Functional Genome Analysis in

Pseudomonas aeruginosa SG17M

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Abstract

Methods of functional genome analysis were applied to the environmental *P.aeruginosa* isolate SG17M. This isolate belongs to clone C, the prevalent *P.aeruginosa* clone isolated from environmental habitats as well as from cystic fibrosis (CF) patients in Western Europe.
This isolate has proven to be unexpectedly virulent and therefore was characterized applying state-of-the-art methods for functional genome analysis.
A library of about 3000 STM mutants was generated as a resource for the analysis of genes that are essential for the survival of *P.aeruginosa* SG17M in diverse habitats. Within this study, the mutant library was screened for mutants that exhibited limited quorum sensing capabilities, and 23 individual mutants were found that did not produce homoserine lactones and were deficient in protease and siderophore secretion. The genes carrying the transposon insertion were identified and sequenced applying a novel protocol for direct genomic sequencing which was established in this study.
The further analysis of transposon mutants necessitates the verification of their phenotypic characteristics by the construction of isogenic mutants by targeted mutagenesis. An easy-to-use and reliable protocol for allelic replacement was established as a further major objective of this thesis in order to provide a tool for fast and easy generation of knock-out mutants in *P.aeruginosa* strains other than the genetic reference strain PAOI. This protocol was applied to strain SG17M and the clinical isolate TBCF 10893 to construct a knock-out mutant in a gene that is not present in *P.aeruginosa* PAOI but unique to a panel of clinical and environmental isolates. The importance of this gene for quorum sensing in two isolates was proven by the generation and phenotypic as well as genotypic characterization of 50 mutants obtained with this method for targeted mutagenesis.
To prove the universal applicability of this allelic replacement method, a second gene was chosen and knocked out the same way, also verifying the phenotype shown by an original STM mutant.
A preliminary characterization of the genes identified as essential for quorum sensing in SG17M was carried out to complete the study.

Key words: *Pseudomonas aeruginosa*, Signature-Tagged Mutagenesis, Allelic Replacement, Quorum Sensing and Virulence.

Kurzzusammenfassung

Methoden der funktionellen Genomanalyse wurden angewendet, um das *P.aeruginosa*-Isolat SG17M näher zu charakterisieren und Ressourcen für weitere phänotypische und genotypische Analysen bereitzustellen.
Dieses Umweltisolat gehört zu Klon C, dem häufigsten *P.aeruginosa*-Klon in Westeuropa, der sowohl aus aquatischen Umwelthabitaten als auch von Patienten mit Cystischer Fibrose (CF) isoliert worden ist.
Eine Mutantenbank aus 3000 Mutanten des Isolats SG17M wurde mittels sequenzspezifizierter Transposonmutagenese hergestellt, um eine Ressource für die Untersuchung von Virulenzfaktoren unter verschiedenen Bedingungen bereitzustellen. Die Mutanten wurden in einer ersten Untersuchung auf ihre Quorum-Sensing-Fähigkeiten überprüft und es wurden 23 Mutanten gefunden, die weder fähig waren, Homoserinlactone zu produzieren noch Protease zu sezernieren und auch nur eingeschränkt Elastase sowie Siderophoren produzierten. Die Gene, in denen eine Transposoninsertion stattgefunden hatte, wurden identifiziert und mittels einer in dieser Arbeit etablierten Methode zur Isolation und Reinigung von genomischer DNA zur direkten genomischen Sequenzierung sequenziert. 18 der in dieser Untersuchung zum Quorum-Sensing gefundenen Gene sind bisher nicht mit Quorum-Sensing in Verbindung gebracht worden und eine vorläufige Kurzcharakterisierung wurde in dieser Arbeit vorgenommen.
Um die veränderten phänotypischen Eigenschaften von Mutanten zu bestätigen, die mittels statistischer Transposoninsertion erzeugt wurden, ist es wichtig, isogene Mutanten mittels gerichteter Mutagenese zu erzeugen.
In dieser Arbeit wurde eine Methode zur schnellen Konstruktion von stabilen Knock-out-Mutanten in klinischen *P.aeruginosa*-Isolaten etabliert und auf die Konstruktion zweier Mutanten angewendet, die einen veränderten Phänotyp in bezug auf Quorum-Sensing zeigten. Etwa 50 Mutanten wurden erzeugt und phänotypisch sowie genotypisch charakterisiert, wobei die jeweiligen Eigenschaften der originalen Transposonmutanten bestätigt werden konnten.
Während der Anfertigung dieser Arbeit war die Autorin an einer Studie einer deutsch-französischen Kooperation beteiligt, die den phänotypischen und genotypischen Vergleich von zwei der virulentesten klinischen *P.aeruginosa*-Isolate zum Ziel hatte und deren Ergebnisse in dieser Arbeit ebenfalls vorgestellt werden.

Schlüsselwörter:
Pseudomonas aeruginosa, Sequenzspezifizierte Transposonmutagenese (STM, Signature Tagged Mutagenesis), gezielte Mutagenese (Allelic Replacement), Quorum Sensing und Virulenz.

