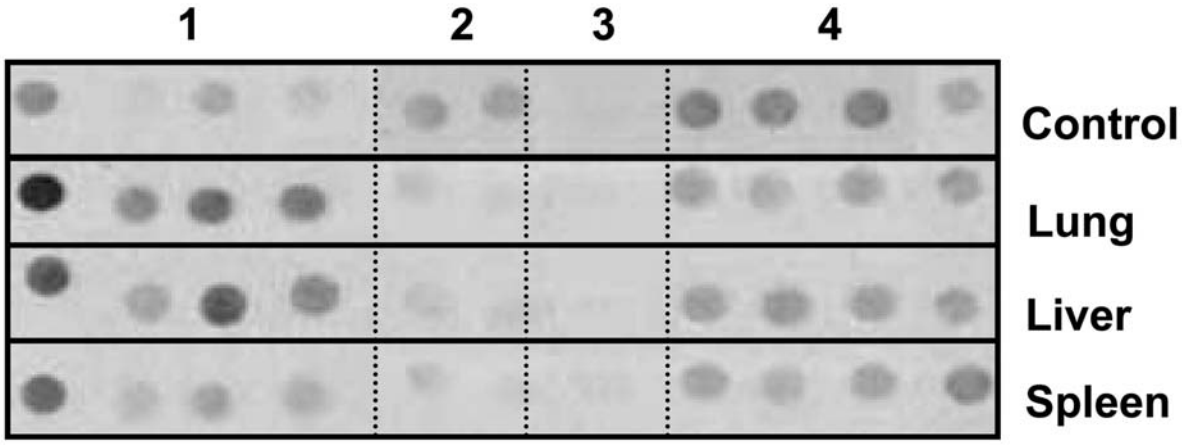

Figure III-9. Dot blot hybridization of oligonucleotide signature tags to determine the survival of individual *P. aeruginosa* TBCF10839 colony morphology mutants in competition experiments. DIG-labeled, *Hin*dIII-digested signal sequences isolated from bacteria not subjected to selection (control) or from bacteria recovered from organs after murine infection experiments (liver, lung and spleen) were hybridized onto dot blots of the signal sequences of the pTnModOGm SigTag. Signal intensities indicate mutants with high (1) or low (2) survival rates *in vivo*, and mutants growing poorly (3) or at similar rates (4) *in vitro* and *in vivo*. Transposon insertions (from left to right) into the TBCF10839 homologs of 1, PA4131; 2, PA4552; 3, PA4954; 4, PA4734; 5, PA0999; 6, PA4640; 7, phage phiCTX ORF37; 8, PA3194; 9, PA5231; 10, PA2388; 11, PA5563; 12, PA1589.

All in all six STM competition experiments were done. After four experiments the mutants with the least and the highest survival were separately tested in a fifth and sixth round (Figure III-10).

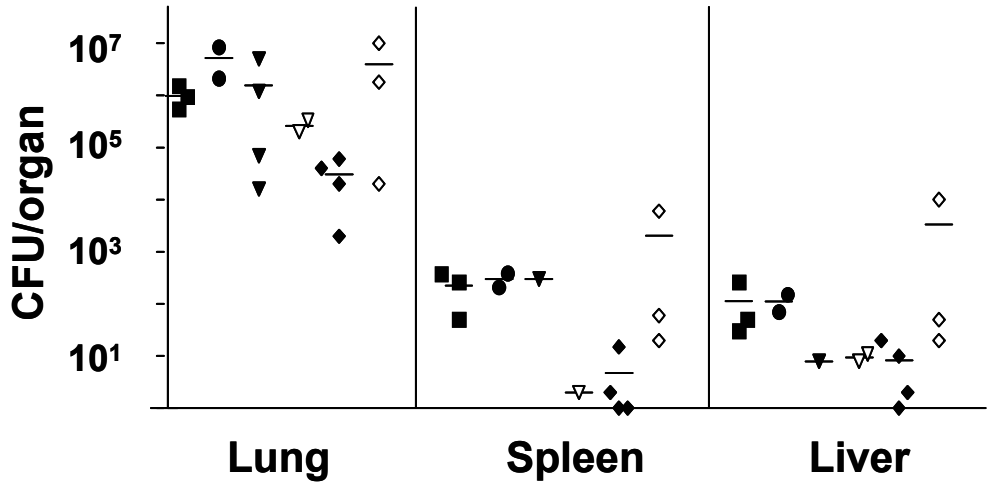

Figure III-10. Total CFU of *P. aeruginosa* TBCF10839 STM colony morphology mutants recovered from murine organs 48 h after intratracheal instillation of up to 25 differentially tagged mutants. The mutants with the lowest (closed diamonds) and highest survival (open diamonds) in the first four experiments (closed square, closed circle, closed triangle, open triangle) were pooled and tested again.

The competition experiments revealed 17 mutants having other than wild type behaviour *in vivo*. Table III-4 lists the 17 colony morphology mutants that grew better (six mutants, category 1) or worse (seven mutants, category 2) than their competitors *in vivo* or that were globally compromised in growth (four mutants, category 3).

Table III-4. STM competition experiments. *P. aeruginosa* TBCF10839 colony morpholoy mutants with higher or lower fitness in acute murine airway infection.

Map position of Tn*5* mutant: gene number in PAO1	**Annotation**
Category 1: "gain of function" (enhanced survival *in vivo*)	
PA4131	Probable iron-sulfur protein
PA4552	*pilW* , type 4 fimbrial biogenesis protein
PA4554	*pilY1*, type 4 fimbrial biogenesis protein
PA4734	Conserved hypothetical protein
PA4954	*motC*, chemotaxis protein
PA5546	Hypothetical protein; predicted cyclopropane fatty acid synthase (Cfa)

Map position of Tn*5* mutant: gene number in PAO1	**Annotation**
Category 2: "loss of function" (reduced survival *in vivo*)	
PA0999	*pqsD*, 3-oxoacyl-[acyl-carrier-protein] synthase III
PA2537	Probable acyltransferase
PA2588	Probable transcriptional regulator
PA2706	Hypothetical protein
PA2838	Probable transcriptional regulator
PA3239	Hypothetical protein; predicted surface lipoprotein (VacJ)
PA4640	*mqoB,* malate:quinone oxidoreductase
Category 3: "non-competitive" (no survival *in vitro* and *in vivo*)	
PA0785	Probable acyl carrier protein phosphodiesterase
PA3194	*edd*, phosphogluconate dehydratase
PA4916	Hypothetical protein; predicted ADP-ribose pyrophosphatase
phiCTX	*Pseudomonas* phage phiCTX, hypothetical protein

Twelve of the 17 mutants belonged to the group with robust morphotypes which included all type D and G, three type C, two type B, two type E and one mutant of the most abundant category A of strains with glossy autolysis.

High fitness *in vivo* was associated with a change of extracellular texture (PA4734, *motC*), impaired swarming and swimming (*motC*) or predisposition to cell lysis (*pilW, pilY1*). The wild-type TB strain is non-piliated due to a deletion in the *pilQ* gene (Chang *et al*., 2007) and hence any secondary mutations in pilin biogenesis genes would be phenotypically silent with respect to pilin production. The autolysis of the mutants, however, points to further features of PilW and PilY1 that are unrelated to pilin biogenesis and should confer the higher fitness of the mutants in the STM infection experiments.

Low fitness in murine airways was observed in metabolic- (PA2537, *mqoB*), regulators of transcription (PA2838, PA2588) or HAQ-deficient (*pqsD*) mutant morphotypes.

In case of the globally compromised mutants, the transposon had disrupted a gene that is essential for survival in a community of isogenic TB strains. Not surprisingly mutants were carrying the transposon in metabolic genes (PA0785, *edd* and PA4916). In the last mutant listed in the Table III-4 the transposon mutagenesis had hit the phiCTX phage that probably made this strain vulnerable to the attack of phage–proficient competitors so that it could not survive. Besides this phage mutant, the susceptibility to autolysis apparently conferred neither an advantage nor disadvantage to the strains to persist in murine airways and to spread to other organs. All non-competitive mutants from the third category were not auxotrophic and could grow in pure cultures, but could not successfully compete for nutrients in a microbial community of isogenic mutants.

Thus, the STM technique used for the generation of the library has an advantage to allow simultaneous examination of a large number of isogenic transposon mutants using unique DNA marker sequences for differentiation. The screening a pool of mutants exposed to a distinct habitat of interest revealed 17 loss- or gain- of function mutants with a worse or better survival. To get an overview of targeted genes, the knocked-out mutants were tested with various bioassays and will be described in the following chapters.

2. Analysis of the targeted genes

After identification of new virulence genes, different assays were performed with knock-out mutants to reveal mechanisms by which one or another gene is responsible for an unusual phenotype. These assays include many parameters: analysis of secreted virulent factors, survival rate *in vivo*, biochemical assays, transcriptome profiles and others. Figure III-11 presents the scheme of the experimental steps from the beginning (screening *in vitro* and *in vivo* was already described in previous chapters) to the detailed analysis of the targeted genes (Figure III-11). The main goal of the analysis of the targeted genes is our understanding of the function of the gene, its role in adaptation, survival and persistence of *P. aeruginosa* in the living organism.

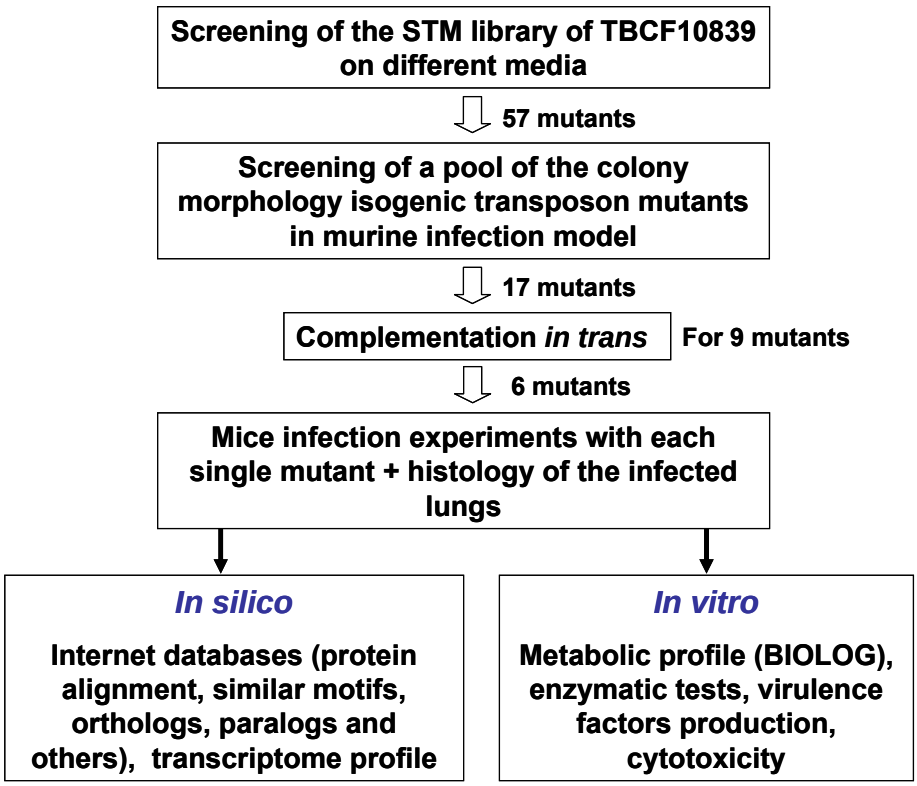

Figure III-11. Scheme of the experimental steps.

First of all, the causative role of the transposon-inactivated gene for morphotype was tested for a subset of nine mutants (Tn*5*::PA3194, Tn*5*::PA4640, Tn*5*::PA4916, Tn*5*::PA0785, Tn*5*::PA4131, Tn*5*::PA4552, Tn*5*::PA4554, Tn*5*::PA0999 and Tn*5*::PA1003) by complementation of the full length gene *in trans.* Complementation

was performed by using plasmids pME6010 (Heeb *et al*., 1988) or pUCP20 (Garrity-Ryan *et al*., 2000). The genes with their own promoter regions were amplified from TBCF10839 by PCR and cloned into one of the vectors.
In the beginning, the pME6010 plasmid was used for the complementation of Tn*5*::*pilW* and Tn*5*::*pilY1* mutants. However, the large size of the pME6010 plasmid (8,3kb) is a disadvantage, especially given the difficulties of cloning and transformation of large fragments into the cell. As an alternative, the smaller 3898 bp shuttle vector pUCP20 was used for the complementation, especially for the long length fragments.

After the complementation, the representative mutants of the three categories were retested separately in mice infection experiments in order to differentiate between the virulence for the host and the fitness within the bacterial community. Infection experiments were carried out for six perspective mutants listed in the Table III-4 (Tn*5*::PA3194, Tn*5*::PA4640, Tn*5*::PA4916, Tn*5*::PA2537, Tn*5*::PA4954, and Tn*5*::PA0785). This non-invasive application technique (Munder *et al*., 2002) via catheter allowed controlling of the delivery of the bacteria to the lungs. During 14 days of infection, the weight and rectal temperature (Appendix 3) of the mice were measured daily and their body condition was determined using a self-developed score (Munder *et al*., 2005). Two mice were sacrificed after 48 hours for the evaluation of lung histology or the determination of CFU in homogenized lungs, liver, spleen and brain.
For the same set of mutants the metabolic profile by testing the bacterial culture on different chemicals was preformed by Phenotypic Microarrays (PMs) analysis. Different chemicals were tested in 96 well microtiter plates (Figure III-12).
For this study, 5 micro (PMs) plates containing major substrates for the assimilation of carbon (181 C-sources, PM1, PM2A), nitrogen (95 N-sources, PM3B), phosphor and sulfur sources (59 P- and 35 S-sources, PM4A) were used. One plate was used to test the stimulation of bacterial growth by nutrient supplements (95 compounds, PM5) (http://www.biolog.com). PMs method allows testing many chemicals simultaneously and comparing a methabolic profile of the mutant with wild type strain(s).

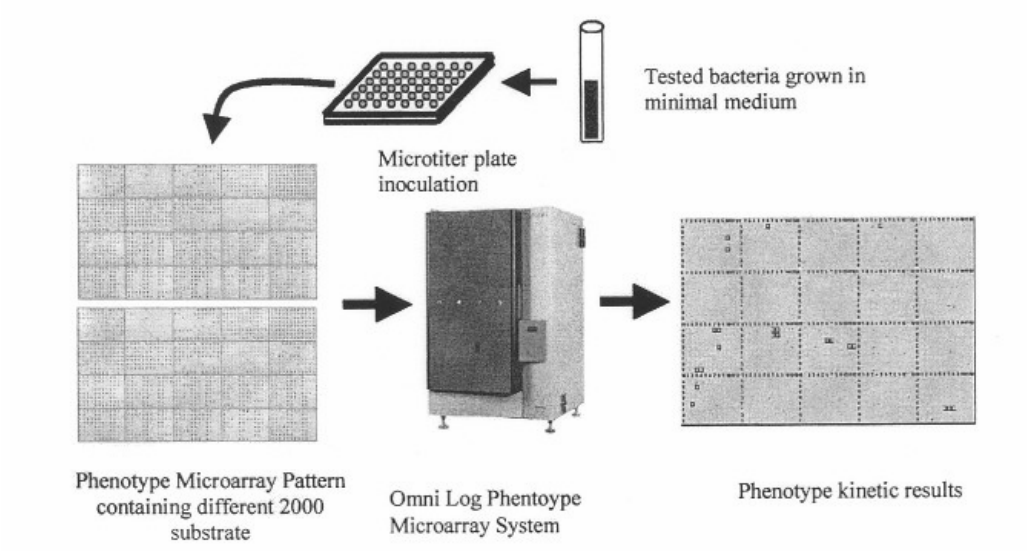

Figure III-12. The procedure of testing of the bacterial cells phenotype (Bochner *et al*., 2001). Aliquots of the microbe are incubated in the microtiter well plates, and respiration is monitored automatically over time via the OmniLog robot. The OmniLog instrument reads and records the color change in PM assays. The instrument cycles microplates in front of a color CCD camera to read 50 in as little as 5 min and provides quantitative and kinetic information about the response of cells in the PMs. Data are stored directly into computer files and can be recalled and compared with other data at any time.

Additionally, other biochemical phenotypic assays were performed. Thus, the testing of metabolic knock-out genes (*mqoB* and *edd*) on different carbon sources helped to get additional information about the phenotype of the mutants.

At the same time, *in silico* analysis of the knocked out genes was performed by application of different internet databases as well as the transcriptome data for the estimation of the gene expression. The microarray technique permits the quantification of specific genes and their expression patterns in a comprehensive genome-wide framework (Ferea and Brown, 1999; Lipshutz *et al*., 1999).

In our study the transcriptome data of different strains previously generated by Salunkhe (2003) in our laboratory was used. The transcription activity of the targeted genes was provided by the mRNA microarray expression of different *P. aeruginosa* strains: pairs of clonal variants TBCF10839 and 892 (Tümmler *et al*., 1991), the reference strain PAO1 (Stover *et al*., 2000), the CF isolates CHA (Dacheux *et al*., 1999), LES400 and LES431 (Salunkhe *et al*., 2005).

For the transcriptome analysis, the strains had been grown under different growth conditions and the absence or presence of stressors such as low iron, exposure to hydrogen peroxide, paraquat or human neutrophils (Salunkhe *et al*., 2005) (Appendix

2). To confirm the transcriptome data, quantitative PCR of the knocked out genes of Tn*5*::PA4640, Tn*5*::PA3194 and Tn*5*::PA4916 mutants was performed.
Additionally, other bioassays were performed for the targeted genes: cytotoxicity of the mutants, enzymatic assay and virulence factors production.

In the following chapters, the phenotype of the most striking isogenic colony morphology variants: Tn*5*::PA4131, Tn*5*::PA3194, Tn*5*::PA4640, Tn*5*::PA4916, Tn*5*::PA0785 and PA4954 will be described after testing with the various bioassays in detail. Additionally, a separate chapter will describe the phenotype of the HAQ deficient mutants being essential for the production and regulation of quinolones and phenazines known as major compounds involved in the pathogenicity of *P. aeruginosa*.

2.1. Features of "gain of function" mutants

Six mutants listed in Table III-4 were higher in fitness after the STM competition experiments *in vivo.* To get an overview about targeted genes, two (PA4131 and PA4954 (*mot C*)) out of six were chosen for the detailed analysis.

2.1.1. Tn*5*::PA4131, predicted iron-sulphur protein

One of the mutants with an enhanced survival in lungs (Table III-4) has a PA4131 gene knock-out, which encodes for an iron- sulphur protein related to genes involved in energy production and conversion. The protein harbours 4Fe-4S ferredoxins sequence signature and has orthologs in many species. PA4131 possesses a conserved 4Fe-4S ferredoxin domain including four cystein residues that bind to a [Fe_4S_4] cluster. PA4131 is a redox protein integrated in cytoplasmic membrane and is predicted to be one of the nitrogen fixation polyferredoxin proteins NapH. Nap proteins have been found in many different organisms, where they fulfill different physiological roles depending on the species. They can function as electron sinks, e.g. during photosynthesis in *Rhodobacter sphaeroides* (Richardson and Ferguson, 1992; Reyes *et al*., 1996); they are used for redox balancing during aerobic respiration on highly reduced carbon sources, e.g. during growth of *Paracoccus* with butyrate as the main source of carbon and energy (Sears *et al*., 2000); and they may be used during anaerobic respiration, as in *E. coli* (Potter *et al*., 2001).

The gene seems to play an important role for TBCF10839 according to the high level of transcription activity (Appendix 3) being not involved in the metabolism of the cell (Table III-5).

Table III-5. PMs data of *P. aeruginosa* TBCF10839 Tn*5::*PA4131*.*

Test[a]	**Difference**[b]		**Mode of action**
	TBCF10839	**PAO1**	
L-Malic Acid		-60	C-source
Dihydroxy-Acetone	-142		C-source
Phosphoryl Choline		-71	P-source

(***a***) Chemicals were tested in 96-well PMs. (***b***) The OmniLog-PM software generates time course curves for respiration (tetrazolium color formation) and calculates differences between the areas for mutant and control cells. The units are arbitrary. Positive values (see below) indicate that the mutant showed greater rates of respiration than the wild type strains (TBCF10839 and PAO1). Negative values indicate that the control showed greater rates of comparisons respiration than the mutant. The differences are averages of pairwise comparisons. All assays were performed in duplicate.

As shown in Table III-5 the mutant was compromised to utilize one substrate (dihydroxy-acetone) only in comparison with wild type.

PA4131 is a redox protein and seems to be important for *P. aeruginosa.* The protein might be an enzyme of the secondary metabolism, not being involved in key metabolic pathways according to the PMs data. If gene is inactivated, the other compensatory mechanisms will be switched on to stabilize the cell state in order to increase the adaptive properties of the pathogen under the stress conditions present in the lung.

2.1.2. Tn*5*::PA4954, *motC*

The *P. aeruginosa* genome contains dual sets of *motAB*-like genes PA1460-PA1461 and PA4954-PA4953 (*motCD*), as well as another gene, *motY* (PA3526), which is known to be required for motor function in some bacteria (Doyle *et al*., 2004). The MotC protein was first identified as RpmA encoding membrane proteins that use membrane potential to conduct ions are required for the rotation of the flagellar motor. The phenotype of the *motC* mutant of the *P. aeruginosa* CF isolate 4020 was shown to be resistant to nonopsonic phagocytosis (Simpson and Speert, 2000). The *motCD* locus was required for the ingestion of *P. aeruginosa* by murine macrophages which represent the primary defense against *P. aeruginosa* in airways (Simpson and Speert, 2000).

The mice infection experiments showed the *motC* mutant was most virulent and most heavily growing in murine organs (Figure III-12 A, B). The histology pictures (Figure III-12 C) indicate significant abnormality in lung architecture and strong inflammatory response by the infected cells. Both the mutant and wild type showed a strong purulent inflammation with intra- and peribronchial infiltrates of leucocytes. Animals infected with TBCF10839 wild type developed a profound, diffuse, necrotic, purulent and fibrinogenous pneumonia. The inflammation was characterised by perivascular oedema and intravascular thrombi. The pneumonia generated by the mutants was similar but less pronounced compared to the wild type, but much stronger compared to the vehicle control (Figure III-13 C, D, E).

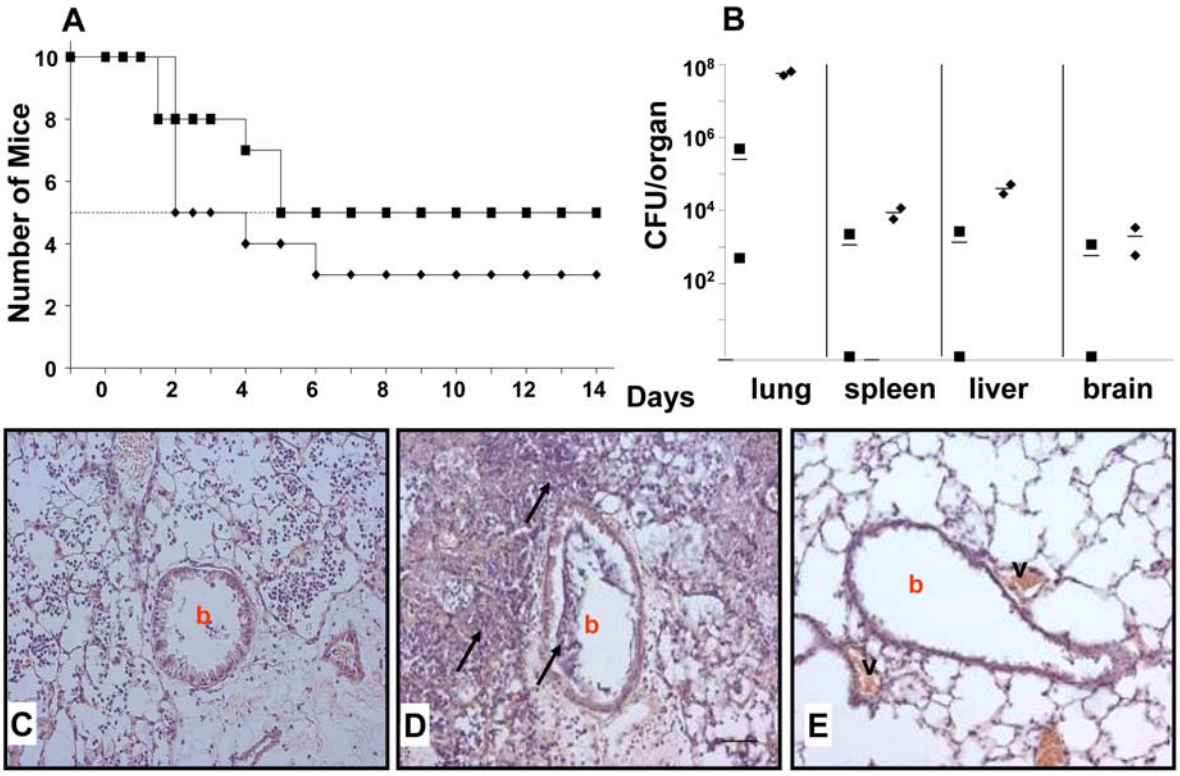

Figure III-13. (A) Survival of mice whose airways had been inoculated at day 0 with 5x10^7 CFU of *P. aeruginosa* TBCF10839 (closed square) and Tn*5*::PA4954 (closed diamond). (B) CFU recovered from murine organs 48 h after intracheal instillation of TBCF10839 (closed square) and Tn*5*::PA4954 (closed diamond). (C, D, E) Lung histology 48 h after infection with Tn*5*::PA4954 (C), TBCF10839 wild type (D) or vehicle control (30 µl PBS) (E). Hematoxylin-eosin stain; original magnification x 200, bar 50 µm. b, bronchus; v, vessel; inflammatory infiltrates are marked by arrows.

On top of that, an unexpected faster turnover of nutrients by the *motC* mutant was noted (Table III-6). The mutant more efficiently metabolized glucose, malate, aromatic amino acids, ammonia and biogenic amines than the wild type strain.

Table III-6. PMs data of *P. aeruginosa* TBCF10839 Tn*5::motC.*

Test[a]	**Difference**[b]		**Mode of action**
	TBCF10839	**PAO1**	
p-Hydroxy-Phenylacetic Acid	83		C-source
L-Malic Acid	68		C-source
D-Glucose		85	C-source
p-Hydroxy-Phenylacetic Acid		76	C-source
L-Glutamine	104		N-source
Acetamide	98		N-source
L-Phenylalanine	66		N-source

Test[a]	Difference[b]		Mode of action
	TBCF10839	PAO1	
Histamine	51		N-source
Ammonia	50		N-source

(***a***) See Table III-5, footnote ***a***

(***b***) See Table III-5, footnote ***b.***

Thus, the *motC* gene was shown to enhance survival of the pathogen in the lung. Such unusual phenotype of the mutant might be explained by a reciprocal cross-control between the type III secretion (TTSS) process and flagellum assembly in *P. aeruginosa* described recently (Soscia *et al*., 2007). The expression of the *P. aeruginosa* TTSS regulon had been shown to be upregulated in a nonflagellated background, which results in an increase of TTSS effector secretion and of cytotoxicity on macrophages.

Taking into account that the antiphagocytic TTSS is dependent on close contact with the host, it might be appropriate for the bacteria to shut down the flagellum required on the one hand for movement and on the other hand for phagocytosis stimulation. At the same time, the inactivation of *motC* gene increases the ability of the mutant to process various nutrients, which also has a positive impact on pathogen survival.

Hence, the inactivation of the MotC/MotD torque generator is beneficial for *P. aeruginosa* to inhabit the atypical niche of the mammalian lung and to reinforce virulence. It is advantageous for the bacterium to stop producing this appendage at some stages of the infection process in order to escape the host nonspecific immune response.

2.2. Features of “loss of function” mutants

Seven mutants after the STM competition experiments in mice showed different behaviour to the wild type and were less in fitness. Four of these mutants belong to genes of energy metabolism. In this chapter two targets: predicted acyltransferase (PA2537) and malate:quinone oxidoreductase (*mqoB*) were chosen for detailed analysis. Two genes related to the HAQ regulation and biosynthesis (PA0999 and PA2588) were contributed together with other genes involved in HAQ metabolism and will be described in the separate chapter (see chapter III-2.3).

2.2.1. Tn*5*::PA2537, predicted acyltransferase

One of the mutants with reduced survival *in vivo* had the PA2537 gene knocked out which encodes the enzyme acyltransferase (fatty acid and phospholipid metabolism). PA2537 has orthologs in many species due to conserved acyltransferase domain. The enzyme is probably 1-acyl-glycerol-3-phosphate acyltransferase accordingly to the information from the pseudomonas website (http://www.pseudomonas.com).

The Tn*5*::PA2537 mutant is one of the mutants compromised for the binding of Congo red dye was less competitive in the STM competition experiments and impaired in mice infection experiments (Figure III-14 A).

During infection, the mutant was less disseminated in the liver and spleen, but not in the lung (Figure III-14 B). The histology images did not show significant abnormality in lung architecture. Compared to a strong purulent inflammation induced by the wild type, only slight peribronchial and perivascular inflammation was detected in Tn*5*::PA2537 infected cells (Figure III-14 C, D, E).

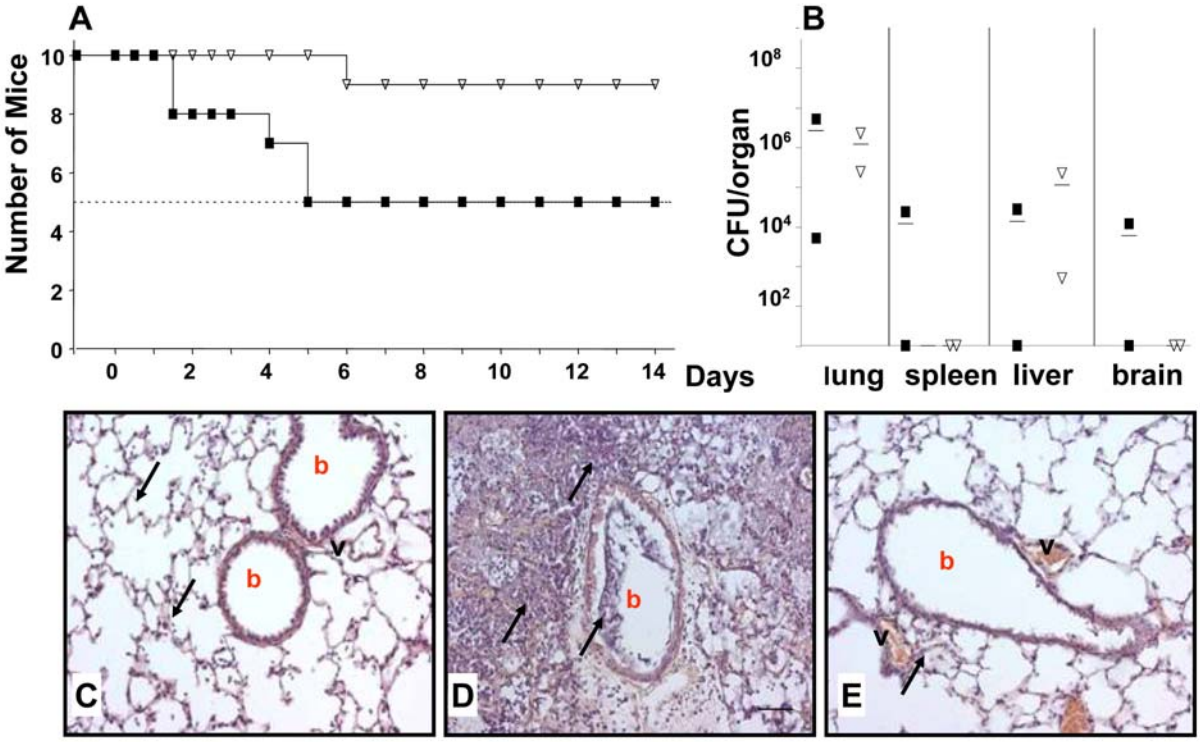

Figure III-14. (A) Survival of mice whose airways had been inoculated at day 0 with $5x10^7$ CFU of *P. aeruginosa* TBCF10839 (closed square) and Tn*5*::PA2537 (open triangle). (B) CFU recovered from murine organs 48 h after intracheal instillation of TBCF10839 (closed square) and Tn*5*::PA2537 (open triangle). (C, D, E) Lung histology 48 h after infection with Tn*5*::PA2537 (C), TBCF10839 (D) or vehicle control instilled with 30 µl PBS (E). Hematoxylin-eosin stain; original magnification x 200, bar 50 µm. b, bronchus; v, vessel; inflammatory infiltrates are marked by arrows.

The metabolic profile of the mutant did not reveal any essential phenotypic differences compared with wild type strains TBCF10839 and PAO1.

Table III-7. PMs data of *P. aeruginosa* TBCF10839 Tn*5*::PA2537.

Test[a]	Difference[b]		Mode of action
	TBCF10839	PAO1	
Dihydroxy-Acetone		53	C-source
L-Malic Acid		-99	C-source
Allantoin	-68		N-source

(***a***) See Table 3.5, footnote ***a***

(***b***) See Table 3.5, footnote ***b.***

Based on the reported phenotype, it is difficult to predict a function of the enzyme. If the enzyme belongs to the group of proteins involved in energy metabolism, we were expecting more substrates on which the mutant cannot properly grow. The lesser

binding of Congo red dye also might indicate that the mutant might be defective in the production of exopolysaccharides and biofilm formation. However, PMs data revealed that the mutant is compromised for utilization of one substrate only compared with wild type. It means that the reduced survival of the mutant is not linked with key metabolic processes and depends on either secondary metabolism or other unknown mechanisms.

2.2.2. Tn*5*::PA4640, malate:quinone oxidoreductase (*mqo*)

MQO is a FAD-dependent membrane-associated protein that catalyses the oxidation of malate to oxaloacetate. The electrons are donated to quinones of the electron transfer chain and NAD will not be accepted as electron acceptor (Kather *et al*., 2000).

The *P. aeruginosa* genome encodes one poorly expressed cytosolic NAD-dependent malate dehydrogenase (EC 1.1.1.37; PA1252) and two membrane-bound malate-quinone oxidoreductases (MqoA, MqoB). The two latter FAD-dependent enzymes catalyze the conversion of malate to oxaloacetate whereby the electrons are donated to quinones of the electron transfer chain.
MQO activity in *P*. *aeruginosa* PAO1 has been described by Mizuno and Kageyama (1978) as a membrane-associated malate dehydrogenase (MDH) activity that uses dichlorophenolindophenol and phenazinemethosulfate as electron acceptors. MQO has previously been studied in *Corynebacterium glutamicum* (Molenaar *et al*., 1998), *Helicobacter pylori* (Kather *et al*., 2000) and *Escherichia coli* (van der Rest *et al*., 2000). In *C*. *glutamicum* and *E*. *coli*, both acytoplasmic NAD-dependent MDH and a membrane associated MQO are present. In *C*. *glutamicum*, a deletion in *mqo* resulted in a mutant whose growth defects could be eliminated by growth in the presence of nicotinamide; apparently, the NAD-dependent MDH was able to take over the function of MQO (Molenaar *et al*., 2000). In *E*. *coli*, the deletion of *mqo* did not result in an observable phenotype (van der Rest *et al*., 2000).

The morphotype of *mqoB* mutant was described as a small size colony (not SCVs). Another feature of the *mqoB* mutant is that it cannot grow on minimal medium with ethanol as a carbon source. The indicated phenotype was also described for the

mqoB mutant of *P. aeruginosa* ATCC17933 strain. The wild type growing aerobically on ethanol was able to use a pyrroloquinoline quinone-dependent ethanol oxidation system, whereas the mutant with an interrupted putative *mqo* gene was defective, showed a severe growth defect on ethanol and was unable to grow on acetate (Kretzschmar *et al*., 2002).

The measurement of MQO activity was performed to confirm previous results that the activity of the enzyme is associated with the membrane fraction. Wild-type *P. aeruginosa* TBCF10839, as well as mutant Tn*5*::*mqoB*, were grown on glucose as carbon source. Cells were collected by centrifugation at the late-exponential phase and disrupted by sonication. The membrane fraction was separated from the soluble cytoplasmic and periplasmic fractions by ultracentrifugation at 100000 x *g*, and tested for MQO activity. The activity of the enzyme is shown in Figure III-15. The membrane fraction of the wild-type cells exhibited MQO activity higher than the membrane fraction from *mqoB* mutant. The residual activity by mqoB mutant might be explained by the activity of a second *mqo* gene (*mqoA*).

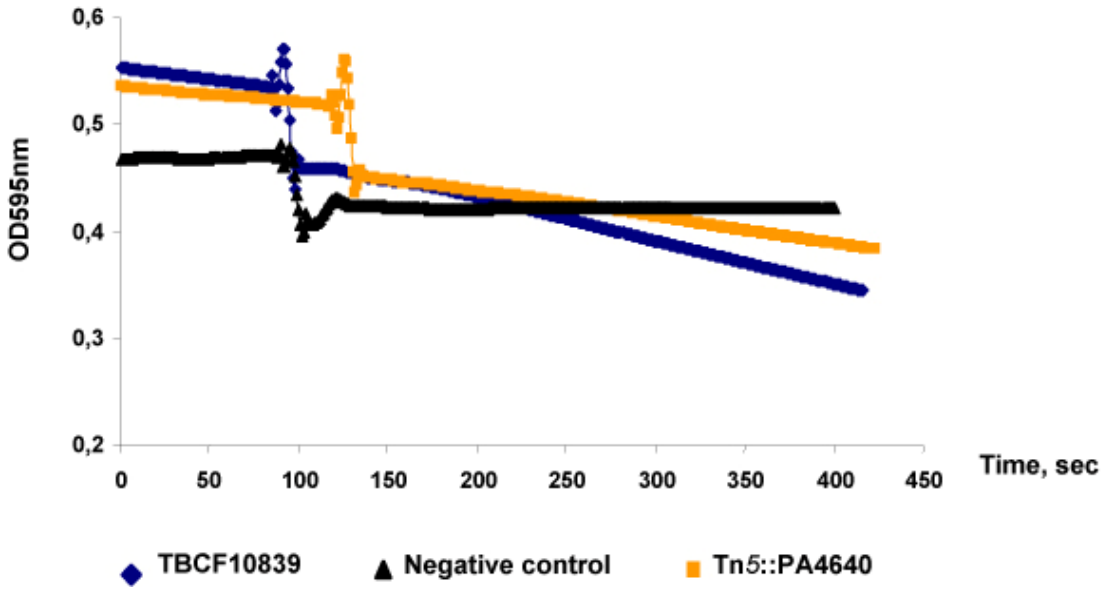

Figure III-15. MQO activity by TBCF10839 and *mqoB* mutant was measured by a decrease in absorbance of 2,6-dichlorophenol indophenol (DCPIP) at OD_{595nm}. A membrane suspension was mixed first with L-malate, DCPIP, KCN and ethylamine; the other chemicals phenazinemethosulfate and FAD were added after 100 sec indicating the beginning of the reaction. In the negative control no membrane fraction was added. MQO activity was determined in the samples by measurement of the slopes of the reaction kinetics.

To confirm the observed phenotype of *mqoB* mutant, a complementation was performed. The PA4640 gene with its own promoter was amplified from the TBCF10839 genome and ligated into the pUCP20 vector. The vector bearing the *mqoB* gene was then introduced into *mqoB* mutant by electroporation. The efficacy of the complementation was checked by growth of the positive clones on M9 medium with ethanol (Figure III-16).

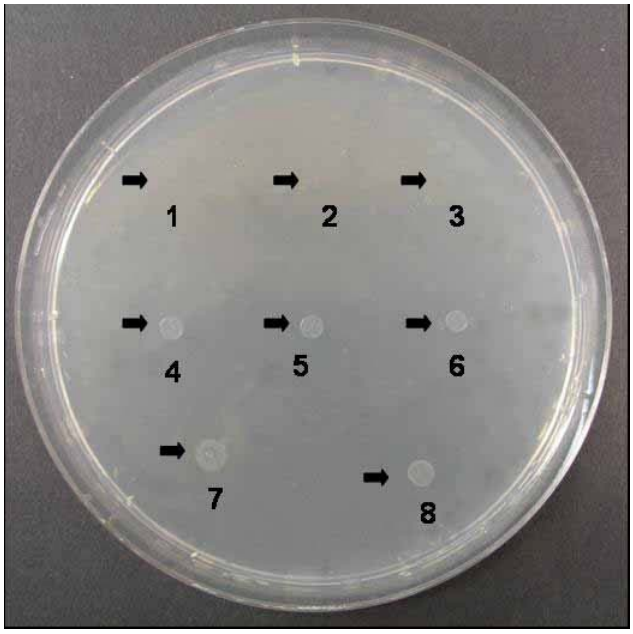

Figure III-16. Growth on minimal medium with 25 mM ethanol after 24h of incubation of *P. aeruginosa* TBCF10839 transposon insertion mutants Tn*5*::TB*mqoB* (1), *P. aeruginosa* PA14 MrT7::PA14*mqoB* (2), *P. aeruginosa* TBCF10839 Tn*5*::TB*mqoB* transformed with pUCP20 plasmid (vector control) (3), *P. aeruginosa* TBCF10839 Tn*5*::TB*mqoB* mutant complemented with pUCP20::TB*mqoB* (carrying the *Hin*dIII/*Sac*I PCR product bearing the *mqoB* gene) (4, 5, 6), TBCF10839 (7) and PA14 (8) wild type strains.

For better understanding of the metabolic function of the MQO enzyme, the phenotype analysis was carried out by PMs. The phenotype difference between the mutant and TBCF10839 or PAO1 wild types is described in Table III-8. Compared with wild-type TBCF10839, the *mqoB* mutant was compromised in the utilization of sulfonic compounds, fumarate, malonate and acetate, but interestingly not in that of malate. All substrates are oxidized in TCA cycle to CO_2 and energy.

Table III-8. PMs data of *P. aeruginosa* TBCF10839 Tn*5::mqoB.*

Test[a]	Difference[b]		Mode of action
	TBCF10839	PAO1	
L-Malic Acid		-111	C-source
Acetic Acid	-76	-74	C-source
Malonic Acid	-54		C-source
Fumaric Acid	-52		C-source
L-Asparagine	-66		N-source
Adenosine	-66		nutrient stimulation
Positive Control*	-61		nutrient stimulation
Tween 80	-57		nutrient stimulation
Inosine + Thiamine	-50		nutrient stimulation
2-Hydroxyethane Sulfonic Acid	-70		S-source
L-Methionine Sulfoxide	-62		S-source
Methane Sulfonic Acid	-58		S-source

(a) See Table 3.5, footnote **a**

(b) See Table 3.5, footnote **b**.