1. Introduction

1.1. Pseudomonas aeruginosa

The genus *Pseudomonas*, created by Migula in 1895, has comprised a wide range of non-fermenting, aerobic, non-sporulating rod-shaped bacteria since its establishment. Later this century, as more and more refined techniques for genetic analysis became available, this genus has been extensively refined and is now made up of five groups. These have been based on the identification of five distinct rRNA groups within the genus[1], of which group I is referred to as the "true *Pseudomonads*". These rRNA genes are highly conserved genes and the 16S-rRNA sequences serve as the major genetic marker molecules in bacterial phylogeny with additional information provided by the 23S-rRNA genes as well as the sequences of genes coding for highly conserved proteins. Former *Pseudomonads* can today be found for example in the *Burkholderia*[2] or the *Stenotrophomonas* genus[3].
Pseudomonads, based on their 16S-rRNA sequences, are classified as members of the group of γ-proteobacteria[4]. *P.aeruginosa* is most possibly the best characterized species of this genus and presumably the most clinically relevant one. It was first isolated from the dressing of a festering wound that was colored slightly greenish, hence it was given its name: aerugo=green, but it is almost ubiquitously distributed in nature. Many *Pseudomonads* have been attributed pathogenic potential[5] but *P.aeruginosa* has been proven to be one of the major opportunistic pathogens infecting immunocompromised hosts such as cancer, transplant, HIV and burn patients. About 10% of the hospital-acquired nosocomial infections[6] and a great many resulting deaths are ascribed to *P.aeruginosa*[7]. Furthermore, infections of the urogenital tract, the respiratory tract, the ear and local eye infections are linked to *P.aeruginosa*. The lower respiratory tract of CF patients is at one time or another in their life colonized with *P.aeruginosa* which leads to the destruction of the lung tissue and thus to the reduced life span of these patients, especially when co-colonization with *Burkholderia cepacia* occurs[8].
This species produces several bacterial pigments such as pyocyanin[9] (blue-green), pyoverdine (Fluorescein, fluorescent green), pyorubin (red) and pyomelanin (brown)[10]. *P.aeruginosa* has been isolated from habitats as diverse as soil[11], plants[12], and different aquatic environments[13]. One of the most common sources for the isolation of *P.aeruginosa* is human wastewater[14].
Metabolically, *P.aeruginosa* is a very versatile bacterium that can utilize a number of carbon sources-even aliphatic, halogenated and non-halogenated aromatic carbon compounds can be metabolized. This trait makes it an interesting bacterium for bioremediation and detoxification of contaminated soil and aquatic systems, its virulence however being a major drawback for environmental applications.
P.aeruginosa tolerates a wide temperature range and can grow in temperatures from 4°C to more than 43°C, in pure and deionized water, and it has even been isolated from disinfectants[15]. It often forms

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biofilms during growth which are very resistant to adversative environmental factors such as nutrient depletion, physical forces and especially toxic substances such as antibiotics[16]. Furthermore, it is microaerophilic so that it can grow both in aerobic habitats as well as under regionally restricted anaerobic conditions, for example in biofilms in the CF lung.
This broad ecological tolerance is determined by the unusually versatile genome of this bacterium and a tight regulation of the gene expression: 7.2% of the genes are transcription regulators, 2.1 % serve as two-component regulators[17]. These two-component sensors, consisting of a sensor kinase and an effector, allow for the optimal adaptation of the organism to changes in its environment. Its genome with an average size of 6.3 Mb is one of the largest and most G+C rich bacterial genomes known to date, and with a predicted number of 5570 open reading frames (ORFs) it nearly reaches the genetic complexity of a simple eukaryote such as *S.cerevisiae* (6000 ORFs).

1.1.1. *P.aeruginosa* SG17M and TB

P.aeruginosa **SG17M** is an environmental isolate from a riverside in the Ruhr area, Western Germany. It belongs to the clone C lineage whose genotype has been isolated in great numbers from the environment as well as from areas close to hospitals. The genome of SG17M has a size of about 6.8 Mb and the strain harbours a 102 kb plasmid. This plasmid is present episomally as well as in an integrated form on the chromosome in a hypervariable region near the *pilA* gene[19]. The integrated form, however, can be mobilized again, especially after subculturing. Another insertion of 99 kb can be found in the hypervariable region after the lipase gene *lipH*, and the third SG17M-specific insertion of 85 kb is located near the terminus of replication. Except for the hypervariable regions, the chromosomal backbone and the gene order are highly conserved between PAO I (DSM 1707) and SG17M.
P.aeruginosa **TBCF 10839 (TB)** is a highly virulent clinical isolate from Hannover, Germany. It was isolated in 1983 from a CF patient with severe acute hypoxemia and proved to be persistent in the lung of this patient even under antibiotic therapy. It could finally be eliminated with i.v. ceftazidim therapy and was consecutively replaced by another *P.aeruginosa* clone. TB is a strongly mucoid isolate that has retained a variable surface so that phage typing is possible. The most striking phenotypical difference to other *P.aeruginosa* isolates is its capability of survival and replication within polymorphonuclear neutrophils (PMN), which is not commonly observed among this bacterial species. This fact suggests that this particular *P.aeruginosa* isolate has certain unique mechanisms that allow for survival in this extremely hostile environment which might also have been responsible for the persistence of this isolate in the CF patient's lung. To investigate this trait, a transposon library of this isolate was constructed and the mutants tested for intracellular survival. In this study, the cytotoxicity towards monocyte-derived human macrophages was assessed, and in addition, a phenotypic comparison between TB and another highly virulent isolate was carried out.