*****The positive control contained LB broth without any supplement.

Downregulation of the genes from the TCA cycle was earlier reported for *Corynebacterium glutamicum mqo* mutant by transcription analysis and have not been reported for *P. aeruginosa*. For *P. aeruginosa* ATCC 17933 malate and fumarate supported growth of the *mqo* mutant *(*Kretzschmar *et al*., 2002)

Under standard growth conditions *in vitro*, MqoA is 3.5 times less expressed than MqoB both at the transcriptional and translational levels, but is 9 times higher expressed under oxidative stress in presence of H_2O_2 (Figure III-17, Appendix 2).

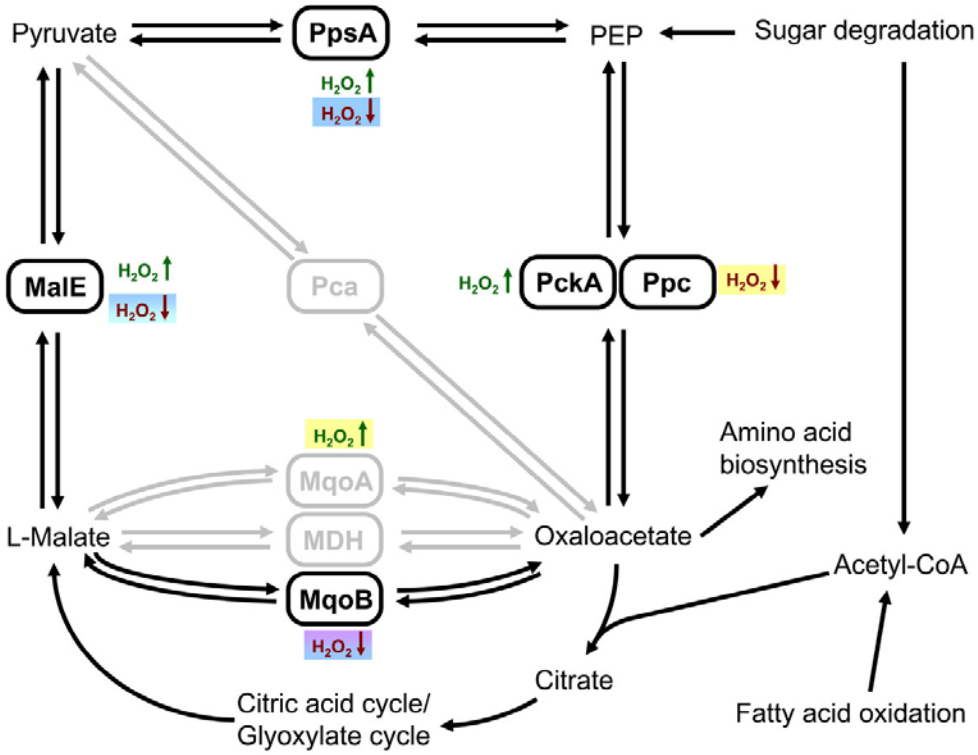

Figure III-17. The malate-oxaloacetate pathway. H_2O_2 (arrows up and down) indicate up and downregulation of genes. Strains are indicated by their colors: **TBCF10839**, **PAO1**, **CHA** and **LES431** (see Appendix 2).

The higher expression of the *mqoB* gene was also confirmed by quantitative PCR (qPCR), where compared to *mqoB* no expression by *mqoA* gene after 30 PCR cycles was detected (Figure III-18).

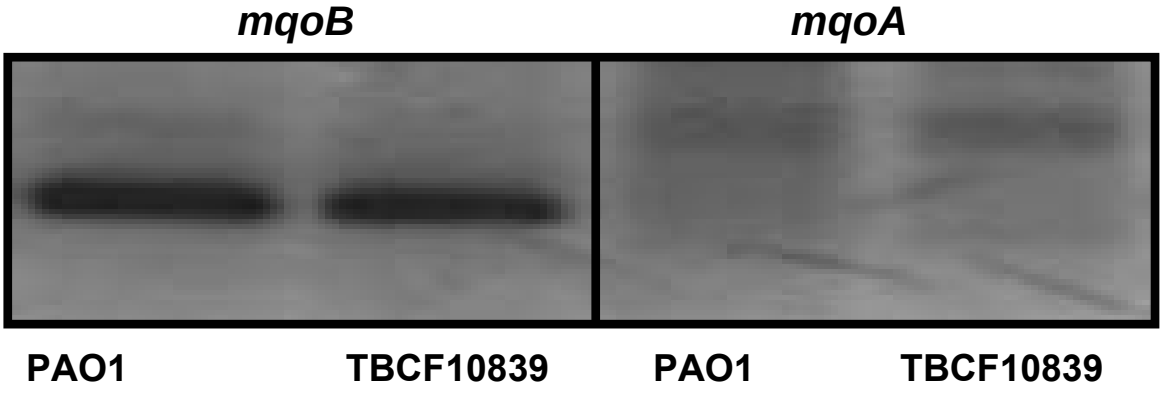

Figure III-18. The qPCR of *mqoB* and *mqoA* genes. cDNA was obtained by reversed transcription of total cell mRNA extracted from cultures grown until late exponential phase. In this Figure, 8µl of the PCR product was taken after 30th cycle and loaded into the gel,

The loss-of-function phenotype of *mqoB* mutant was proven by chronic murine infection model. The survival of mice whose airways had been inoculated by single *mqoB* mutant was higher in comparison with wild type and caused less inflammation in murine lungs. The *mqoB* mutant was recovered in wild type frequencies from lungs, liver, spleen and brain (Figure III-19 A, B). The histology images did not show significant abnormality in lung architecture (Figure III-19 C, D, E).

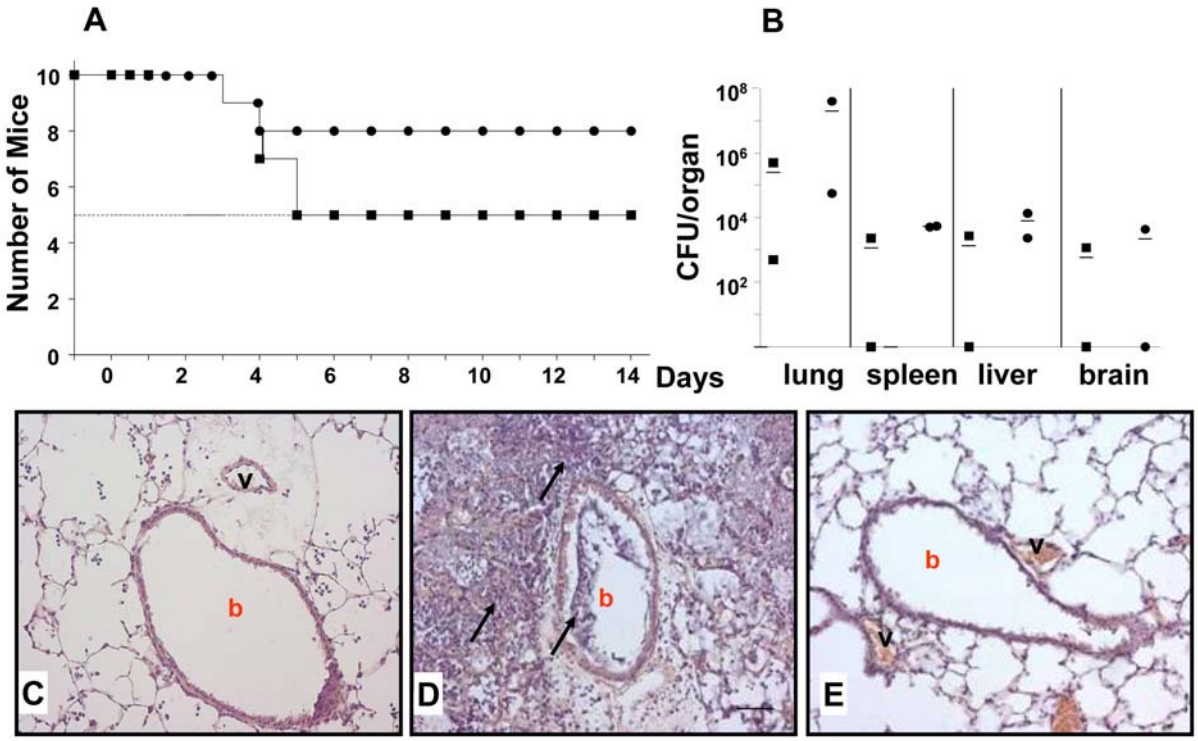

Figure III-19. (A) Survival of mice whose airways had been inoculated at day 0 with 5x10^7 CFU of *P. aeruginosa* TBCF10839 (closed square) and Tn*5*::*mqoB* (closed circle). (B) CFU recovered from murine organs 48 h after intracheal instillation. (C, D, E) Lung histology 48 h after infection with Tn*5*::*mqoB* (C), TBCF10839 (D) or vehicle control (30 µl PBS) (E). Hematoxylin-eosin stain; original magnification x 200, bar 50 µm. b, bronchus; v, vessel; inflammatory infiltrates are marked by arrows.

Thus, Mqo is the key enzyme for the production of oxaloacetate which is a precursor for the synthesis of amino acids and citrate. Malic enzyme and pyruvate carboxylase may bypass the lack of Mqo but this pathway of oxaloacetate synthesis is insufficient for growth if C2-compounds like acetate or ethanol are provided as sole carbon sources. The *mqoB* mutant grew normally on LB within the microbial community of isogenic strains, but it grew slower on plates, was less virulent than wild type and was out-competed in murine lungs. This data suggests that the proficient production of oxaloacetate is necessary for airway colonizing capacity and virulence of *P. aeruginosa*. In other words, biological fitness of *P. aeruginosa* requires that the

turnover of acetyl-CoA, the key intermediate of sugar and fatty acid degradation, is not restricted by the insufficient supply of oxaloacetate.

2.3. Features of the mutants affected in the biosynthesis and regulation of HAQs

The intercellular communication of bacteria to cooperate and coordinate their behavior in a cell density-dependent manner plays an important role in lifestyle of the pathogen. (Camara *et al*., 2002; de Kievit *et al*., 2000). A common feature of intercellular communication is the transcriptional activation of quorum-sensing-controlled genes when the bacterial signal molecules reach a certain threshold. In addition to the two acyl-homoserine lactone-type autoinducers, signal molecules belonging to the family of 4-hydroxy-2-alkylquinolines (HAQs) (Deziel *et al*., 2004) have been identified, which include in addition to N oxides (exhibiting antimicrobial activities) 3,4-hydroxy-2-heptylquinoline (PQS) (Pesci *et al*., 1999) and 4-hydroxy-2-heptylquinoline. Both molecules have been shown to be involved in intracellular communication.

Out of six mutants Tn*5*::*pqsD*(PA0999), Tn*5*::*mvfR*(PA1003), Tn*5*::*pqsL*(PA4190), Tn*5*::PA2361, Tn*5*::PA4915 are predicted to be involved in the biosynthesis and regulation of HAQs, the role of the gene products had so far only been investigated for PqsL, PqsD and MvfR. They all are involved in the biosynthesis or regulation of HAQs (D'Argenio *et al*., 2002; Deziel *et al*., 2004; Gallagher *et al*., 2002; Wade *et al*., 2005; Diggle *et al*., 2003). The transcription regulator MvfR is required for the production of several secreted compounds, including virulence factors and PQS (Cao *et al*., 2001: Rahme *et al*., 1997). PqsD being a part of pqsABCD operon, which encodes several putative enzymes required for the synthesis of HAQs is an analogue of FabH transacetylase (Gallagher *et al*., 2002).

2.3.1. Characteristics of PA4915 and PA2361 genes

PA4915 and PA2361 harbour sequence signatures of metalloproteinase and chemotaxis proteins and both have orthologs in almost all sequenced *Pseudomonas*

genomes and numerous other gamma-proteobacteria with an overall sequence similarity of 30-40% according to information from the websites http://www.tigr.org and http://www.genome.ad.jp.

The first PA4915 is annotated as a methyl-accepting chemotaxis protein related to genes of cell motility, secretion and signal transduction mechanisms. The gene contains a methyl-accepting chemotaxis protein (MCP) signaling domain, which is thought to transduce the signal to CheA since it is highly conserved in very diverse MCPs (Figure III-20).

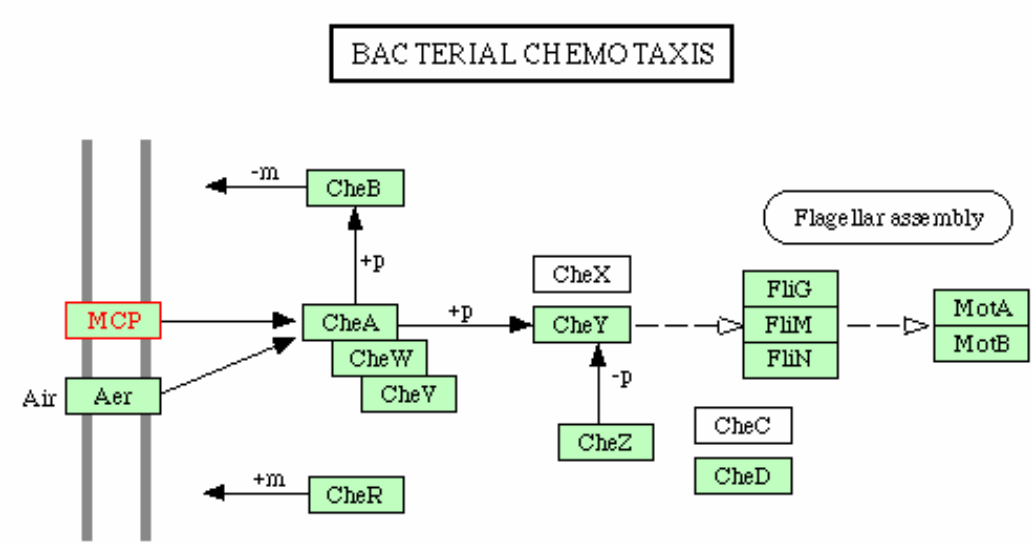

Figure III-20. The bacterial chemotaxis of *P. aeruginosa* (http://www.genome.ad.jp.).

The proteins involved in chemotaxis, have a complicated structure and localization. Many other bacteria possess numerous MCPs, including both membrane-bound and soluble species. In the genome of *P. aeruginosa*, there are five loci containing clusters of chemotaxis-like genes (Cluster I to Cluster V) (Ferrandez *et al*., 2002). Cluster I and Cluster V are involved in chemotaxis (Masduki *et al*., 1995; Stover *et al*., 2000), Cluster III is involved in the regulation and function of type IV pili and twitching motility (Whitchurch *et al*., 2004), and Cluster IV is involved in autoaggregation (D'Argenio *et al.,* 2002). The *P. aeruginosa* genome encodes 26 MCPs.

The second gene PA2361 is annotated as an uncharacterized protein conserved in many bacteria with unknown function (http://www.Pseudomonas.com). The gene from 25 to 1251 bp contains the region IsmF responsible for the type VI protein

secretion system component VasK (intracellular trafficking, secretion, and vesicular transport) (http://www.ncbi.gov), but no more information is available for the gene so far.

2.3.2. Characteristic of PA2588 gene

PA2588 is annotated as a probable transcription regulator with an AraC-type DNA-binding domain-containing protein (http://www.pseudomonas.com).

The AraC family of proteins contains over a hundred activators that are related to one another by sequence homology, especially between their carboxy-termini, which constitute their DNA-binding domains (Gallegos *et al*., 1997). However, despite their similarity to each other, these proteins regulate the expression of genes or operons that encode diverse functions. Some AraC family members activate the expression of genes or operons involved in the metabolism of carbon compounds that range from sugars to herbicides. Others allow the cell to respond to external conditions, such as oxidative stress, heavy metal toxicity or the presence of antibiotics. Still other AraC family members are required for the expression of virulence factors by bacterial pathogens (Martin *et al*., 2001).

2.3.3. Phenotypic characteristics of the HAQ deficient mutants

The type B colony morphology mutants are an illustrating example that knock-outs in unrelated pathways or regulons may converge to the same morphotype. Even the transcriptome profile for all five mutants revealed a similarity of the genes expression. Genes are shown to be down regulated under oxidative stress condition induced by H_2O_2 for all mutants (Table III-9).

Table III-9. GeneChip signal intensities of mRNA transcripts involved in HAQs biosynthesis or regulation.

ORF	Gene name	Gene annotation	Expression of mRNA transcripts			
			TBCF10839		892	
			*LE, LB	**LE, LB+H_2O_2	*LE, LB	**LE, LB+H_2O_2
PA0999	*pqsD*	3-oxoacyl-[acyl-carrier-protein] synthase III	1268	220 (0.2)	244	167 (0.7)
PA1003	*mvfR*	Transcription regulator	239	92 (0.4)	226	50 (0.2)
PA2361	-	Hypothetical protein	100	10 (0.1)	36	15 (0.4)
PA2588	-	Probable transcriptional regulator	350	51 (0.15)	114	23 (0.2)
PA4915	-	Probable chemotaxis transducer	506	27 (0.05)	440	23 (0.05)

Numbers in brackets indicate relative expression values in 10 mM H_2O_2 compared to growth in LB medium.

*late exponential (LE) phase, cells were cultivated in LB and RNA was extracted at cell density OD578 = 3.5. **LE, LB + 10mM hydrogen peroxide (Salunkhe, 2003)

The absence of HAQs produced by mutants (Figure III-5), especially PQS, which is an important molecule for signal transduction had not been found to be crucial for the survival in lungs by Tn*5*::*mvfR*, Tn*5*::*pqsL*, Tn*5*::PA2361 and Tn*5*::PA4915 mutants. In two other cases, the knock-out either in PA0999 or PA2588 genes decreased survival of TBCF10839 in the lung. Tn*5*::PA2588 mutant was also shown to be impaired in the *C. elegans* infection model (personal communication, Garvis S), but the complementation studies to this mutant failed. This may indicate that either the morphotype was induced by secondary mutation event or another strategy for the complementation is recommended.

Thus, our data indicate that the gene products of all five mutants are essential for the production or regulation of quinolones and phenazines that adds a further layer of complexity to the regulation of HAQ production. If the role of MvfR and PqsD is known, the targets of the other two type B mutants (PA2361 and PA4915) are not

directly involved in quinolone or phenazine synthesis. They are probably members of a network that regulates the production and transport of these major secondary metabolites of *P. aeruginosa* some of which exhibit antimicrobial activity, modulate the host defense or act as bacterial signaling molecules.

The knock-out of an essential gene for biosynthesis should be detrimental for fitness, but the knock-out of a regulatory gene may be compensated by other mechanisms of the *P. aeruginosa* signalling network. Consistent with this interpretation, the PqsD knock-out was out-competed by isogenic strains that were capable to synthesize quinolones, whereas the knock-out in the master regulatory gene *mvfR* was not compromised in its fitness *in vivo*.

2.4. Features of “non-competitive” mutants

Four mutants of the last category 3 were shown to be non-competitive in the presence of other mutants after the STM competition experiments in mice. No survival *in vivo* and *in vitro* was detected. Three mutants from the group are related to the energy metabolism enzymes. In the following chapters three mutants will be described in detail, where one is known to be a key enzyme of energy metabolism (*edd*) and the other two are new genes affecting the fitness of *P. aeruginosa in vivo* and have not been reported so far in the literature.

2.4.1. Tn*5*::PA3194, a key enzyme of Entner-Doudoroff (ED) pathway (*edd*)

One of the non-competitive mutants listed in Table III-4 has a knock-out in *edd* gene well known as 6-phospho-gluconatedehydratase (EC 4.2.1.12) which catalyses the first step in the Entner-Doudoroff (ED) pathway. It dehydrates 6-phospho-d-gluconate into 6-phospho-2-dehydro-3-deoxy-d-gluconate (Peekhaus and Conway, 1998).

The colonies of the *edd* mutant are smaller in size than wild type. The major feature of the mutant is its inability to grow on minimal medium with glucose as a carbon source. No growth of the mutant was detected after 24 hours of incubation at 37 ^{o}C (Figure III-21 A). Further, the mutant was successfully complemented and growth on glucose was recovered. Interestingly, the phenotype of TBCF10839 mutant was different from another clinical isolate PA14, where the last one could utilize glucose (Figure III-21 B).

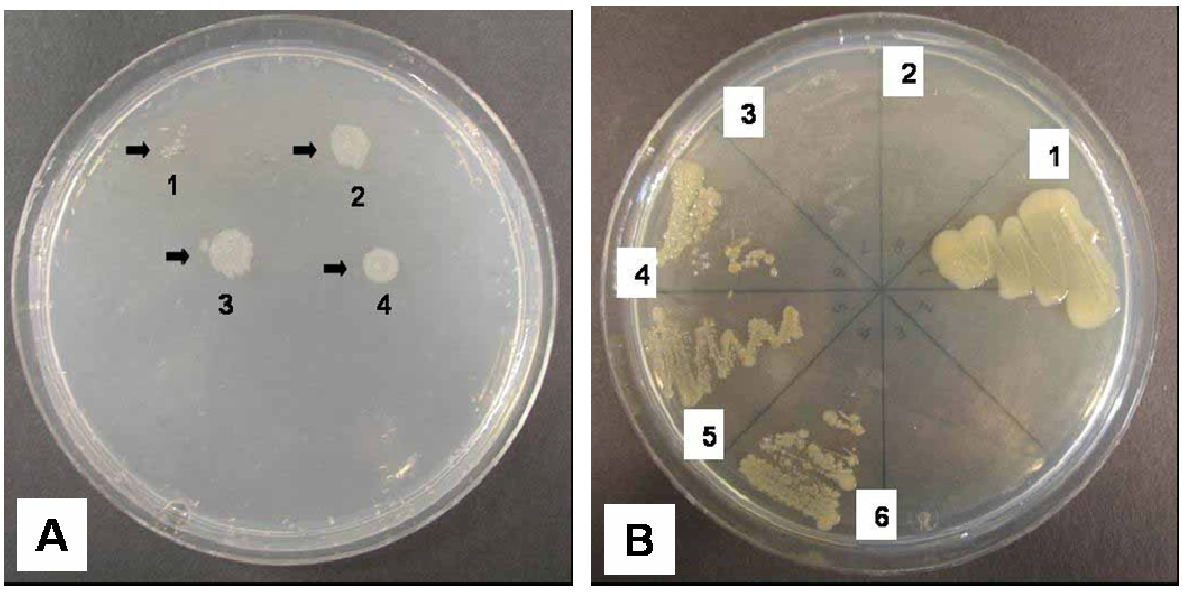

Figure III-21. (a) Growth on minimal medium with 25 mM glucose after 24h of incubation of transposon insertion mutants Tn*5*::TB*edd* of strain TBCF10839 (1), MrT7::PA14*edd* of strain PA14 (2), TBCF10839 (3) and PA14 (4) wild type strains. (b) Growth on minimal medium with 25 mM glucose after 48h of incubation of *P. aeruginosa* strain TBCF10839 (1) and the isogenic transposon insertion mutants Tn*5*::TB*edd* (2), Tn*5*::TB*edd* transformed with pUCP20 plasmid (vector control) (3), Tn*5*::TB*edd* mutant complemented with pUCP20::TB*edd* carrying the *Hin*dIII/*Sac*I PCR product bearing the *edd* gene (4, 5, 6).

Many bacteria possess genes for the ED pathway, but the extent of its role in glucose catabolism varies considerably (Cuskey *et al*., 1985).

In our experiments, the *edd*⁻ mutant was out-competed both *in vivo* and *in vitro*. The mutant was attenuated in virulence in the mice infection experiment, caused less inflammation in murine lungs and was more efficiently eliminated than the wild type (Figure III-22 A, B). The histology images did not show significant abnormality in lung architecture (Figure III-22 C,D,E).

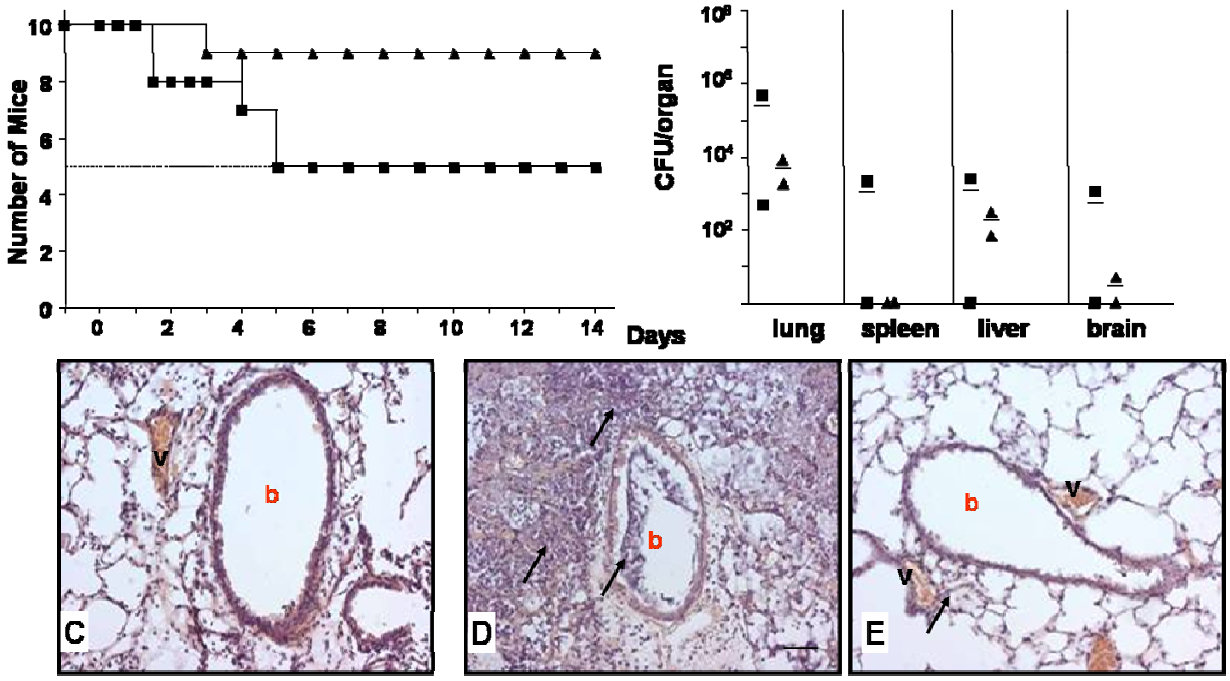

Figure III-22. (A) Survival of mice whose airways had been inoculated at day 0 with 5x10^7 CFU of *P. aeruginosa* TBCF10839 (closed square) and Tn*5*::*edd* (closed triangle). (B) CFU recovered from murine organs 48 h after intracheal instillation. (C, D, E) Lung histology 48 h after infection with Tn*5*::*edd* (C), TBCF10839 (D) or vehicle control (30 µl PBS) (E). Hematoxylin-eosin stain; original magnification x 200, bar 50 µm. b, bronchus; v, vessel; inflammatory infiltrates are marked by arrows.

The similar phenotype was reported for *P. chlororaphis,* where *edd*$^-$ knocked out mutant also failed to grow on glucose and displayed reduced root colonization (Kim *et al*., 2007). In this context, a mutation in *zwf* of another CF isolate *P. aeruginosa* strain FRD1, encoding glucose-6-phosphate dehydrogenase, leads to a 90% reduction in alginate production (Silo-Suh *et al*., 2005).

The metabolic profile showed the *edd* mutant was compromised in utilization of different compounds (Table III-10).

Table III-10. PMs data of *P. aeruginosa* TBCF10839 Tn*5::edd.*

Test[a]	Difference[b]		Mode of action
	TBCF10839	PAO1	
L-Malic Acid		-108	C-source
D-Gluconic Acid	-106		C-source
D-Glucose	-76		C-source
L-Aspartic Acid	-68		C-source

Test[a]	Difference[b]		Mode of action
	TBCF10839	PAO1	
Xanthine	-55	-76	N-source
Adenosine	-57	-73	N-source
Uric Acid		-65	N-source
Xanthosine		-61	N-source
Acetamide	-111		N-source
Guanosine	-71		N-source
N-Acetyl-D-Glucosamine	-68		N-source
Cytidine	-61		N-source
D-Alanine	-55		N-source
L-Asparagine	-51		N-source
D-(+)-Glucose		-120	nutrient stimulation
N-Acetyl D-Glucosamine		-107	nutrient stimulation
Deferoxamine Mesylate		-103	nutrient stimulation
Riboflavin		-101	nutrient stimulation
Thiamine		-88	nutrient stimulation
Putrescine	-82		nutrient stimulation
Positive Control*	-65		nutrient stimulation
2`-Deoxy-Adenosine	-56		nutrient stimulation
L-Asparagine	-55		nutrient stimulation
Tween 80	-54		nutrient stimulation
Phosphoryl Choline		-143	P-source
D-Glucose-6-Phosphate		-70	P-source
D-Glucose-1-Phosphate		-68	P-source
Pyrophosphate	-64		P-source

(a) See Table 3.5, footnote **a**

(b) See Table 3.5, footnote **b**.

*****The positive control contained LB broth without any supplement.

The transcriptome profile did not reveal the differences in the gene expression among different strains under two different conditions. To have a full picture of the carbohydrates degradation by prokaryotic cells the Figure III-23 was made to exhibit the pathways and features in the regulation of genes involved in Entner-Doudoroff pathway (Figure III-23).

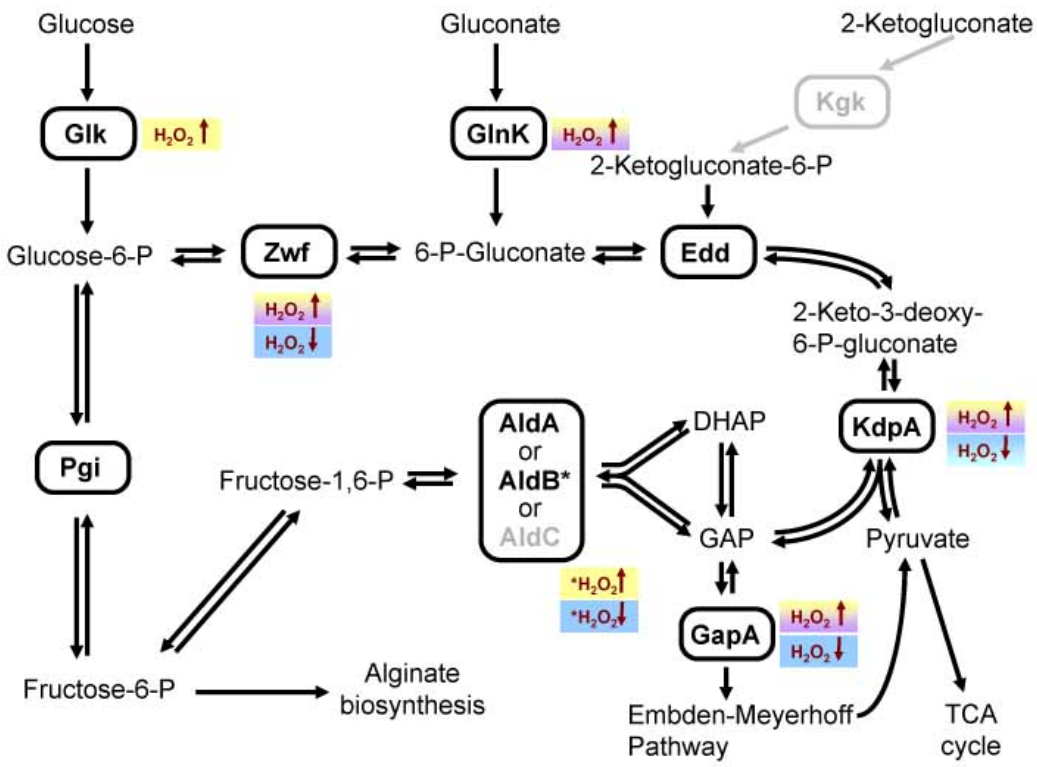

Figure III-23. The metabolic pathway of carbohydrates (Entner-Doudoroff pathway). H_2O_2 (arrows up and down) indicate up and down regulation of genes. Strains are indicated by their colors: **TBCF10839**, **PAO1** and **CHA** (see appendix 2).

An explanation of the mechanism by which the mutant is out-competed *in vitro* and *in vivo* is based on a biochemical property of the enzyme. As shown, the Edd is involved in the carbon flow from glucose and gluconate (PMs data) into pyruvate and alginate (Banerjee *et al*., 1983). The knock-out in the gene leads to the reduction of carbon metabolic process. By this, the colonies of the mutant are smaller in size than wild type and less mucoid. The reduction of alginate production leads to the decreasing of biofilm foramtion. All these factors are negatively influence on colonization of the pathogen and further its adaptation. It means that genes involved in energy metabolism are essential for the colonization, adaptation and dissemination of the pathogen, the genes of key metabolic pathways especially.

2.4.2. Tn*5*::PA0785, predicted acyl carrier protein phosphodiesterase

PA0785 encodes a protein annotated as an acyl carrier protein phosphodiesterase. The gene has a flavodoxin like fold domain conserved among different species. The flavodoxin family includes bacterial and eukaryotic NAD(P)H dehydrogenase (quinine, EC:1.6.99.2). These enzymes catalyse the NAD(P)H-dependent two-electron reductions of quinones and protect cells against damage by free radicals and reactive oxygen species (Fischl and Kennedy, 1990). This enzyme uses a FAD co-factor. The family also includes acyl carrier protein phosphodiesterase (EC:3.1.4.14). This enzyme converts holo-ACP to apo-ACP by hydrolytic cleavage of the phosphopantetheine residue from ACP (Li *et al*., 1995).

The enzyme cleaves acyl-[acyl-carrier-protein] species with acyl chains of 6-16 carbon atoms length although it appears to demonstrate a preference for the unacylated acyl-carrier-protein (ACP) and short- chain ACPs over the medium- and long-chain species (http://www.expasy.ch).

The phenotype of the Tn*5*::PA0785 mutant possessed a high cytotoxicity compared to wild type (Figure III-24).

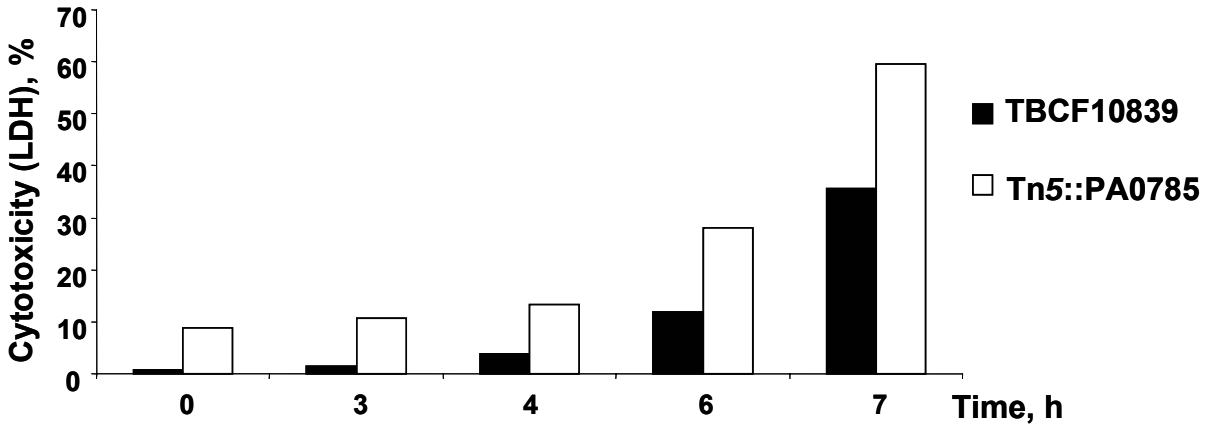

Figure III-24. Cytotoxicity of TBCF10839 and Tn*5*::PA0785 toward CHO cells. The cytotoxicity was assayed by measuring the lactate dehydrogenase (LDH) released into culture supernatants. CHO cells were infected with TBCF10839 or Tn*5*::PA0785 at a MOI of 10, and supernatants were collected at different time points. Each mutant/strain was tested triplicate. % was calculated from three independent experiments, 100 % value represented the LDH released from cells lysed by 0.1 % (v/v) TritonX-100.

In spite of high cytotoxisity, the mutant was shown to be out-competed both *in vitro* and *in vivo,* attenuated in mice infection experiment (Figure III-25). During infection, the mutant was less presented in the liver, spleen and in the lung (Figure III-25 B). The histology images did not show significant abnormality in lung architecture (Figure III-25 C, D, E).

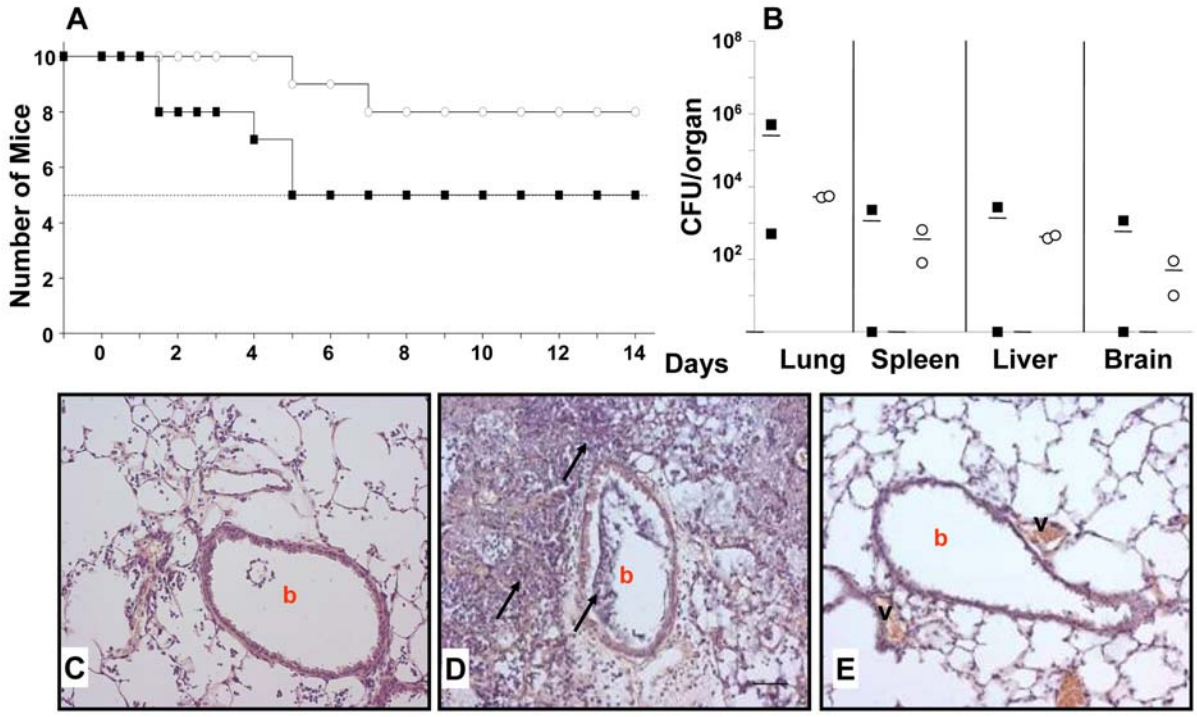

Figure III-25. (A) Survival of mice whose airways had been inoculated at day 0 with 5x10^7 CFU of *P. aeruginosa* TBCF10839 (closed square) and Tn*5*::PA0785 (open circle). (B) CFU recovered from murine organs 48 h after intracheal instillation of TBCF10839 (closed square) and Tn*5*::PA2537 (open circle). (C, D, E) Lung histology 48 h after infection with Tn*5*::PA0785 (C), TBCF10839 (D) or vehicle control was instilled with 30 µl PBS (E). Hematoxylin-eosin stain; original magnification x 200, bar 50 µm. b, bronchus; v, vessel; inflammatory infiltrates are marked by arrows.

The metabolic profile revealed that the mutant was compromised for utilization of a high amount of chemicals that testify about a complex correlation and participation of the enzyme in various metabolic processes (Table III-11).

Table III-11. PMs data of *P. aeruginosa* TBCF10839 Tn*5*::PA0785.

Test[a]	Difference[b]		Mode of action
	TBCF10839	PAO1	
L-Malic Acid		-67	C-source
g-Amino-N-Butyric Acid		-134	N-source
L-Arginine	-75		N-source
Cytosine	-68		N-source
Uracil	-64		N-source
Histamine	-55		N-source
Allantoin	-52		N-source
N-Acetyl D-Glucosamine		-80	nutrient stimulation
Tween 20		-66	nutrient stimulation
p-Amino-Benzoic Acid		-65	nutrient stimulation
D;L-Mevalonic Acid		-64	nutrient stimulation
Guanine		-60	nutrient stimulation
Tween 60	-102	-85	nutrient stimulation
b-Nicotinamide Adenine Dinucleotide	-94	-60	nutrient stimulation
d-Amino-Levulinic Acid	-90	-61	nutrient stimulation
Tween 40	-88	-80	nutrient stimulation
Cyano-Cobalamine	-86	-66	nutrient stimulation
Riboflavin	-85	-76	nutrient stimulation
Thiamine Pyrophosphate	-79	-68	nutrient stimulation
Deferoxamine Mesylate	-77	-82	nutrient stimulation
Choline	-75	-66	nutrient stimulation
D-(+)-Glucose	-72	-90	nutrient stimulation
Thymine	-67	-98	nutrient stimulation
Thiamine	-65	-66	nutrient stimulation
Menadione	-59	-83	nutrient stimulation
Folic Acid	-62	-64	nutrient stimulation
Glutathione (reduced form)	-59	-74	nutrient stimulation
Hematin	-58	-76	nutrient stimulation
Nicotinic Acid	-111		nutrient stimulation
D-Biotin	-103		nutrient stimulation
Tween 80	-99		nutrient stimulation
Quinolinic Acid	-96		nutrient stimulation
L-Ornithine	-93		nutrient stimulation
L-Serine	-90		nutrient stimulation
L-Proline	-87		nutrient stimulation

Test[a]	Difference[b]		Mode of action
	TBCF10839	PAO1	
L-Glutamine	-86		nutrient stimulation
Glycine	-86		nutrient stimulation
Putrescine	-85		nutrient stimulation
Spermidine	-83		nutrient stimulation
Adenosine-3';5'-Cyclic Monophosphate	-81		nutrient stimulation
L-Citrulline	-79		nutrient stimulation
p-Amino-Benzoic Acid	-79		nutrient stimulation
Positive Control*	-78		nutrient stimulation
Pyridoxine	-76		nutrient stimulation
L-Alanine	-76		nutrient stimulation
Chorismic Acid	-74		nutrient stimulation
L-Cysteine	-74		nutrient stimulation
L-Histidine	-72		nutrient stimulation
Pyrrolo-Quinoline Quinone	-71		nutrient stimulation
L-Glutamic Acid	-71		nutrient stimulation
Nicotinamide	-70		nutrient stimulation
Negative Control	-70		nutrient stimulation
Inosine + Thiamine	-69		nutrient stimulation
Spermine	-68		nutrient stimulation
L-Aspartic Acid	-67		nutrient stimulation
Adenosine	-66		nutrient stimulation
L-Tyrosine	-65		nutrient stimulation
L-Arginine	-64		nutrient stimulation
L-Tryptophan	-62		nutrient stimulation
L-Leucine	-59		nutrient stimulation
Adenine	-58		nutrient stimulation
Uracil	-58		nutrient stimulation
m-Inositol	-58		nutrient stimulation
Pyridoxal	-56		nutrient stimulation
D-Pantothenic Acid	-56		nutrient stimulation
D-Glutamic Acid	-53		nutrient stimulation
L-Threonine	-52		nutrient stimulation
Guanosine-3';5'-Cyclic Monophosphate	-51		nutrient stimulation
Phosphoryl Choline	-62	-129	P-source
Thymidine- 5'-Monophosphate		-67	P-source
O-Phosphoryl-Ethanolamine		-60	P-source
Dithiophosphate	-62		P-source

Test[a]	Difference[b]		Mode of action
	TBCF10839	PAO1	
Thiophosphate	-60		P-source
O-Phospho-L-Threonine	-57		P-source
D-Mannose-1-Phosphate	-56		P-source
Phospho-L-Arginine	-54		P-source
O-Phospho-D-Tyrosine	-53		P-source
2-Hydroxyethane Sulfonic Acid	-57		S-source
Methane Sulfonic Acid	-54		S-source

(a) See Table 3.5, footnote **a**

(b) See Table 3.5, footnote **b**.