1.1.2. Genome Organization and Functional Genomics in *P.aeruginosa*

Early genome analyses of the genetic reference strain *P.aeruginosa* PAOI relied on techniques such as restriction mapping followed by PFGE and Southern hybridization[20,21]. Nowadays, the sequence of the PAOI genome is available and of great use to the *Pseudomonas* community, represented by the fact that annotation of many genes is still an ongoing process.

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In general, in spite of a conserved core genome[22,23], the *P.aeruginosa* genome exhibits great plasticity, several hotspots for the integration of gene islands and novel genetic material have been identified. Within a flagellar gene cluster, a 16 kb island has been detected which contains genes that are essential for flagellin glycosylation and replaces the PAOI genes 1088-1091[24] which might explain why PAOI does not express type a flagellin which is otherwise common in *P.aeruginosa*, but type b flagellin instead. A site where horizontal gene transfer frequently occurs is a region of 7 kb around the phospholipase gene *pldA* , PA3487 as well as a homologue of the *vgr* gene which is known to be associated with rearrangement hotspots in the *E.coli* genome[25] and marks an insertion point of a 60 bp stretch of conserved DNA.
All in all, 38 islands have been detected within the chromosome by microarray analysis, some of which encode gene products that have been proven to play a key role in *P.aeruginosa* biology, as well as products of hitherto unknown function[26] with one known island being absent in all examined strains. Also, several deletions of different sizes have been found by the same experimental approach; especially large deletions have been linked to *P.aeruginosa* evolution in the lung of cystic fibrosis patients[20]. Perhaps the most prominent example of this kind is the loss of the antisigma factor *mucA* through mutation which leads to the deregulation of an alternative sigma factor σ^{22}, AlgT or AlgU required for the expression of the alginate biosynthesis operon. This operon is then consecutively expressed and marks the transition of non-mucoid *P.aeruginosa* to the mucoid phenotype[27] which grows in a biofilm *in vivo* and is almost impossible to eradicate from the lung. It has been shown that this mucoid conversion is triggered by superoxide stress inflicted on the bacteria by PMN in the lung[28] and may serve as a defense mechanism of the bacteria towards the host defenses.
Another example of gene loss that confers a selective advantage is the loss of *mutS* and changes in the *mutY* gene leading to hypermutator phenotypes, which exhibit strongly enhanced antibiotic resistance[29].
The conserved core genome of *P.aeruginosa* consists mainly of the essential genes which are distributed in the region of the replication origin, whereas catabolic and non-essential genes can be found mostly in the other half of the chromosome[30], an organization that has been known also from *P.putida*. An interesting fact is that genes of one metabolic pathway are often located all over the chromosome instead of being organized in a polycistronic gene cassette, regulated by a common promoter and transcribed as a single mRNA as it is the case, for example, in *E.coli*[31]. However, these genes share a common regulation. This separation could be due to the integration of newly acquired DNA like for example plasmids into a once smaller genome[32,24]. It has been shown that genome organization in *P.aeruginosa* does not correlate with habitat or relation to infections. 573 isolates originating from habitats as different as the lung of a cystic fibrosis patient and a riverine ecosystem from a completely different geographical area have been subjected to macrorestriction analysis and subsequent PFGE which revealed that the dominant clone C was isolated from patients as well as from the environment[33]. Isolates that differed in less than 20% of their restriction bands were termed “clonal variants” as this similarity could not be appointed to statistical variations, and showed that clonal variants of one isolate could be found in isolates from completely different habitats[34]. This similarity of strains from completely different habitats can be appointed to a genomic conservation that is independent of the strain’s habitat and lifestyle and might be one reason for the large and diverse genome of *P.aeruginosa*. Another explanation could be that the large and diverse genome enables this bacterium to survive and thrive in virtually every habitat which might also account for the large

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number of regulatory genes. Specialists such as *M.tuberculosis* or *H.pylori*, which are restricted to certain habitats and lifestyles, content themselves with 3% or 1% of regulatory genes respectively, while the genomes of *E.coli* and *H.influenzae* for example, two ubiquitously spread bacterial species that can also adapt to numerous habitats, contain 5.3% and 5.8% genes with regulatory function, respectively[35].
Although the sequence of *P.aeruginosa* has been published and enables the *Pseudomonas* community to conduct further *in vitro*, *in vivo* and *in silico* analyses, there are still a great many facts that need further exploring and research, thus laying the ground for functional genome analysis of the post-genomic era.