*****The positive control contained LB broth without any supplement.

The obtained results indicate that the PA0785 gene knock-out reduces the ability of *P. aeruginosa* to survive in lungs because of its inability to utilize many substrates. At the same time the PA0785 gene knock-out increases the secretion of virulence factors that may play a role as compensatory mechanisms for better invasion and adaptation.

The high cytotoxicity induced by the mutant towards to CHO cells might be explained by upregulation of the TTSS system to break down of the eukaryotic cells to have additional access of nutrients. The TTSS system is an important mechanism for *P. aeruginosa* survival throughout infection to overcome unique challenges that the bacteria face at specific stages (Hauser *et al*., 2002) however, this mechanism is not sufficient for bacterial cell without joint action of many metabolic enzymes which are obligatory for survival.

In our case, the inability of the mutant to utilize many compounds is reflecting on competitive properties of the mutant. The mutant cannot compete with other mutants/clones in a mixture population and was also impaired in virulence in mice. Based on the reported phenotype it is an essential enzyme involved in energy metabolism, inactivation of which decreases the adaptation, dissemination and survival of *P. aeruginosa* in the lung.

2.4.3. Tn*5*::PA4916, predicted ADP - ribose pyrophosphatase

The Tn*5*::TBPA4916 mutant is one of the non-competitive mutants listed in Table III-4. According to the annotation from the pseudomonas website, the gene has a predicted function ADP-ribose pyrophosphatase belonging to the genes of nucleotide metabolism.

The gene has more than 200 orthologs (http://www.genome.jp) in many species with an overall sequence similarity not less then 30%.

The blast search revealed that PA4916 harbours a sequence well known as a NUDIX motif (from 6 to 140 aminoacids, Figure III-26) that shares a strong homology between many species according to the information stored in the website (http://www.genome.jp).

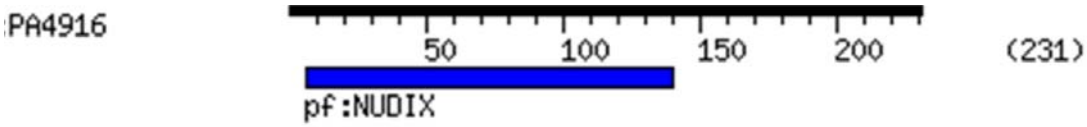

Figure III-26. The nudix motive (blue color) of PA4916 gene.

The nudix (*nu*cleoside *di*phosphate linked to another moiety, *X*) hydrolases are nucleoside diphosphate pyrophosphatases known to be important in degrading toxic intracellular compounds and in the virulence of several different bacteria (Bessman *et al*., 1996, 2001; Gaywee *et al*., 2002; Lundin *et al*., 2003; McLennan *et al*., 1999).

The Nudix hydrolase family of proteins consists of approximately 800 members found in more than 200 prokaryotic and eukaryotic species (Bessman *et al*., 1996; Xu *et al*., 2002). The hydrolases may be divided into subfamilies based on the substrate specificity: dinucleoside oligophosphate pyrophosphatases, NADH, ADP-ribose, nucleotide sugars, or ribo- and deoxyribonucleoside triphosphates (Dunn *et al*., 1999). Catalytic activity is located within the Nudix motif, which has in many cases well conserved however, how this activity relates to cellular function is less understood. (Bessman *et al*., 2001; Cartwright *et al*., 1999; Conyers *et al*., 1999).

The colonies of the mutant are smaller in size than TBCF10839 wild type and non-mucoid under ambient temperature. The mutant was not compromised in production of the virulence factors such as pyocyanine and protease (Figure III-27 A, B).

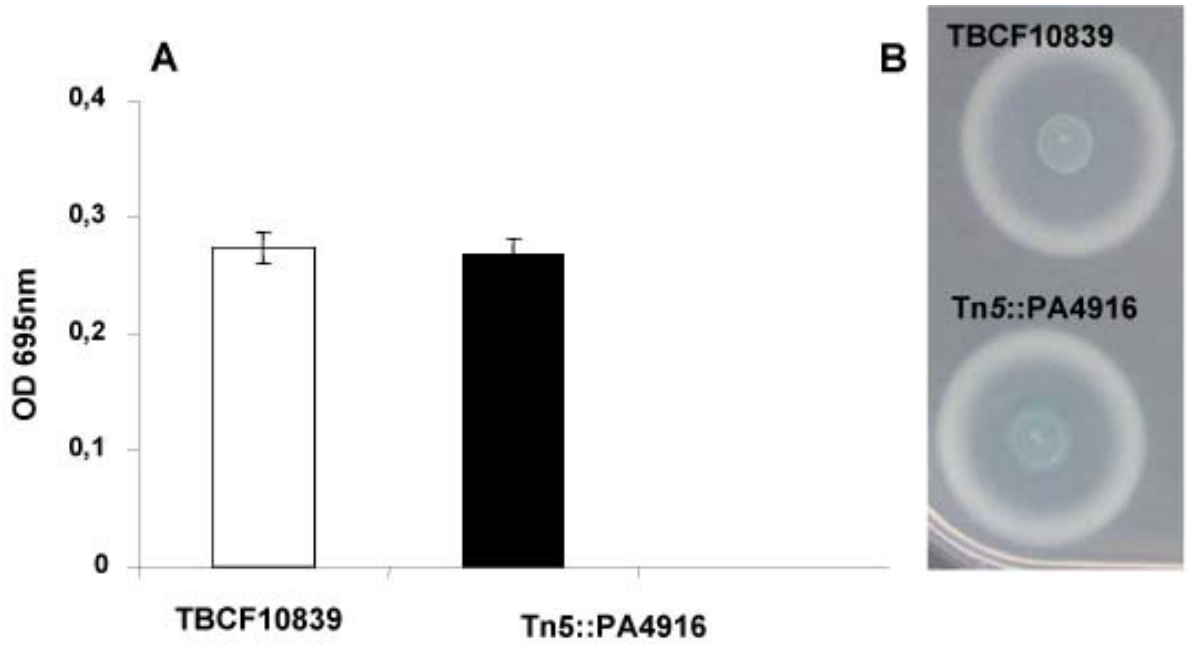

Figure III-27. (A) The pyocyanine and (B) protease production activities by TBCF10839 and Tn*5*::PA4916 after 16 hours of incubation in King A (pyocyanine) and casein agar (protease).

The mutant was subjected to cytotoxicity test on CHO cells. The results showed that the mutant possessed less cytotoxicity than the wild type after 7 hours of incubation (Figure III-28).

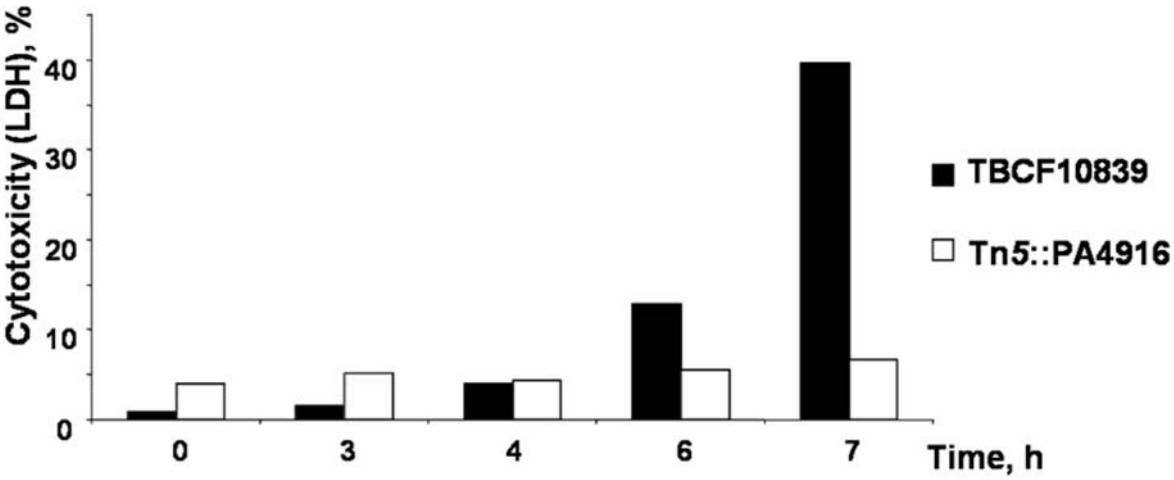

Figure III-28. Cytotoxicity of TBCF10839 and Tn*5*::PA4916 toward CHO cells. The cytotoxicity was assayed by measuring the lactate dehydrogenase (LDH) released into culture supernatants. CHO cells were infected with TBCF10839 or Tn5::PA0785 at a MOI of 10, and supernatants were collected at different time points. Each mutant/strain was tested triplicate. % was calculated from three independent experiments, 100 % value represented the LDH released from cells lysed by 0.1 % (v/v) TritonX-100.

The main feature of the mutant is its complete avirulence in mice infection experiment (Figure III-30 A). After 14 days of infection the survival of mice consisted 100%, the mutant was completely eliminated in brain, liver and spleen, and had a minor amount in lungs (Figure III-29 B). Similar to other attenuated mutants, the histology did not show any significant abnormality in the lung architecture and strong inflammatory response by infected cells (Figure III-29 C, D, E).

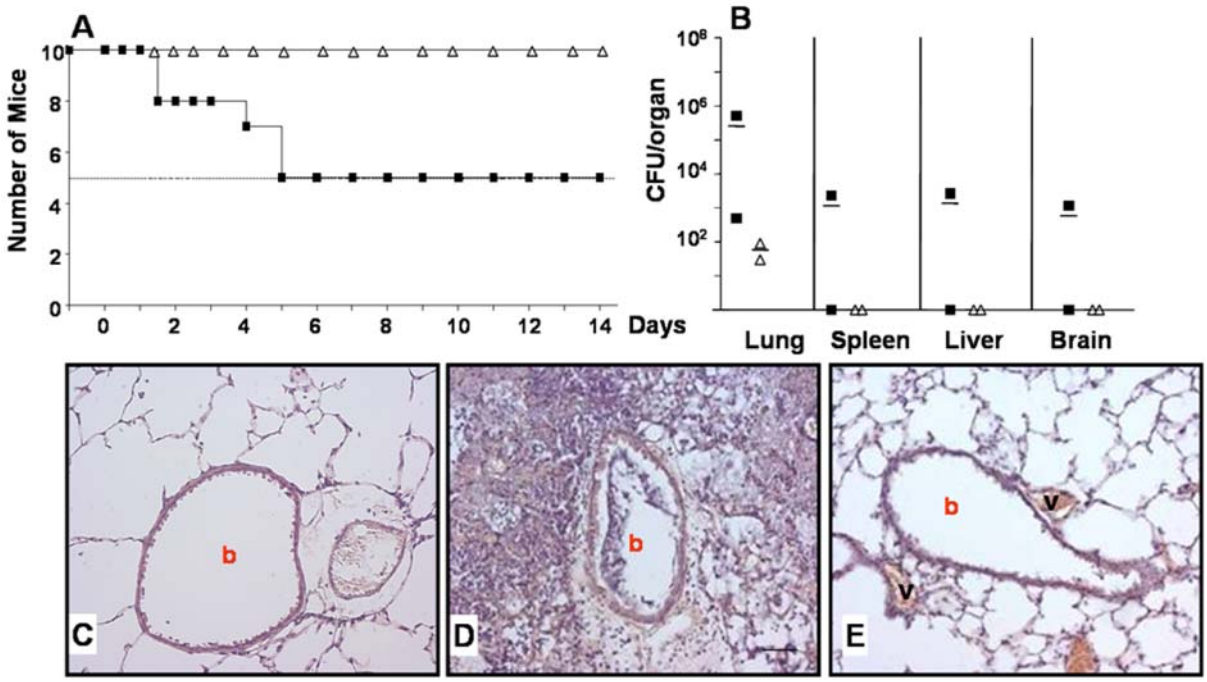

Figure III-29. (A) Survival of mice whose airways had been inoculated at day 0 with 5x10^7 CFU of *P. aeruginosa* TBCF10839 (closed square) and Tn*5*::PA4916 (open triangle). (B) CFU recovered from murine organs 48 h after intracheal instillation. (C, D, E) Lung histology 48 h after infection with Tn*5*::PA4916 (C), TBCF10839 (D) or vehicle control instilled (30 µl PBS) (E). Hematoxylin-eosin stain; original magnification x 200, bar 50 µm. b, bronchus; v, vessel; inflammatory infiltrates are marked by arrows.

In many cases genes having a nudix motif are involved in the virulence process of the pathogens. Thus, the nudA gene of *L. pneumophila* was described as a major importance for resisting stress in *L. pneumophila* and is a virulence factor (Edelstein *et al*., 2005). Similar to our case, the nudA mutant also showed a smaller colony size than its parent and was out-competed both in competition studies in macrophages and in the guinea pigs infection model in the lungs and spleen.

The TBCF10839 strain had acquired a robust mucoid morphotype in the CF lungs, and transposon insertion in PA4916 had led to a non-mucoid strain. To confirm that the observed unusual phenotype of the mutant was induced by Tn*5* insertion in the PA4916 gene, the complementation of the gene was performed. The DNA fragment of the genes PA4916-PA4917 with their own promoter were amplified from the TBCF10839 genome and ligated into pUCP20 plasmid. The vector bearing the PA4916-PA4917 genes was then introduced into Tn*5*::TB4916 mutant by electroporation. The efficacy of the complementation was checked by growth of the positive clones on blood agar at room temperature. After complementation, the mutant switched back to the mucoid phenotype of the wild type strain (Figure III-30).

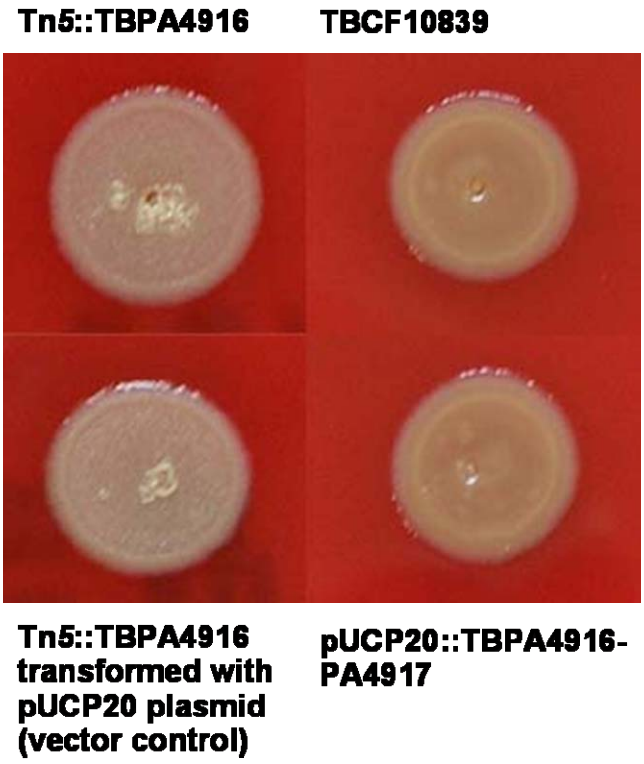

Figure III-30. The complementation of Tn*5*::PA4916 mutant by pUCP20::TBPA4916 carrying the *Hin*dIII/*Sac*I PCR product bearing the PA4916-PA4917 genes.

Compared with TBCF10839 wild type, the mutant was compromised in the utilization of carboxylic acid, triose carbohydrate and aminoacids (Table III-12). Since the gene has been predicted to have a function of nucleotide metabolism, it was interesting to notice that the phenotype of the mutant was compromised on adenine, thymine and adenosine-3'; 5'-cyclic monophosphate (cAMP) being a second messenger in signal transduction of the cell.

Table III-12. PMs data of *P. aeruginosa* TBCF10839 Tn*5*::PA4916.

Test[a]	Difference[b]		Mode of action
	TBCF10839	PAO1	
L-Malic Acid		-94	C-source
Dihydroxy-Acetone	-55		C-source
g-Amino-N-Butyric Acid	-59	-121	N-source
Thymine	-68		N-source
L-Arginine	-67		N-source
Adenine	-67		nutrient stimulation
Adenosine-3';5'-Cyclic Monophosphate	-63		nutrient stimulation
Positive Control*	-53		nutrient stimulation
L-Leucine	-51		nutrient stimulation
Phosphoryl Choline		-79	P-source
Pyrophosphate	-76		P-source

(a) See Table 3.5, footnote **a**

(b) See Table 3.5, footnote **b**.

***The positive control contained LB broth without any supplement.

The protein sequence of the gene contains an EAL domain (Figure III-31). It is known that enzymes possessing the EAL domain control the levels of c-di-GMP being a chemical messenger present uniquely in bacteria (Ross *et al*., 1991; Simm *et al*., 2004; Kulasakara *et al*., 2006).

MSSAEVLASVDIVALRLNPGHGLELLLIRRAQAPFAGQWALPGVLVNGRSADHSLD
DAAVRALRDKARLEPAYIEQVATVGNAVRDPRGWSLSVFYLVLVGPDTQVEDDDLD
FVPLRDVRSERFALPFDHAQLVQQACERLASKSVYSALPLFLLAPRFTVA**EAL**KAFE
CAIGQEVQHSSLRGRLERMKEAGWVEDTGERQRPPMGRPQHVLHFTPKPGGAFV
FDRSLLAS

Figure III-31. The protein sequence of PA4916 keeping an EAL domain (http://www.pseudomonas.com)

c-di-GMP is a novel global second messenger in bacteria the metabolism of which is controlled by GGDEF and EAL domain proteins. Cyclic nucleotides (cAMP, c-di-GMP) represent second messenger molecules in all kingdoms and the signalling by cyclic nucleotides seems pretty simple. In bacteria, mass sequencing of genomes detected the highly abundant protein domains GGDEF and EAL. Both these domains are involved in the turnover of cyclic-di-GMP (c-di-GMP) in vivo where by the GGDEF

domain stimulates c-di-GMP production and the EAL domain c-di-GMP degradation (Ross *et al*., 1991; Simm *et al*., 2004; Kulasakara *et al*., 2006).

Signal intensity compared among different strains during late exponential growth showed that the transcription activity of PA4916 gene is down regulated under oxidative stress in TBCF10839, 892 and LES431 strains, but not in PAO1. Interestingly, the transcription activity of the gene in TBCF10839 was different from the reference strain PAO1, as shown in Figure III-32 after qPCR.

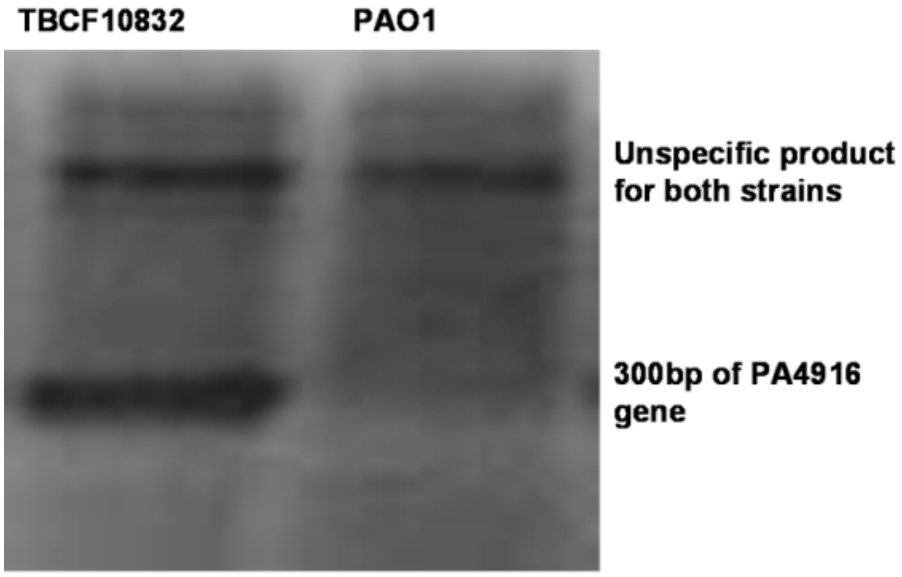

Figure III-32. qPCR of PA4916 gene. cDNA was obtained by reversed transcription of total cell mRNA extracted from cultures grown until late exponential phase. In this Figure, 8µl of the PCR product was taken after 30th cycle and loaded into the gel.

The mechanism by which the enzyme exerts such an unusual phenotype is unknown. It might be explained by the belonging of the enzyme to the family of Nudix proteins. It is known that Nudix hydrolases perform a “housecleaning” role by hydrolyzing potentially toxic nucleotides and by preventing the unbalanced accumulation of normal metabolites. It means that in the cell cycle or during periods of stress the signalling molecules or metabolic intermediates whose concentrations require modulation during changes, increase. High concentrations may become toxic to the cell. The fact that the mutant is unable to utilize cAMP and nucleotides indicates that *in vivo* condition during the bacterial growth the accumulation of these compounds will lead to a toxic effect for the cells. Presumably, the enzyme plays a role in both

extracellular and intracellular survival of the bacterium and has a high impact on the life style of the pathogen.

Based on presence of the EAL domain in the protein sequence, the enzyme seems to have a phosphodiesterase activity in relation to different nucleotides, and hydrolyze c-di-GMP those increased concentration in the cells share a toxic effect together with other metabolites.

Hence, results of the metabolic profile of the mutant demonstrate the association of the predicted function of enzyme and described phenotype. The PA4916 protein is a novel gene which has not been reported earlier for *P. aeruginosa*, and is predicted to be involved in the mechanism of c-di-GMP signal transduction.

IV. Conclusion and perspectives

Pseudomonas aeruginosa is a metabolically versatile ubiquitous microorganism and an opportunistic pathogen for plants, animals and men. *P. aeruginosa* is a serious problem in patients with CF. Chronic airway infection and the accompanying inflammatory response are clearly the major clinical problems for CF patients today.
Strains of *P. aeruginosa* from CF patients with chronic lung infections display high phenotypic diversity. High frequency of phenotype switching is often the result of adaptive genetic diversification resulting in increased chances of bacterial survival in their niche. During chronic *P. aeruginosa* infection there is a progressive anatomical deterioration of the CF lung, which provides spatial compartmentalization associated with diversification of the organism into morphotypes.

Colony morphology is an easy-to-follow phenotypic trait and hence the morphotype is used as a major criterion in the clinical microbiology laboratory to select isolates for further analysis. The colony is a microbial community of genetically identical organisms whereby the individual cell faces a continuously changing microenvironment determined by the mass flow of nutrients and metabolites and the signals of its neighbours. Consequently, features of growth and aging, motility, secretion, extracellular matrix composition and cell-to-cell communication will govern the macroscopic appearance of a colony.
In this work, we were interested to know if and if yes, to what extent a single mutation could change both morphotype and biological fitness of *P. aeruginosa* in the atypical niche of mammalian lungs.
A metabolically versatile and pathogenic strain TBCF10839 from chronically infected CF lungs was chosen as the model organism because we hypothesized that such a strain had demonstrated its capability to persist in human airways and thus the perturbation by single hit mutagenesis should unravel the possible next steps of dissociative behaviour during niche adaptation.
Screening of a library of non-auxotrophic transposon mutants under different culture conditions *in vitro* uncovered that a knock-out in about 1% of the 5,500 ORFs of the *P. aeruginosa* genome led to a change of morphotype half of which was robust. In the other half environmental cues could rapidly modulate colony morphology in

accordance with the complex etiology and rapid turnover of this phenotypic trait in nature.
Half of the identified targets encode features of primary or secondary metabolism, and the other half is shared in equal portions by genes assigned to the categories of transport, regulation or motility/chemotaxis.

The TBCF10839 strain has a robust mucoid morphotype and transposon insertion had led to a non-mucoid strain in four mutants (type E), but none of the targets has reported previously to be associated with alginate biosynthesis or its regulation.

Autolysis divided into three subtypes (type A, PqsL, PilY1 and PilW) was the most prominent morphotype in the collection of single mutants, but almost an evolutionarily neutral trait in murine lungs. Cell lysis is supposed to be a disadvantageous phenotype, however, all phage-competent autolytic mutants were not impaired in their fitness to grow in murine airways. Moreover, the soft autolysis induced by *pilY1* and *pilW* mutants even increased the potential of the pathogen for the survival.
For all other morphotypes, the association between colony morphology *in vitro* and survival in *vivo* was rather loose indicating that gene inactivation may modulate fitness by various modes, the change of morphotype being the most visible but not necessarily the most relevant feature *in vivo*.

By several examples: *pqsD* and *mvfR, opmQ* and *fpvR, pilW* and *pilY1* were shown that the knock-outs in related pathways or regulons may converge to the same morphotype. However, despite the similar morphotype, the fitness *in vivo* for some mutants was different. As an example, the genes of the mutants of category B showed to be essential for the production or regulation of quinolones and phenazines. The morphology for *mvfR* and *pqsD* mutants was the same, but the knock-out in an important gene for biosynthesis (*pqsD*) was more essential for fitness then the knock-out of a regulatory gene (*mvfR*).

The metabolic control of morphotype was the main and most unexpected result of the study. Colony morphology variants were caused most frequently by the inactivation of metabolic genes. Two genes from the Entner-Douderoffy (*edd*) and malate-oxaloacetate (*mqoB*) pathways were shown to be necessary for airway colonizing

capacity and virulence of *P. aeruginosa*. Thus, the *mqoB* was less virulent than wild type and was compromised to utilize various compounds such as sugars and fatty acids that decrease the fitness of the pathogen.
The similar phenotype shown for the *edd* mutant was explained by its involvement in the carbon flow from glucose and gluconate into pyruvate and alginate. The reduction of the production these compounds reasonably explains why the mutant is less mucoid and out-competed both *in vitro* and *in vivo*.
By these two examples, the biological fitness of *P. aeruginosa* has been shown to require the enzymes that will turnover the acetyl-CoA, pyruvate and alginate, the key intermediate of sugar and fatty acid degradation.

In this work, the important role of the genetic background on genotype-phenotype associations was demonstrated. The colony morphology of the TBCF10839 strain in many cases was not reproducible by other PAO1 and PA14 strains. In spite of 99.8 % or more identity in the sequence of TBCF10839 with the respective PAO1 genes, the phenotype of TBCF10839 strain was not reproduced by the insertion of a transposon into the homologous gene of the PAO1 strain. As was shown by *edd* mutants of TBCF10839 and PA14 strains, the mutant of the last one could utilize glucose that indicates another mechanism of sugar degradation. Also, as an example, the reversion of a mucoid to a non-mucoid phenotype that affected the fitness of the TBCF10839 strain was not be accessible in a non-mucoid ancestor such as PAO1.

Thus, the STM technique used for the generation of the library has an advantage to allow simultaneous examination of a large number of isogenic transposon mutants using unique DNA marker sequences for differentiation. In our study, the screening a pool of mutants exposed to a distinct habitat of interest revealed 17 loss- or gain-of-function mutants with a worse or better survival. One of the mutants was out-competed in survival both *in vivo* and *in vitro.* The phenotype of the mutant was similar with those showed for the *nudA* mutant of *L. pneumophila* being described as a flagellated Gram-negative human pathogen. The mutant was not compromised in production of the virulence factors such as pyocyanine and protease, but was avirulent in the mice infection experiment. The enzyme encoding by the PA4916 gene possesses a phosphodiesterase activity and performs a “housecleaning” role by

hydrolyzing potentially toxic nucleotides and by preventing the unbalanced accumulation of normal metabolites. Presumably, it is the new non-described enzyme that plays a role in both extracellular and intracellular survival of the bacterium and has a high impact on the life style of the pathogen.

Hence, the targets identified in this combined *in vitro* and *in vivo* screening already provide ample opportunity to analyze in further studies the complex interplay between dissociative behaviour, adaptive radiation, lifestyle and fitness of *P. aeruginosa*.

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VI. Abbreviations

AHL	acylated homoserine lactones	Max.	Maximum
approx.	approximately	min	Minute
bp	Base pair	MOPS	Morpholinopropanesulfonic acid
^{o}C	Degree Celsius	n	nano- (10^{-9})
c-di-GMP	cyclic-di-guanosine monophosphate	OD	Optical Density
CDS	Coding sequence	ORF	Open reading frame; possible gene
CF	Cystic fibrosis	p	pico- (10^{-12})
cfu	Colony forming units	PAGE	Polyacrylamide gel electrophoresis
dATP	Deoxyadenosine triphosphate	PQS	2-heptyl-3-hydroxy-4-quinolone
DEPC	Diethypyrocarbonate	PCR	Polymerase chain reaction
dGTP	Deoxyguanosine triphosphate	QS	Quorum sensing
DIG	Digoxigenin	rpm	Revolutions per minute
DNase	Deoxyribonuclease	RNase	Ribonuclease
dNTP	Deoxynucleotide triphosphate	RT	Room temperature (23 ^{o}C)
dTTP	Deoxythymidine triphosphate	s	Second
EDTA	Ethylenediaminetetraacetic acid	SDS	Sodium dodecyl sulfate
EPS	Extracellular polysaccharides	t	Time
e.g.	For example	T	Temperature
et. al.,	et alteri (and others)	TE	Tris-EDTA
i.e.	That means	µ	micro- (10^{-6})
FCS	Fetal calf serum	Mb	Megabases
x g	Centrifugal acceleration	max.	Maximum
h	Hour	Tris	Tris(hydroxymethyl)aminomethane
HAQ	4-hydroxy-2-alkylquinolines	TTSS	Type III secretion system
HHQ	4-hydroxy-2-heptylquinoline	U	Unit (unit of enzymatic activity)
I.E.	Injection units	UV	Ultra violet
kBp	1000 Base pairs	V	Volt
LB	Luria-Bertani	Vol.	Unit volumes
M	Molar	% v/v	Percentage volume per total volume
m	milli- (10^{-3}); Meter, Mass	% w/v	Prcentage by weight per total volume

VII. Appendices

Appendix 1

Nucleotide Sequence of the genes surrounding the Tn*5* insert of 6eB10 mutant

Gene	Strand	Left End	Right End
A1	+	181	387
A2	+	507	1220
A3	-	1248	1490
A4	+	1744	2040
A5	+	2037	2738
A6	+	2735	3418
A7	+	3415	3621
A8	+	3618	3989
A9	+	4419	4628
A10	+	4666	5340
A11	-	5498	5932
A12	-	5979	6161
B1	+	6359	6691
B2	+	6688	7044
B3	+	7047	7304
B4	+	7304	7543
B5	+	7540	8061

A1 dir* [182-388]

TGAACATCACTGAAGCCATTGAGCATTTTGGCTCCAAGAAAAAGCTGGCTGACGCCTTGGGGATT
CAGCCAAGTGCTGTCACCCAGTGGGGCGATTCGATACCTGTCGGCCGCCAGTACCAAATTCAGG
TGATTAGCAAGAACAGGCTGAAGGCCGAGTCTGGAGCAGCTCGTGATGAAGAGACTACGGGGG
CGGCGGAAGCCTAA

A2 dir [508-1221]

TGTCACAAGTTGCAGTCATCCAACAAGGCCCGGTCCTGGCGATGAGCAGCCGCGAGATTGCGGC
GCTGGTCGAGTCCCGACACGACAACGTGAAGAGGACCATCGAGCGCCTCGGCGAGAAGGGGGT
CATCAGGTTTACTCCGTCGGAGGAAACCTCCCACGCTGGCGCTGGGGCGCGGCCCGTGAGTGT
TTATCTCGTCGACAAGCGCGACAGCTTCGTTGTTGTTGCGCAGCTCAGCCCGGAATTCACTGCG
CGCCTGGTGGACCGCTGGCAGGAGTTGGAGTCTCAGCTAGCGCATGGTGTGCCCGCCGTCCCT
ACGAATCTGGCGGATGCACTGAGGCTGGCTGCTGATCAGGTCGAGAAGAATCAGGCGCTGCAGT
TGGTCATCAGCGAGCAGGCGCCCAAGGTCCAGGCCCTGGAGCGGCTCAGCGGTGCGGCCGGC
ACGATGTGCATAACCGACGCAGCGAAGCACCCTCAGATCAACCCCGCCCGGCTCTTCGACTGGC
TCCAGCAGAACCGATGGATCTACCGCCGGAGCGGCTCTGCTCGCTGGATCGGCTATCAGCCAC
GAATCCCAGACGGCTGGATCATGCACAAGGTGACGGTTCTCGTTCGTGACGACCAGGGCGACGA
GCGTGCTGCGAGCCAGGTACGCATCACTGCCAAGGGGCTGTCGGTGCTGGCGCGGAAGATCGA
GGAGGGCAAGCTGTGA

A3 rev [1249-1491]**

CACGAATCAAGAAAATGTGTCGCCGGAGGTGAGCCAGTGAGCACGATCATCATGTCGGCCTGCT
GGCCGCTGCAGGGTTTGACGCCGGCGCAGAAGGCTGTGCTGATCAGCTTGGCGGACAACGCGA
ACGATGAGGGCGTGTGCTGGCCTTCGGTGGCGAAGATCGCCGAGCGCACCTGCCTTTCCGAAC
GTGCCGTGCAGCAGGCCATCAAGGTGCTGAACGAGTGCAAGGCGCTGAGCAT

A4 dir [1745-2041]

TGCAGGAGGCTTGCCGGAATGTGTGGGCAGCGTACCGGGCAGCCTACGAGGCGCGCTGGAGT
GTTCAGCCGGTGCGAAACGCCAAGGTCAACTCCCAGGTGAAGCAACTGGTGGCCGCCCTCGGC
GGCGAGGCTCCAGCGGTAGCGGCGTTCTTCGTCGGGCTGGATGACAAGTTCCTGGTCGACAGT
TGCCATGAGTTCGGGTTGCTACTGGCCAAGGCTGGCGCTTACCGGACGAAGTGGGCGACAGCC
GGTTCCGCGCCGTCGGCCGATTGGACTGATCAGGTGCAGCTATGA

A5 dir [2038-2739]
TGACCCGCAGGCAGTTCGAACCGCAATCGGTCGGTGCTGTGCTGGCGCATGTGAATCAGGACG
CGGGGCTGCGCCCCTTGTCCCAGCCGGCGGTGAAGGTCGATCCCCAGACGAGAAGCGAGGTC
GACCGGTTGTTCTTGCGGATCAAGGCGATCTGCCCTGGATGGAGAAGCTCCTGGCCCAACGATG
AGGTCGAGAACGCAGCGAAGGCGGAGTGGCTGGCAGAGATCGTCCGGCAACAGGTTACGCGCC
GCGAGCAACTGCAGGCCGGGGTAAGAGCGTTGAGCGCGCAGGCAAGGCCGCTTGTTCCATCTG
CCGGCCAGTTCTGCGCCTGGTGCTGGGCTCCTGAGGTCTTCGGCCTGCCATCCCTTGATGACGC
ATATCGCGAGGCGCTGGCCAATACCCACCCAGCCATGGTCGGAGCCGCGAAATGGAGTTGCCC
TGCGGTGTATTGNGCAGCCGCTGGCGCTGGATTCAGCCGGCTGCAGGCTCTGGCAAGAAAGGA
TGGGCTTGCGGCGCTGGAGATCTCCTATCGACAGATCATCAAGAAGCTGGCGCGTGGCGAGGC
GCTCGGGAAGGTCCGGATCGACAGTTGCCATGAGTTCGGGTTGCTACTGGCCAAGGCTGGCGC
TANCCGGACGAAGTGGGCGACAGCCGTTTCCGCGCCGTCGGCCGATTGGACTGATCAGGTGCA
GCTATGA

A6 dir [2736-3419]
TGACCCGCAGGCAGTTCGAACCGCAATCGGTCGGTGCTGTGCTGGCGCATGTGAATCAGGACG
CGGGGCTGCGCCCCTTGTCCCAGCCGGCGGTGAAGGTCGATCCCCAGACGAGAAGCGAGGTC
GACCGGTTGTTCTTGCGGATCAAGGCGATCTGCCCTGGATGGAGAAGCTCCTGGCCCAACGATG
AGGTCGAGAACGCAGCGAAGGCGGAGTGGCTGGCAGAGATCGTCCGGCAACAGGTTACGCGCC
GCGAGCAACTGCAGGCCGGGGTAAGAGCGTTGAGCGCGCAGGCAAGGCCGCTTGTTCCATCTG
CCGGCCAGTTCTGCGCCTGGTGCTGGGCTCCTGAGGTCTTCGGCCTGCCATCCCTTGATGACGC
ATATCGCGAGGCGCTGGCCAATACCCACCCAGCCATGGTCGGAGCCGCGAAATGGAGTTGCCC
TGCGGTGTATTGGGCAGCCGCTGGCGCTGGATTCAGCCGGCTGCAGGCTCTGGCAAGAAAGGA
TGGGCTTGCGGCGCTGGAGATCTCCTATCGACAGATCATCAAGAAGCTGGCGCGTGGCGAGGC
GCTCGGGAAGGTTCCGGAGGGAGAGGTCACCCACCAGAAAGCGCGAACCCAATCCGTTGGAAT
TGCTGCGCTTGCGCAGCTTCGAAAACAACTCAAAGGAGGAGATCGCTCATGA

A7 dir [3416-3622]
TGAAGTGGAGCGTACTCAACGACTATCTGATGGTTAGCGACACCCAGCCGCCCTACAAGGTCTG
CAAGCTCCTGGTCGCCGGCGAGGCTCACTACCGGGCCAGTGTTCAGGGTGAATTCATTTGCACC
CCGGTTGCGACTGCGAAGGAGGCGTGCGGTGTTTGCGAGCGCCATCACCAGATCACCTATCCG
CGGGAGGTCGCGTGA

A8 dir [3619-3990]
TGAAGGGGCGGGCCGTTACTTCGGAGCAGAAGCGCTGGCACGACCTGCTGGCGCGCCATGTGG
GGTGCATCGCCTGTCGGGTGTCCATGGGGATCGTGAACACCTATTGCAGCATTCACCACGTCGA
CGGCAGGACGAAGCCCCACGCGCATTGGTATGTGCTGCCGCTGTGCGCTGGGCATCATCAAAA
CGGCTACGGCGGTGCGGGCTTCACCGGGGTCGCCGTTCACCCGTACAAGGCGCGCTTTGAGGC
TGAGTACGGAACCCAATCGGACCTGCTTTCGAAATGCGCCTCGATCTTGGCGGAGGAGGGGCAC
GACATACCGGCGGGGTTCCTCGCATGGCTAGACGGTGGCGAGGTGGAAGCATGA

A9 dir [4420-4629]
TGTTCCACGAGATCGATTGCGCCGCTTGTGGAGCAGCGGGGTTCGTCGATGGCGTGACGGGGC
TGGCGCTGGAGCAGCGGGATGCGGTGGTGCAACTGCGGATGTGGGTAAAGCGGCTGCTGGAAG
AGCAGCGACGCCAGGCGAGCAGGCTGGCGCGAGAAGAGAGCAACCGGAAGGGCGCTGGCGGC
GCTCACTTTCGAGGGGATTGA

A10 dir [4667-5341]
TGATTTACGAAAGCGTTTCAAGTGCGGTCGTTTCGGCGCTGGCAGCAGACTGCATCGACAACAC
AAGCAAGCAGGCATGGCAAAAGCTCTATCAGGCCGGCGAGCCTGGTCGTCGTGGCGGAGTGAT
GGTATCCGCTGATCTCAGGCAGCAAATCGATTGCTGGGTACATGCTCGCTTGCATGACCAACTTA
TTCCGCGCCACTGGGCGGCGCTGGTGGCGAAGTACAGCACTCACCAGGCTAAGAAAGTCCAGG
CGATCTCTCTTCTGCGGTCGGTGGTCGCAACGCCGGCTCCTGCTCTTTTCCTCTACAAGGCCATA
ACGACTTGGGCGATTCCGAAACTGAAGGGTGTCCAGCCGGCGCTGCGAAAAACCGTCTCTGTCG
AAATCCCAGTGGACGGATCACCAGAAAAGCAAGCCAGGGCCGTGCGCGCCGCGCTGGAGGCAG
AGCGAGTGAAGCGGAAGCGCCTTATGGCTCGATCTTCTGGAATGATCGTCCTGCCGGATGAGTT
CTACGACATGAACACATGGGATCTCGATGGGAAGCCCGAGTCGACTCGGCGTGAGTGGCGCAG
GAAGATTCATCGTGTTCTCGACGAAATGGTCGACGAGGCGCTGGTGGCGGCGGAGCAGATCCTC
AACGCAGAGGGATTGCTGGCCAAGGATGCGGCATAG

A11 rev [5499-5933]
TACGTCTTTGTCCCGCTCGATCTGAACTCGCAGCCGAAGGCCGAGAAAATCAAGCGCATTTTCCA
GCGCTTCCATTTTGGTGTTGTGGAGGAAGTCAACGAGCCTGTCGCCCTGAGTTTGTGCGATACC
GAGCAGCCGGCAGAGGTCAGCCTTCGCATATCTCGCCGGACCATTTCGTTCCAGAGCGCGATCT
TCGCAACGGTAACCGCCGGCAGATGGATAACGCGCTCGCCTGGTTGCGCCTGGCTGGCTGCGG
GGATTTCTCGGCGCTGATCAACGTACAGCGAGAGGGTTGACTCGATGGCGTCAACCGCCTCGCT
GATTGCATGGGCCTTATCGTCACCGTAGCTGTTCAGTTCCGGCAGGTCTCTGCAGAAAACGGCG
ACGCCAGGAGCGCTATCGTCCTGTTCGAAACGGATTGCATAGTCGTACAT