1.1.3. *P.aeruginosa* virulence, Cytotoxicity and Cystic Fibrosis

Early in the 20th century, *P.aeruginosa* was mostly known as an organism with a saprophytic lifestyle but in recent years, however, it has been more and more linked to infections especially in CF patients and immunocompromised individuals[36]. The pathogenic potential of *P.aeruginosa* is determined by a number of pathogenicity factors which in concerted action are responsible for the clinical symptoms of a *Pseudomonas* infection.
The first stage of a *Pseudomonas* infection is the attachment to the host cell via type IV pili that mediate contact to the eukaryotic cell[37]. Upon invasion, the bacterium secretes a number of enzymes such as lipases and proteases as well as toxins into the host cell that cause significant damage. The third stage is the establishment of the infection that goes hand in hand with the evasion and suppression of the host defenses.
Three different types of secretion systems are responsible for secreting the numerous virulence factors into the host cell. The Type Three Secretion System (TTSS), which can be found in many gram-negative bacteria, seems to be the key player in the secretion of virulence factors associated with lung tissue damage in CF. It is thought to be important for the ability of *P.aeruginosa* to avoid innate immune clearance in mammals which is a prerequisite for establishing chronic infections. This secretion system is dependent on cell-to cell contact of host and pathogen and uses a needle-like structure to deliver the effector proteins either to the cell membrane or directly into the cytoplasm of the eukaryotic cell[38]. Four of these effector proteins have been identified to date: ExoS, T, Y and U. These effectors are believed to be the main culprits in *P.aeruginosa*-associated cytotoxicity, and in fact all of them have cell-altering properties.
ExoS, a 49 kD protein, has been proven to be a bifunctional cytotoxin. Its aminoterminal moiety has GTPase activating properties and mediates actin reorganization of the host cell without cytotoxicity, while its carboxyl terminus codes for an ADP-ribosyltransferase domain which, if expressed in cultured cells, is cytotoxic by ADP-ribosylating *Ras* and interfering with cell signaling processes that depend on *Ras-Rho* crosstalk[39]. It is proposed that ExoS plays a major role in interfering with the wound healing process and thus maintaining sites of infection and establishing a chronical *P.aeruginosa* infection. ExoS has also been linked to the disturbance of other cellular processes such as inhibition of DNA synthesis[40] as well as interference with cell matrix adherence[41]. ExoS also has been proven to be the key player in the cell death of macrophages upon phagocytosis of several clinical isolates of *P.aeruginosa*[42]. But ExoS does not only play a major role in pathogenicity towards eukaryotic cells *in vitro*; it has also been proven to mediate rapid killing of adult *Drosophila melanogaster* accompanied by bacterial multiplication to high titers although very low MOIs were applied[43].

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ExoU, a 72 kDa protein, is found in strains that lack ExoS genes[44] and is mainly responsible for mediating cytotoxicity of these strains. Controlled expression of ExoU in yeast and mechanistic studies of exoU-mediated cytotoxicity indicated that ExoU possesses phospholipase activity; later, structural studies and Conserved Domain Database search revealed that ExoU has a patatin-like phospholipase domain, patatin being a plant enzyme that is activated upon environmental stress or pathogenic infection[45]. ExoU uses a catalytic dyad, and the catalytic domain is located in the aminoterminal half of the protein. However, the function of the carboxyterminal moiety remains unknown. Like ExoS and ExoT, it requires activation by an eukaryotic factor for *in vitro* enzymatic activity, thus suggesting that this mechanism protects the bacterium from its own toxin and ensures that the phospholipase activity is directed towards the host cell[46]. A possible role of ExoU during infection is due to its enzymatic activity: Phospholipases cleave phospholipids and release fatty acids, including arachidonic acid, the precursor of proinflammatory lipid mediators such as prostaglandins and leukotrienes. The activation of ExoU could play a role in eicosanoid-mediated proinflammatory responses, and recent microarray results have supported this hypothesis: The transcription of several genes involved in cellular transcription and signal regulation processes is induced upon intoxication with ExoU.[47]

ExoY and ExoT are the other two effectors secreted via the TTSS but are much less characterized and their functions mainly remain elusive. ExoY is an extracellular adenylate cyclase[48] which shows a certain homology to extracellular adenylate cyclases of *B.pertussis* and *B.anthracis*. However, it is not stimulated or activated by calmodulin as are the *B.pertussis* and *B.anthracis* homologs, and it has been suggested that a eukaryotic factor activates ExoY.

ExoT has been studied by using the heterologous TTSS of *Yersinia* to deliver the exotoxin into HeLa cells[49]. It has been found that ExoT can modify and inactivate host cell proteins involved in maintaining the actin cytoskeleton *in vivo* by two different mechanisms: Both wild-type ExoT variants that expressed GAP-activity as well as ADP ribosylating activity disrupted the actin microfilaments of infected cells but the effect was reversed with GAP-activity expressing bacteria once the infection of the cells was terminated by killing the bacteria. The cytoskeleton changes due to ADP-ribosylating activity, however, were irreversible. Studies are currently ongoing to elucidate the functions of these two TTSS effectors and their role in pathogenicity.

The *toxA* gene product exotoxin A is also an ADP ribosyltransferase[50], its target protein is eEF2 (eukaryotic elongation factor 2) and one of the most potent toxins known to date[51]. High levels of antibodies to ExoA are commonly found in CF patients[52], suggesting frequent exposure to the antigen. Also, a strong correlation between high serum antibody levels to ExoA and increased mortality in CF patients exists[53] and ExoA is currently being discussed as an important virulence determinant in patients with CF[54].