A12 rev [5980-6162]
CATTTGAGCCCCAGTTGCTTGATGATCGCCTTGCGGGTCGGTTCTGGCATTTCCTTAGTTCCGTG
GTCCGCGAAGGTGGTCTGTTTGCCGTTCGGGGCGGTGATCTTGAAGTGGCTTCCCTTGCCGGCT
TCGAAGGTCACCCCTTGGGCCTTCAACCATCGTCTGAATTCGCTGAACTTCAT

B1 dir [6360-6692]
TGAAGATGCCTGACAAACCCGACACCTGGGCGGCCCTGCTCGCTTGGCTGAGCCAGCATGCGC
CTATCATCTGTGCCTCCCTCCTGTCGTGGGCCATGGCTATTGCCAGGATCATTTATGGGGGCGGT
ACGCGCAGACAGGCCTTATGGGAAGGCGCGCTTTGCGGCGGGCTGGCACTGACGGTTATCAGT
GGGTTCGAGTTCTTCGGCGTTCCGCAGAGCATGGCCACCTTCATTGGTGGCTGGATCGGATTCC
TGGGTGTCGAGAAGATCCGCGACCTGGCTGACCGTTACGCAGGGATCAAGCTGCCGCGTCGAG
GGTCTGGCGAATGA

B2 dir [6689-7045]
TGATGATTACCGCCGATCAACTCGACCGTGCTACCGGCTGCGGCGGCGCTACTGCTTCGGTCTG
GGTCGAACACATCAACGGCGCCATGGATCGGTTCGAGGTCAACACGCCTGAGCGCGTGGCGAT
GTTCCTGGCTCAGGTCGGGCACGAAAGCCAGAGCATCAAGCGCCTGGTCGAGAACCTGAACTAC
TCCGCCGATGGCTTGCTNCAGACGTGGCCGATACGGTTACGCCTGCCGAGGCGAAGCAGTACG
CCCGCCAGCCAGAGCGCATCGCGAACCGCGTCTATGCAAACAGGATGGGCAATGGGTCGCCGG
ATACGGGCGATGGGTATCGATACCGGGGCCGCGGCCTGA

B3 dir [7048-7305]
TGATCACCGGCCACGACAACTACGCCGAAGCCGCCCGCGCCCTGGCGCTGCCACTGGTGGCGC
AACCGGAGTTGCTTGAGCAACGGACCTGGGCTGCCATCGCGTCGGCATGGTGGTGGAAGTCGA
GGGGTTTAAACGAACTGGCCGACCAGGGTCGCTTCGAGCGGATCACCCTCAAGATCAACAGTGG
CTACAACGGCGCAGATGACCGTGCGGCTCGCCTCGAGTGGGCGCGTGCTGCGCTCAAGGGGGA
ATGA

B4 dir [7305-7544]
TGCTCGGGTTCACGACGAAAGCTGAGGCGCGACGCATCGGCGCCTCGCACCACGGGAGCTATT
ACGGCATTCCGATGTGGCTAGGGGATGTCGATAGCGATTGCCCGCTAGCGTTCGCAAAGTGGGC
GCCGCTTGAGCTGGTCGTCTCCCTGCTCTCGGTCATTGAGGGCATCGTCAACTCGATGCTCGAT
CAAGAGCAGACGTTCATGTTCAAGGTTGGTCGGAGGATCGACCAGTGA

B5 dir [7541-8062]
TGACCTGGCGGCCCTGGTTGGTGGTCGCCCTGGTAGGCGCGCTTGTGTTCTGGCGCCTCGATC
ACGTGACCGCTCAGCGTGACGACCTGCAGGCCGCCGTCGAGCAATCCGCCGAGACGATCACCG
CAATGGCCCAGCAGGCCAAGCGCGACACCCAGGCACAGGTCGAGGCCGATGCCCTGGCCCGAA
CCTACCAAGCAGCACTACAGGCCTCCCATGAAGAAAACCAATTGCGCCGCGATGCTATCGGCAC
TGGTGCTCGCGTCGTGTACGTCAAAGCCCGCTGTCCCGCAGACGGAGTGCACCCGGCTCCCGG
AGCCTCCGGCAGCGCTGATGCAGGAAGAGCCGTCCTTGCTGCCGCTGATGGACAAGTTGTTTCT
GATCTCCGAGCCGGAGTCGAGCGACGCGAATTGATGATTGAGGCGCTGCGTAAGCACATCGCC
GCCCTGCCGAGGATTGCAGAAGATGATCAGTATCAAGCCGGNAAGGGTTCCAGCAACANCCTCG
CCCGACCTGACTGA

***direct sequence**
****reverse sequence**

Appendix 2

The transcriptome data

Table IV-1 The transcriptome profile of genes with impact on morphotype.

					Strains indicated by different color		TB		892		CHA		PAO1		LES400		LES431	
PAO1 gene number	**Gene name**	**Gene annotation**	***Morphotype**	****Survival**	**[a] Growth condition**		[1] late exponential		late exponential		late exponential		late exponential		late exponential		late exponential	
					Chip- No.		MHH01	MHH01	MHH01	MHH01	MHH03	MHH03	MHH03	MHH03	MHH03	MHH03	MHH03	MHH0
PA0413	*chpA*	Chemotaxis p	E				161	180	256	241	155	123	428	379	148	105	108	181
PA0415	*chpC*	Chemotaxis p	E				114	98	165	175	192	219	472	444	116	155	160	178
PA0424	*mexR*	Multidrug resi	A				139	105	211	246	263	377	153	128	2353	2065	1162	835
PA0482	*glcB*	Malate synthase G					521	510	232	370	455	431	706	849	860	887	967	1091
PA0728		Probable bacteriophage integrase					6	4	4	9	43	31	4	20	7	2	7	9
PA0785		Probable acyl carrier protein ph		N			11	14	5	8	9	37	44	42	12	19	60	15
PA0920		Hypothetical membrane protein					86	99	63	95	131	81	33	15	50	44	73	94
PA0999	*pqsD*	3-oxoacyl-[acy	B	L			1392	1144	208	281	648	742	74	87	66	40	407	451
PA1003	*mvfR*	Transcription	B				254	224	245	208	121	114	100	39	71	2	191	243
PA1589	*sucD*	Succinyl-CoA synthetase					1809	1952	1629	1829	1868	2550	1575	1799	1816	2064	1723	1451
PA1633	*kdpA*	Potassium-transporting ATPase					32	17	22	10	140	104	85	144	129	150	28	33
PA1823	*nudC*	NADH pyrophosphatase					280	268	71	108	115	178	213	204	208	262	149	170
PA1846	*cti*	Cis/trans isom	E				54	48	44	52	74	56	15	146	93	39	15	148
PA2028		Probable trans	A				28	31	39	39	37	22	32	43	53	38	14	82
PA2122		Hypothetical p	A				9	32	5	5	7	6	9	11	5	8	7	8
PA2361		Hypothetical p	B				104	96	42	31	14	13	35	67	83	25	121	154
PA2388	*fpvR*	Transcriptiona	F				152	184	104	125	21	36	125	154	73	84	156	202
PA2391	*opmQ*	Probable oute	F				31	17	31	31	94	57	155	91	65	54	117	120
PA2537		Probable acyl	C	L			84	90	40	40	21	23	134	115	569	511	178	208
PA2579	*kynA*	Tryptophan 2,	C				93	118	140	228	126	107	143	176	178	199	131	140
PA2706		Hypothetical protein		L			152	142	125	133	217	184	185	210	189	149	206	335
PA2838		Probable trans	C	L			12	19	12	13	17	6	37	14	10	24	19	16
PA2946		Hypothetical protein; predicted integral membrane protein					471	365	336	323	382	383	755	641	821	651	569	564
PA3012		Hypothetical protein					62	66	111	154	67	77	16	5	46	47	77	80
PA3194	*edd*	Phosphoglucd	G	N			196	202	194	187	1797	933	382	484	378	207	458	518

Continued Table IV-1.

TB		892		CHA		PAO1		LES400		LES431		TB		TB		TB		TB		892	
[2] late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		[3] late exponential + PMNs		[4] early exponential, iron depleted		[5] late exponential, iron depleted		[6] late exponential + paraquat		late exponential + paraquat	
MHH00	MHH00	MHH01	MHH01	MHH03	MHH03	MHH01	MHH01	MHH03	MHH03	MHH03	MHH03	MHH00	MHH00	MHH00	MHH00	MHH01	MHH01	MHH00	MHH00	MHH01	MHH01
196	245	217	173	173	133	775	668	54	168	76	131	190	136	106	136	133	133	349	261	380	261
129	158	142	103	239	87	212	223	238	72	132	185	208	173	335	240	218	191	180	168	229	167
335	302	210	245	1579	1344	149	156	1395	1319	1357	1292	1018	841	101	35	135	111	415	334	192	248
399	359	328	294	78	106	765	644	287	413	813	718	372	442	379	418	374	331	508	445	595	402
5	5	6	9	25	35	15	4	10	11	3	9	13	25	30	26	18	39	10	13	14	9
5	4	11	13	54	243	25	39	65	10	33	56	8	11	158	40	24	10	4	9	10	17
70	69	96	94	92	56	43	47	128	150	43	57	58	74	108	83	130	148	45	60	111	82
187	253	176	158	310	149	56	86	31	28	348	363	388	556	747	706	651	1161	109	145	444	387
86	98	63	38	157	120	12	36	20	94	51	70	433	479	200	93	188	216	365	420	143	188
3481	3044	3091	3490	412	522	3359	3409	394	809	1775	1389	1258	1435	2268	2056	2338	2755	1497	1225	3074	2359
52	53	15	13	118	162	43	47	84	29	61	67	51	26	57	193	99	153	23	28	36	26
167	167	204	166	133	21	282	304	138	19	166	82	85	82	209	173	172	142	122	87	152	120
85	81	82	81	19	134	98	83	41	10	69	86	20	43	105	7	65	76	38	34	69	48
39	46	50	45	67	6	26	22	7	31	45	52	59	62	143	53	75	45	36	39	38	33
3	3	3	3	26	25	17	6	21	7	9	9	9	21	14	12	9	10	8	9	5	5
12	9	13	18	92	17	14	7	41	13	42	48	37	24	16	56	38	24	28	42	32	24
216	202	200	221	80	30	196	239	103	100	225	116	733	663	143	213	238	184	293	283	166	146
88	88	79	62	44	31	42	45	163	185	54	94	64	38	98	237	323	368	34	35	49	32
64	47	46	34	104	49	37	47	85	160	128	81	46	64	98	169	85	100	49	44	40	38
366	354	225	225	125	55	73	55	76	143	204	164	82	83	134	162	107	56	239	226	279	226
108	147	124	109	301	37	99	105	282	230	125	134	130	160	223	147	104	82	107	118	150	136
4	11	15	15	25	20	4	9	71	108	6	9	14	16	21	40	38	58	6	12	14	13
641	763	627	504	9	21	765	662	319	326	452	657	175	207	98	208	287	205	348	409	312	285
62	71	67	62	12	69	44	35	5	123	80	86	55	76	86	79	54	86	121	90	192	159
365	345	267	302	635	759	773	571	602	341	402	594	253	163	361	365	309	342	293	318	147	151

Continued Table IV-1.

					Strains indicated by		TB		892		CHA		PAO1		LES400		LES431	
PAO1 gene number	**Gene name**	**Gene annotation**	***Morphotype**	****Survival**	**[a] Growth condition**		[1] late exponential		late exponential		late exponential		late exponential		late exponential		late exponential	
					Chip- No.		MHH01	MHH01	MHH01	MHH01	MHH03	MHH03	MHH03	MHH03	MHH03	MHH03	MHH03	MHH0
PA3238		Hypothetical protein					122	127	50	55	140	123	51	21	131	98	83	16
PA3239		Conserved hypothetical protein		L			322	355	98	102	292	322	277	153	270	277	263	204
PA3462		Probable sens	A				45	47	63	46	27	26	17	17	6	8	26	15
PA3748		Conserved hy	A				154	142	122	144	185	231	241	227	223	208	130	168
PA3804		Hypothetical protein					340	326	124	200	430	391	381	306	358	498	274	124
PA4131		Probable iron-sulfur protein		G			2810	2598	331	463	119	173	660	954	96	93	805	750
PA4190	*pqsL*	Probable FAD	A				142	158	44	52	54	46	103	102	7	4	77	39
PA4489		Conserved hy	A				79	82	83	75	89	62	159	161	240	168	105	114
PA4552	*pilW*	Type IV fimbri	D	G			101	99	88	72	66	99	120	182	90	68	139	127
PA4554	*pilY1*	Type IV fimbri	D	G			80	99	74	84	149	114	230	188	75	55	89	131
PA4640	*mqoB*	Malate:quinon	G	L			212	231	177	240	389	458	346	392	389	360	325	323
PA4703		Hypothetical protein; predicted regulator of competence-specific gene					114	131	281	225	21	23	9	4	50	53	42	30
PA4734		Hypothetical p	C	G			103	125	109	139	178	145	104	108	111	85	68	65
PA4915		Probable cher	B				518	494	558	322	78	86	78	58	105	78	160	97
PA4949		Conserved hypothetical protein; predicted sugar kinase					81	90	91	86	199	275	159	94	138	140	97	174
PA4951	*orn*	Oligoribonuclease					342	315	473	397	249	253	322	355	157	186	130	184
PA4954	*motC*	Chemotaxis p	E	G			89	107	170	165	201	155	130	223	207	95	134	93
PA5121		Hypothetical membrane protein; predicted small-conductance mechan					23	34	31	38	102	88	20	15	75	105	40	28
PA5231		Probable ATP-binding/permease fusion ABC transporter					356	317	705	582	91	36	207	142	41	100	237	208
PA5524		Probable shor	A				30	40	36	54	98	110	85	86	30	56	66	41
PA5546		Conserved hypothetical protein		G			1168	1349	975	629	400	376	183	238	232	239	691	365
PA5563	*soj*	Chromosome partitioning protein					224	224	470	576	422	486	232	276	510	497	244	253
PA4797		not spotted on Affymetrix- Chip																

Continued Table IV-1.

TB		892		CHA		PAO1		LES400		LES431		TB		TB		TB		TB		892	
[2] late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		[3] late exponential + PMNs		[4] early exponential, iron depleted		[5] late exponential, iron depleted		[6] late exponential + paraquat		late exponential + paraquat	
MHH00	MHH00	MHH01	MHH01	MHH03	MHH03	MHH01	MHH01	MHH03	MHH03	MHH03	MHH03	MHH00	MHH00	MHH00	MHH00	MHH01	MHH01	MHH00	MHH00	MHH01	MHH01
129	161	164	147	318	61	165	149	451	459	237	221	129	138	37	112	163	225	63	46	73	74
250	283	317	342	374	240	325	359	824	1409	670	609	144	157	391	297	423	438	59	65	121	103
16	21	22	4	59	93	15	5	14	7	11	10	60	51	29	17	10	11	57	51	27	46
195	196	230	235	88	154	254	258	327	48	281	262	139	121	77	63	212	208	127	115	258	150
496	427	257	303	188	20	544	536	25	207	327	447	131	153	229	247	262	274	241	272	244	209
145	150	351	219	268	239	74	52	21	45	401	405	203	242	306	347	715	566	117	106	785	506
110	82	82	41	42	139	22	20	29	16	53	56	69	69	122	173	115	135	43	34	80	62
98	74	76	52	75	27	57	75	141	170	84	104	57	51	51	51	78	90	54	85	84	65
62	79	64	93	173	98	197	117	81	93	54	46	113	102	159	208	123	118	89	106	110	110
68	80	134	91	145	208	182	198	35	34	85	78	93	98	270	260	179	187	67	71	114	88
262	235	224	269	172	10	111	114	206	356	302	289	161	163	122	222	295	311	222	163	427	264
31	29	30	25	73	116	15	30	189	206	83	65	230	293	92	92	84	75	219	309	97	157
166	203	180	159	141	62	132	115	228	88	73	74	145	155	135	143	200	195	101	112	165	127
26	27	26	19	18	116	70	76	168	177	83	92	407	342	260	261	427	346	301	456	113	297
106	95	84	100	427	252	130	131	266	204	151	229	122	104	184	211	138	109	96	111	104	85
304	282	302	311	239	253	329	373	59	182	183	109	422	453	357	291	314	298	397	504	296	333
92	124	90	77	274	220	92	68	27	166	167	272	78	146	81	83	110	164	150	147	140	135
76	64	55	62	111	14	43	41	86	87	95	42	33	23	111	28	40	53	36	27	29	36
109	118	177	54	6	127	371	345	222	30	54	63	551	522	29	180	114	112	546	622	343	610
53	65	68	75	211	144	101	95	14	43	87	89	38	54	9	4	42	24	49	66	43	45
327	343	512	240	4554	2176	939	871	2664	3898	923	852	683	502	458	589	522	584	715	961	278	452
1045	1108	817	1007	237	294	370	430	448	273	462	446	343	360	112	253	317	236	604	499	589	587

* See Table III-3 and Figure III-2 for definition of colony morphology types

** Survival rate: G, gain of virulence; L, loss of virulence; N, non-competative (no survival *in vivo* and *in vitro*).

[a] Various growth conditions used for RNA extraction:

[1] Late exponential (LE) phase, cells were cultivated in LB and RNA was extracted at cell density OD578 = 3.5

[2] LE, LB + 10mM hydrogen peroxide

[3] LE, LB + polymorphonuclear neutrophils from the blood of healthy donors (separated from bacteria by Ø1 µm dialysis membrane)
[4] Early exponential phase, LB iron depleted by Chelex-100 beads (Sigma)
[5] LE, LB iron depleted by Chelex-100 beads (Sigma)
[6] LE, LB + 0.5mM paraquat

	called as "absent" by the Affymetrix- software
	called as "marginal expressed" by the Affymetrix- software

Table IV-2 The transcriptome profile of genes involved in malate-oxaloacetate pathway.

				Strains indicated by different color	TB		892		CHA		PAO1		LES400		LES431	
PAO1 gene number	**Gene name**	**Gene annotation**	**Catalyzed reaction**	**[a] Growth condition**	[1] late exponential		late exponential		late exponential		late exponential		late exponential		late exponential	
				Chip- No.	MHH01	MHH01	MHH01	MHH01	MHH03	MHH03	MHH03	MHH0	MHH0	MHH0	MHH03	MHH03
PA1252	*(mdh)*	probable L-ma	(L)-Malate + NAD+ <=> Oxaloacetate + NADH		99	114	43	33	60	81	71	115	55	61	124	87
PA1400	*(pca)*	probable pyruv	ATP + Pyruvate + HCO3- <=> ADP + Orthoph		16	16	31	29	73	33	5	7	43	62	62	72
PA1770	*ppsA*	phosphoenolpy	ATP + Pyruvate + H2O <=> AMP + Phosphoe		399	392	339	454	201	317	476	444	162	211	167	243
PA3452	*mqoA*	malate:quinone	(L)-Malate + Acceptor <=> Oxaloacetate + Rec		79	84	62	97	269	246	99	74	62	50	18	60
PA3471	*(malE)*	probable malic	(L)-Malate + NAD+ <=> Pyruvate + CO2 + NA		211	200	120	133	292	281	375	308	246	266	198	125
PA3687	*ppc*	phosphoenolpy	Orthophosphate + Oxaloacetate <=> H2O + P		248	269	119	118	172	259	165	153	129	112	137	123
PA4640	*mqoB*	malate:quinone	(L)-Malate + Acceptor <=> Oxaloacetate + Rec		212	231	177	240	389	458	346	392	389	360	325	323
PA5046	*(malE)*	malic enzyme	(L)-Malate + NAD+ <=> Pyruvate + CO2 + NA		495	409	347	375	562	673	997	814	1006	1288	622	561
PA5192	*pckA*	phosphoenolpy	ATP + Oxaloacetate <=> ADP + Phosphoenol		439	402	295	322	507	817	760	721	1542	1596	1031	993

Continued Table IV-2

TB		892		CHA		PAO1		LES400		LES431		TB		TB		TB		TB		892	
[2] late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		late exponential + H_2O_2		[3] late exponential + PMNs		[4] early exponential, iron depleted		[5] late exponential, iron depleted		[6] late exponential + paraquat		late exponential + paraquat	
MHH00	MHH00	MHH01	MHH01	MHH03	MHH03	MHH01	MHH01	MHH03	MHH03	MHH03	MHH03	MHH00	MHH00	MHH00	MHH00	MHH01	MHH01	MHH00	MHH00	MHH01	MHH01
35	30	42	35	72	161	37	41	163	55	57	69	71	105	255	166	101	144	37	21	40	32
24	20	17	21	102	171	18	18	59	107	25	53	27	35	21	42	35	20	26	24	35	23
741	705	784	729	26	42	774	713	145	10	324	193	310	256	270	356	287	312	416	387	765	559
447	448	221	342	207	177	91	105	18	83	99	157	58	54	263	295	238	268	71	65	64	67
227	249	416	207	89	7	388	321	76	124	230	242	123	121	247	170	182	130	98	112	268	202
87	89	131	89	254	293	220	209	116	28	133	162	263	284	65	246	204	216	119	155	119	116
262	235	224	269	172	10	111	114	206	356	302	289	161	163	122	222	295	311	222	163	427	264
1342	1255	1311	1236	274	206	1707	1718	159	222	812	763	333	287	306	380	403	428	348	338	537	397
1378	1235	1200	1288	196	87	1001	940	335	468	1325	1369	193	157	499	269	304	291	299	261	526	377

called as "absent" by the Affymetrix- software

called as "marginal expressed" by the Affymetrix- software

[a] See Table IV-1, footnote (a) various growth conditions used for RNA extraction

Table IV-3 The transcriptome profile of genes involved in the carbohydrates metabolism (Entner-Doudoroff pathway).