Other virulence factors among the massive arsenal employed by *P.aeruginosa* are for example type IV pili that play a key role in bacteria-host cell contact. Pilus function mediated by *pilU* and *pilT* is required for *in vitro* adherence and cytotoxicity toward epithelial cells and has been identified as an important virulence determinant *in vivo*[55].

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Important mediators of host tissue destruction that are secreted independently of the TTSS by *P.aeruginosa* are elastase, alkaline phosphatase and the LasA-fragment which not only destroy host tissues but also several determinants of the host immune response[56]. Other secreted virulence factors are pyocyanins[57], which damage lung tissue most likely due to their redox cycling and resultant generation of reactive oxygen species. Tissue destruction is also caused by Phospholipase C and rhamnolipids, the latter of which are heat-stable cytotoxins with detergent-like properties[58].
It has been demonstrated that many if not most of the virulence factors of *P.aeruginosa* mediating cytotoxicity and tissue destruction are regulated by the quorum sensing system, the latest addition to these findings being the demonstration that the expression of *P.aeruginosa exoS* is controlled by quorum sensing and RpoS[59] and is downregulated during bacterial growth in biofilms. This is just another example of the tight regulation of virulence factor expression to inflict maximum damage upon the host and to insure evasion of the host's immune defense systems.
Now that the sequence of *P.aeruginosa* is available and numerous transposon mutant libraries have been constructed, more new virulence factors are likely to be discovered. In order to act in the most concerted and thus efficient manner, the expression of these virulence factors has to be tightly regulated. This is achieved by a process called quorum sensing (QS), the "bacterial crosstalk" which can take place in cultures of single species as well as in mixed populations.

1.1.4. Quorum Sensing in *P.aeruginosa*

For a long time, it was commonly accepted that bacteria were individual organisms that existed only to feed and multiply: Coordinated behavior and intercellular signaling was believed to be exclusive to higher eukaryotic organisms. When it was discovered that *V.fischeri,* a marine microorganism that colonizes the light organ of several fish could regulate light production according to cell density[60], the mechanisms for this phenomenon were investigated. Light production was found to be caused by a luciferase activity that was only expressed at a high cell density. It was also discovered that the expression of the luciferase was dependent on the concentration of a small molecule identified as a 3-oxo-C_6 homoserine lactone (C_6-HSL): Low concentrations of this molecule did not promote luciferase expression while higher concentrations above a certain threshold concentration did. Molecules of this kind were later found to be produced by other bacteria as well and were termed autoinducers. These autoinducer molecules are constitutively expressed in a low concentration, but after a certain cell density is reached, the concentration of autoinducers reaches the threshold and for example light production by luciferase activity is induced.
The gene products of *luxR* and *luxI* were found to be involved in the transcriptional regulation of coordinated and cell-density-dependent light production[61] and later, *LuxR* was identified to encode a transcriptional activator of the bioluminescence operon and the LuxI gene product was revealed to be the autoinducer synthase that directs the synthesis of 3-oxo-C_6-homoserine lactones. When the LuxR-protein binds the autoinducer, it is activated and enabled to induce transcription of the *lux* operon, including *luxI* so that a positive feedback loop is generated. Soon after these findings, similar systems were discovered in other species, and it was found that they also relied on LuxR and LuxI homologs. The activation mechanism of the LuxR-like protein by an autoinducer molecule and subsequent expression of the lux-homolog operon as well as the creation of a positive feedback loop was also found to be the same. This cell-density-dependent mechanism was termed *quorum sensing* (QS) and represents the first step towards organized behavior and probably towards a multicellular organism.
In *P.aeruginosa*, three QS systems have been identified: the LasR-, RhlR and *Pseudomonas* Quinolone Signal (PQS) system. These systems each have their own autoinducer molecules which are rather specific for the respective system[62]: The LasR system is activated by 3-oxo-C_{12} -HSL while the RhlR system utilizes a C_4 -HSL. Not much is known about the PQS. Its signal molecule has been

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identified as 2-heptyl-3-hydroxy-4-quinolone[63] which is structurally completely different from all known autoinducers to date. Experiments have shown that the PQS system is controlled by the LasR system but its activity requires the RhlR system as well, so it could be a link between both QS systems in *P.aeruginosa*. Another link has also been observed as the *las* system positively regulates the gene expression of both *rhlR* and *rhlI*. Furthermore, 3-oxo-C_{12}-HSL is able to compete with C_4 -HSL for binding to RhlR, indicating that 3-oxo-C_{12}-HSL serves as antagonist of the rhlR system. This suggests a hierarchical arrangement with the lasR system being superior to the rhlR system and the PQS requires the function of both systems for active signaling. Figure 1 provides an overview of the QS cascade in *P.aeruginosa*:

LasR gene expression is regulated by the two global regulators GacA and Vfr as well as by the *las* QS system, which regulates both the expression of *lasR* and *lasI* with *lasI* creating an autoinduction feedback loop. The *rhl* system is regulated similarly as that GacA regulates *rhlR* expression, which is to some degree also controlled by the hierarchically superior *las* system. The autoinducer molecule of the las system, 3-oxo-C_{12}HSL, can posttranslationally block RhlR activation by its autoinducer, C_4-HSL, creating another regulatory link between the two systems. The two QS systems both control the third as yet poorly characterized QS system in *P.aeruginosa*, the Pseudomonas Quinolone Signal (PQS). Expression of the PQS is under the control of the *las* system, but RhlR is also required for its activity. All three QS systems are involved in virulence factor production, and the las signal molecule, 3-oxo-C_{12}HSL, is required for biofilm differentiation and exhibits immunomodulatory activity [64].

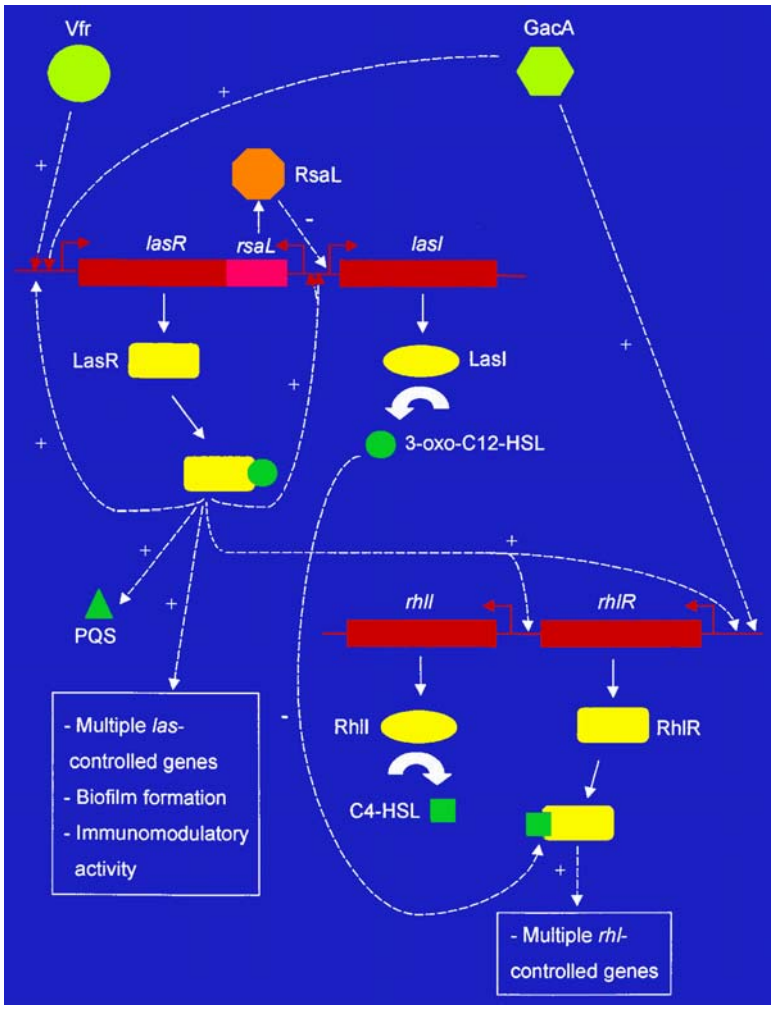

Figure 1a: The QS circuitry of P.aeruginosa. Adapted from de Kievit and Iglewski: *Infect. Immun*. 68(9): 4839-4849, 2000.
Color code: green: positive effectors; orange: repressor; red: affected genes; yellow:proteins

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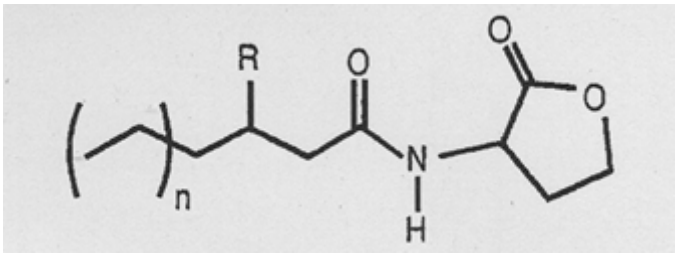

Figure 1b: Homoserine Lactone (HSL)