				Strains indicated by different color	TB		892		CHA		PAO1		LES400		LES431	
PAO1 gene number	Gene name	Gene annotation	Catalyzed reaction	[a] Growth condition	[1] late exponential		late exponential		late exponential		late exponential		late exponential		late exponential	
				Chip- No.	MHH01	MHH01	MHH01	MHH01	MHH03	MHH03	MHH03	MHH03	MHH03	MHH03	MHH03	MHH03
PA2261	*(kgk)*	probable 2-ketogl	ATP + 2-Keto-D-gluconate <=> ADP + 2-Ket		26	24	57	55	78	119	5	14	11	6	8	16
PA2321	*(glkB)*	gluconokinase	ATP + D-Gluconic acid <=> ADP + 6-Phospl		18	14	520	609	780	735	80	139	321	283	320	277
PA3131	*(aldA)*	probable aldolase	2-Dehydro-3-deoxy-6-phospho-D-gluconate		51	63	36	66	138	200	98	120	100	15	97	77
PA3181	*(kdpA)*	2-keto-3-deoxy-6-	2-Dehydro-3-deoxy-6-phospho-D-gluconate		62	74	116	116	413	471	119	67	525	574	323	305
PA3183	*zwf*	glucose-6-phosph	D-Glucose 6-phosphate + NADP+ <=> D-Gl		65	74	97	115	621	641	130	142	904	1155	449	409
PA3193	*glk*	glucokinase	ATP + D-Glucose <=> ADP + D-Glucose 6-p		36	52	49	55	156	191	75	146	109	93	134	93
PA3194	*edd*	phosphogluconat	6-Phospho-D-gluconate <=> 2-Dehydro-3-de		196	202	194	187	1797	933	382	484	378	207	458	518
PA3195	*gapA*	glyceraldehyde 3-	D-glyceraldehyde-3-phosphate + Orthophos		141	130	258	250	1432	1685	785	1028	452	574	532	423
PA4029	*(aldB)*	conserved hypoth	2-Dehydro-3-deoxy-6-phospho-D-gluconate		55	50	343	362	187	295	211	231	402	420	209	203
PA4732	*pgi*	glucose-6-phosph	D-Glucose 6-phosphate <=> D-Fructose 6-p		459	501	212	177	408	462	659	874	542	554	630	708
PA4783	*(aldC)*	conserved hypoth	2-Dehydro-3-deoxy-6-phospho-D-gluconate		12	14	36	36	35	23	83	69	78	24	28	97

Continued Table IV-3

TB	892	CHA	PAO1	LES400	LES431	TB	TB	TB	TB	892
[2] late exponential + H_2O_2	late exponential + H_2O_2	late exponential + H_2O_2	late exponential + H_2O_2	late exponential + H_2O_2	late exponential + H_2O_2	[3] late exponential + PMNs	[4] early exponential, iron depleted	[5] late exponential, iron depleted	[6] late exponential + paraquat	late exponential + paraquat
MHH00 MHH00	MHH01 MHH01	MHH03 MHH03	MHH01 MHH01	MHH03 MHH03	MHH03 MHH03	MHH00 MHH00	MHH00 MHH00	MHH01 MHH01	MHH00 MHH00	MHH01 MHH01
86 81	134 72	26 16	161 181	69 29	29 7	7 5	176 35	15 12	115 83	41 50
1043 972	852 940	401 247	529 542	199 463	915 875	128 80	40 172	189 239	690 614	612 506
99 130	104 113	33 179	101 113	13 58	133 124	53 48	13 56	86 81	58 53	89 60
565 545	262 317	5 48	427 415	230 97	455 394	64 86	48 49	41 65	178 203	49 64
329 304	209 248	151 23	898 944	362 182	1540 1705	51 48	14 17	52 12	168 162	57 77
219 228	145 150	158 31	201 184	163 157	86 53	42 56	19 22	69 57	103 138	45 50
365 345	267 302	635 759	773 571	602 341	402 594	253 163	361 365	309 342	293 318	147 151
640 540	445 379	616 444	2440 2233	322 250	314 337	338 306	228 492	305 325	680 758	156 176
276 261	257 271	88 50	105 108	238 207	358 348	89 104	20 102	86 59	340 263	427 373
374 313	413 329	258 192	406 411	149 351	249 357	475 511	451 569	297 396	242 208	226 188
23 36	25 21	75 18	48 47	141 70	30 72	16 26	11 79	55 67	26 31	31 33

called as "absent" by the Affymetrix- software

called as "marginal expressed" by the Affymetrix- software

[a] See Table IV-1, footnote (a) various growth conditions used for RNA extraction

Appendix 3

Body weight and rectal temperature of mice airways infected by *P. aeruginosa*

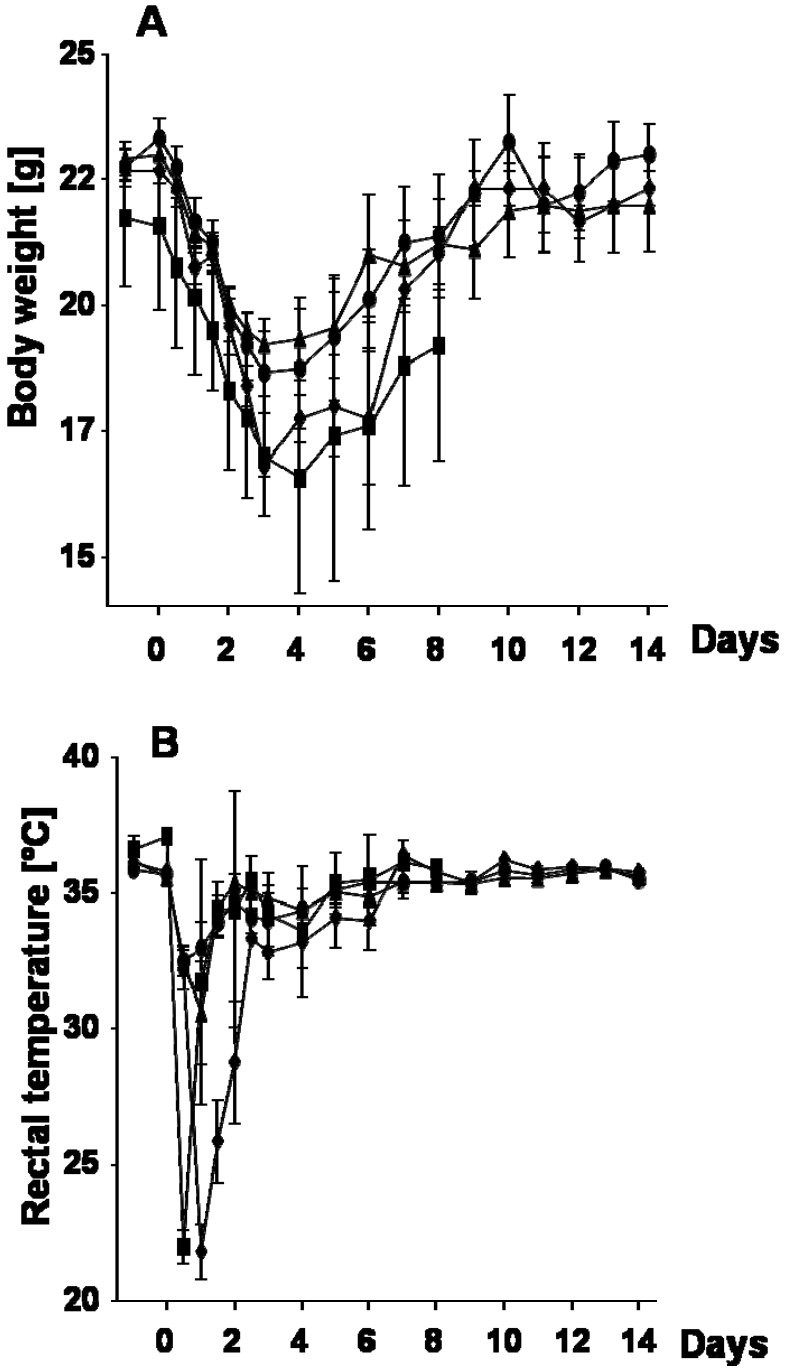

Figure IV-1. Body weight (A) and rectal temperature (B) of mice whose airways had been inoculated at day 0 with $5x10^7$ CFU of *P. aeruginosa* TBCF10839 (closed square), *P. aeruginosa* TBCF10839 Tn*5*::PA4640 (closed circle), *P. aeruginosa* TBCF10839 Tn*5*::PA3194 (closed triangle) and *P. aeruginosa* TBCF10839 Tn*5*::PA4954 (closed diamond).

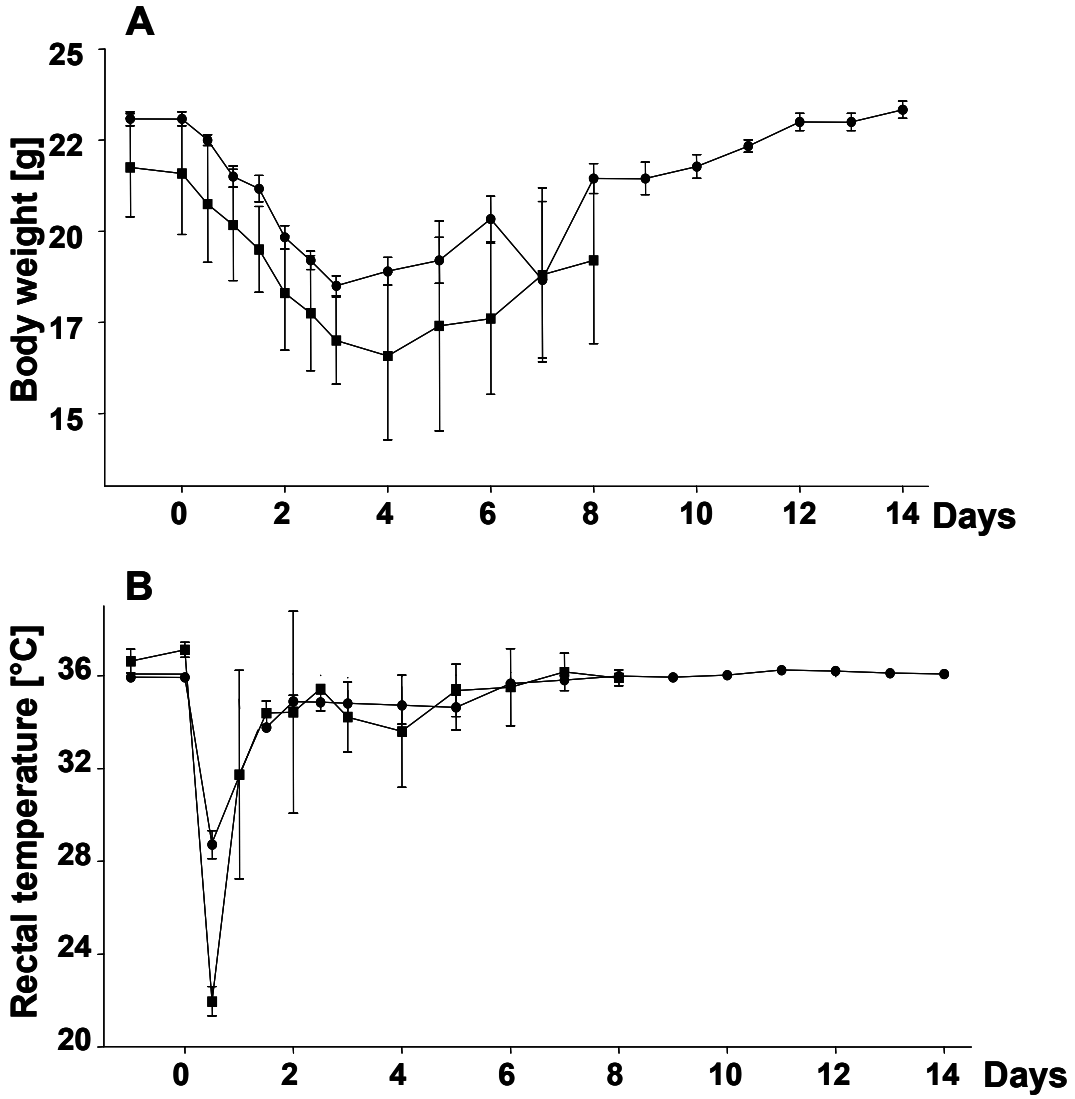

Figure IV-2. Body weight (A) and rectal temperature (B) of mice whose airways had been inoculated at day 0 with $5x10^7$ CFU of *P. aeruginosa* TBCF10839 (closed square), *P. aeruginosa* TBCF10839 Tn*5*::PA2537 (closed circle).

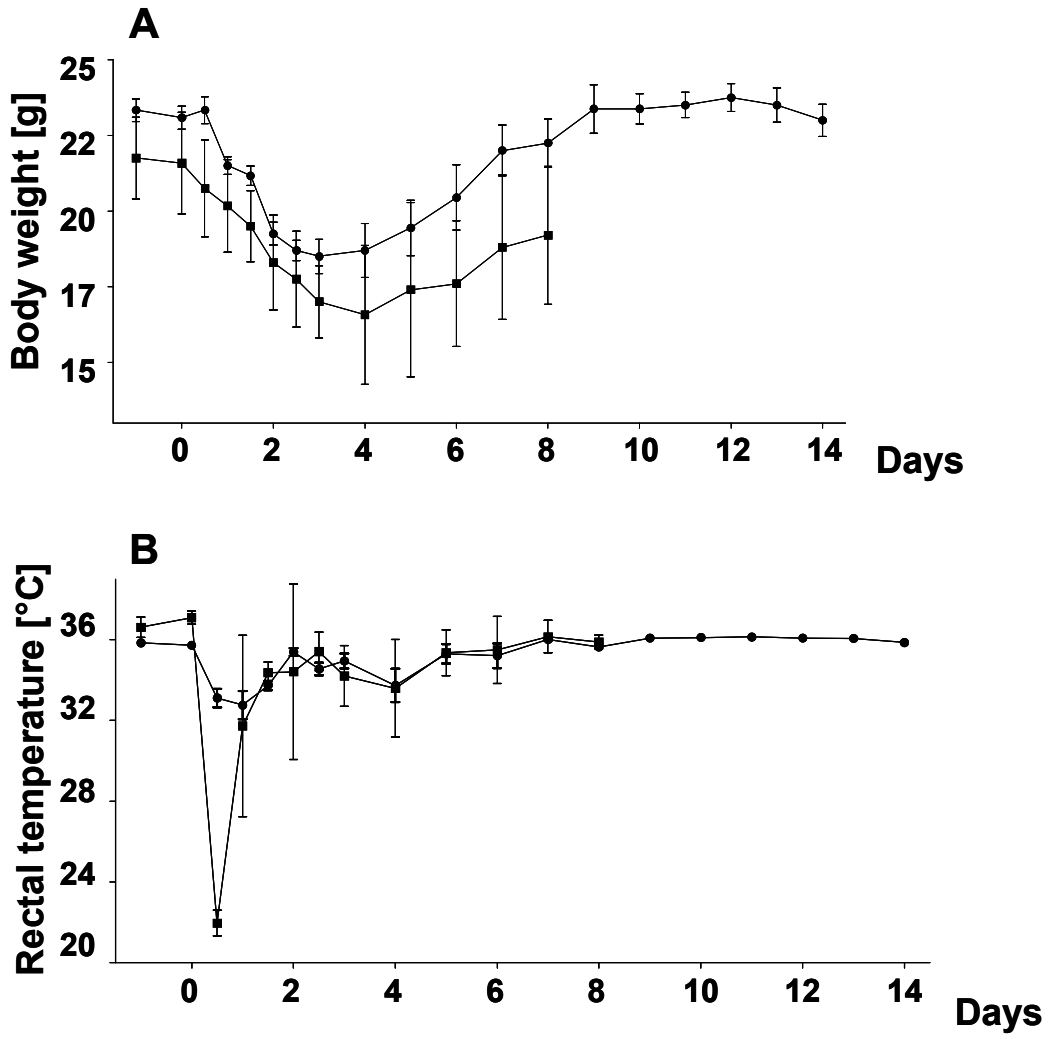

Figure IV-3. Body weight (A) and rectal temperature (B) of mice whose airways had been inoculated at day 0 with $5x10^7$ CFU of *P. aeruginosa* TBCF10839 (closed square), *P. aeruginosa* TBCF10839 Tn*5*::PA0785 (closed circle).

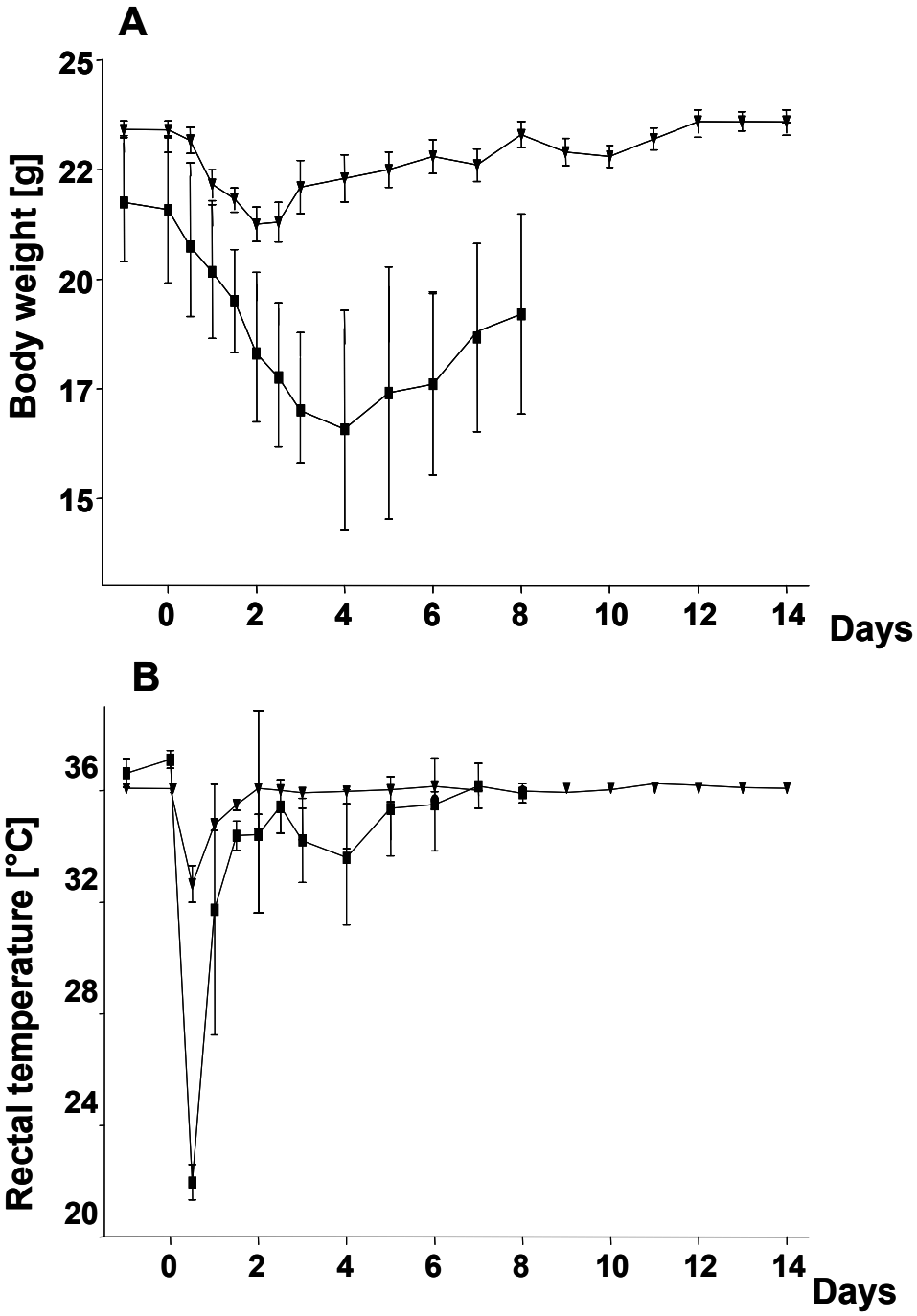

Figure IV-4. Body weight (A) and rectal temperature (B) of mice whose airways had been inoculated at day 0 with $5x10^7$ CFU of *P. aeruginosa* TBCF10839 (closed square), *P. aeruginosa* TBCF10839 Tn*5*::PA4916 (closed triangle).

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2003-2004 Agroecology Institute (FAL), Germany
Young scientific researcher in the laboratory of molecular biology.

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1996-2001 Kazan State Uneversity, Microbiology Department. Master degree. Diploma with honors.

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Publications

1. **Rakhimova E.,** Munder A., Wiehlmann L., Bredenbruch F., Tummler B. Fitness of Isogenic Morphology Variants of *Pseudomonas aeruginosa* in Murine Airway Infection. *Submitted.*

2. **Rakhimova E.R.,** Garusov A.V., Zaripova S.K. Biological activity of oil contaminated soil upon its salinization. 2005. *Eurasian Soil Science (Russia)* 38(4): 425-428.

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7. **Rakhimova E.R.,** Zaripova S.K., Norina E.S., Belova I.V." Microorganisms of soil under condition of oil and salt contamination". 5th. Conf."Biology – science of 21 century", 16-20 April, 2001. Pustchino (Russia).

Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation Genetic traits of *P. aeruginosa* morphotypes affecting virulence *in vivo*

selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den

_____

(Unterschrift)

Name: **Rakhimova Elza**