Quorum Sensing in *Pseudomonas* has been identified to play a key role in the concerted action of bacterial virulence factor expression in infections of mammalian and plant hosts. The type II secretion system and its secreted effectors, elastase and several proteases are regulated by both QS systems[65], the expression of pyoverdines, cyanide and rhamnolipid production is under direct control of the *rhlR* system[66]. It has been shown that QS mutants exhibit a strongly decreased virulence in numerous infection models. Lately, a direct link between quorum sensing activity and the regulation of virulence factor production has been discovered[67]. The *luxR*-type gene *vqsR* proved to be essential for the production of autoinducers as well as for protease and pyocyanin synthesis; in addition, it regulates the expression of genes involved in antimicrobial resistance.
In CF patients, it has been shown that the sputa contained *lasR* transcripts in significant concentrations as well as *lasA*, *lasB* and *toxA* gene products suggesting a concerted regulation of these virulence factors and active regulation of gene expression by the lasR system during chronic lung infections[68]. Although the QS systems in *P.aeruginosa* are very specific for their cognate HSLs, crosstalk between species has also been observed[69]. *B.cepacia*, an organism that was formerly grouped into the *Pseudomonas* genus, often co-colonizes the lungs of CF patients together with *P.aeruginosa*. It has been observed that *B.cepacia* can utilize the exogenous autoinducers produced by the other species to up-regulate its virulence factors[70] and is thus able to profit from the investment that other species made in order to regulate their own virulence.
As many important animal and plant pathogens use quorum sensing to regulate their virulence, it might be possible to design antimicrobial agents that interfere with quorum sensing and allow for the control of pathogens. Also, as quorum sensing plays a major role in biofilm formation, if interference with the cell-to-cell signal could be achieved, the formation of biofilms, for example on indwelling medical devices, could be reduced[71]. Research about the effect of HSLs on mammalian cells is ongoing and this as well as extensive research on the role of HSLs as virulence factors themselves could add yet another part to the puzzle that makes up bacterial intercellular communication.

1.2. Methods for functional genomics in bacteria

In the post-genome sequencing era, it has now come to the point where the vast information gained from the numerous sequencing projects can be ordered and categorized so that it contributes to the ongoing research for example about bacterial lifestyle, host-pathogen interactions, or environmental application of bacteria for bioremediation. Numerous tools for functional genome analysis have been developed over the past years of which Signature-Tagged Mutagenesis, Allelic Replacement and DNA chip analysis shall be discussed here in brief.

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1.2.1. Signature - Tagged Transposon Mutagenesis

Transposon mutagenesis is one of the most important techniques for functional genome analysis which is reflected in the great number of transposon mutant libraries that have been constructed in recent years. This technique relies on the random insertion of a transposon into the genome, statistically disrupting ORFs and knocking out the respective genes. Transposon mutagenesis yields respectable numbers of mutants depending on the properties of the parental strain and also allows for easy identification of the mutants, for example by employing Southern hybridization as the mutants are marked by the transposon insertion. Subsequent cloning and mapping of the genes in question is greatly facilitated this way.

The most popular transposons for application in *P.aeruginosa* are *Tn5*-derived[73] minitransposons[74] which are usually located on a delivery vector that also contains the transposase gene. However, this gene is located outside of the inverted repeats which mark the boundaries for the translocated DNA. This way, multiple transposon insertions into the genome can be prevented which were a major problem with earlier applied transposons, especially when naturally occurring transposons were utilized. Another advantage of minitransposons in comparison with natural transposons is their size. Naturally occurring transposons are often very large and not easy to handle. They also frequently recognize insertion hotspots in the genome and make statistical gene disruption impossible[75].

The ideal delivery vector for a *Tn5*-derived minitransposon is a suicide plasmid that does not replicate within the host organism and an antibiotic cassette for selection which is located within the inverted repeats. This way, in order to survive the antibiotic stress upon selection, the bacterium is forced to translocate the transposon into the genome which also leads to the genomic incorporation of the resistance cassette. The plasmid is lost after transposon insertion so that the transposition remains a singular process and can be reliably located in the genome.

An insertional mutagenesis most often goes hand in hand with a change of phenotype so that mutants can be easily selected, especially when an antibiotic resistance cassette is employed as selection marker. A problem arises nevertheless if the gene that carries the insertion lies upstream in a polycistronic RNA stretch and influences the expression of the downstream genes. This kind of polar effect cannot be ruled out if statistical insertions are generated so the gene responsible for the phenotype has to be verified either by directed mutagenesis or complementation.

The transposon mutagenesis approach has undergone extensive refining in recent years, one of which was the introduction of signal sequences into the transposed part so that each single mutant could be individually identified. This method, termed Signature Tagged Mutagenesis, *STM*[76] , was introduced by M. Hensel in 1995 for the identification of *S.typhimurium* virulence factors. STM combines insertional mutagenesis with the possibility of examining a large number of mutants simultaneously as every mutant is marked by a distinct and individual signature sequence which can be tracked throughout the experiment by hybridization to a specific probe.

The signature sequence consists of a variable region that comprises 40 bp of all 4 nucleotides in a statistical order and is flanked at both sides by 20 bp of a known sequence which serves as template for amplification of the signature sequence by PCR. There are several requirements that have to be met for the signature sequence to allow for easy detection and handling:

- The signature sequence has to be constructed so that the nucleotide arrangement yields the maximum possible variety, two identical signature sequences should occur only once in 2×10^7 molecules at most.
- The formation of secondary structures has to be avoided as the formation of loop and hairpin structures would greatly inhibit the amplification of the signature sequence by PCR.
- Furthermore, the sequences need to be devoid of restriction sites for certain enzymes so that these can be used for cloning.
- The single signature sequences have to be selected for a) strong hybridization signals and b) for as little cross-hybridization with each other as possible.

Signature sequences that meet these requirements are incorporated into a minitransposon with a suitable antibiotic resistance marker and the whole construct is then ligated into a suicide vector. Ideal suicide vectors for STM have a narrow-host origin of replication and cannot be stably replicated in the organism subjected to mutagenesis so that each cell of the host organism carries only one copy of the minitransposon. The vector containing the signature tagged minitransposon is then transformed into

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an appropriate *E. coli* strain and transferred into the host organism via conjugation or - if applicable - electroporated directly into the organism of choice.
Mutagenesis is carried out as optimized for the host organism and resulting mutants are arrayed in 96 well plates. Blots with all signature tags hybridized onto the membrane serve as references for comparing the presence of the signature sequences recovered from mutants subjected to the selection experiments with those of the reference pool. It has been shown that pools of 48 different mutants (46 mutants plus 2 negative controls) are the limit for reasonable handling and optimal detection of signature sequences[77]. The arrayed mutants are subjected to selection; in the original case of Hensel's experiment the bacteria were intraperitoneally injected into BALB/c mice, and after killing the mice, organ homogenates were plated and examined for their bacteria count.
The mutants that did not survive the selection were identified as follows: From the selected bacteria, cultures were inoculated and the DNA prepared so that the signature sequences could be amplified by PCR using the conserved 20 bp part at each side of the variable sequence as template. The amplified signature sequences were then separated from the flanking regions and used to generate, in Hensel´s case, radioactively labeled hybridization probes. Non-radioactive DIG-labeling can as well be employed to label the probes, which in turn are then hybridized to the reference colony blots. Mutants without hybridization signals are not present in the selected population anymore, meaning that they did not survive the selection experiment at one point and most likely carry a transposon insertion in a gene that is absolutely necessary for survival under the applied selection conditions (dominant-negative selection). In this case, these genes could be major candidates for virulence factors in this particular strain for this specific selection model. These genes can then be identified by sequencing the flanking regions of the transposon insertion site and their role in virulence proven by site directed mutagenesis.
One major drawback of STM is the high price for the detection of the DIG-labeled signature tags in the case of non-radioactive detection, in the case of radioactive detection, the disadvantages are obvious. Hence it is beneficial to construct a mutant library that can be used in STM screens; if possible, however, single screens are preferable, for example in 96 well plates.
Figure 2 presents a schematic overview of the generation and selection of mutants of an STM library.

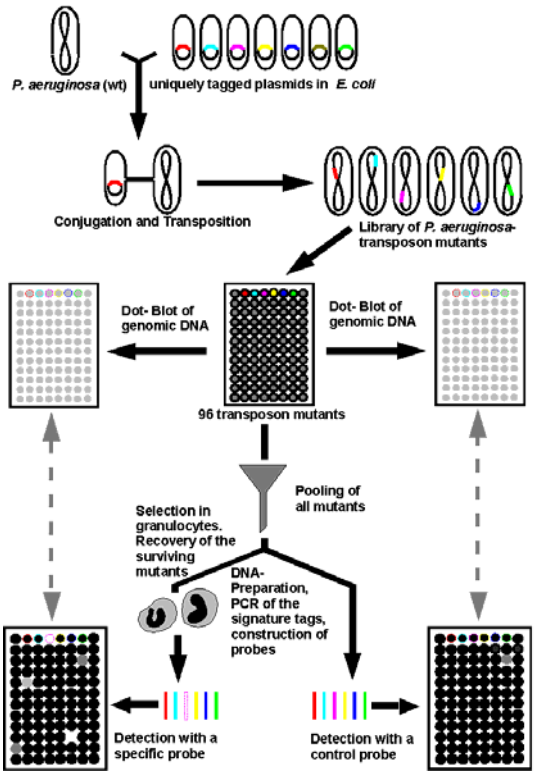

Figure 2: Signature tagged mutagenesis, figure courtesy of L. Wiehlmann, Dissertation, 2001[78].
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[77] Wiehlmann L: Personal communication, 2001
[78] Wiehlmann L: Sequenzspezifizierte Transposonmutagenese (STM) in *Pseudomonas aeruginosa*. Dissertation, Universität Hannover, 2001

1.2.2. Directed Mutagenesis via Allelic Replacement

Allelic replacement is the method of choice to analyze gene functions by replacing an intact gene by an artificially constructed homolog which carries a mutation. This homolog is introduced into the genome of an organism of choice, homologous recombination between the genomic copy of the targeted gene and the artificial construct takes place and the gene is knocked out. Phenotypic analyses of the mutant can then shed light onto the function of the mutated gene. This method is widely applied to all sorts of organisms, in *P.aeruginosa*, it is however not easily achieved due to several reasons.

First of all, the arsenal of suitable vectors to deliver the mutated allele is rather limited; all the available vectors serve the same gene replacement strategy: Insertion of a target gene sequence disrupted by a suitable antibiotic resistance gene that allows for positive selection, homologous recombination of this sequence into the genome of the organism of interest and curing of the plasmid via negative selection employing the *sac*B gene[79].

This technique has been well established and numerous mutants have been obtained for the genetic reference strain, *P.aeruginosa* PAOI. Nevertheless, the mutagenesis in clinical isolates proves to be difficult[80]. The recombination frequency in *P.aeruginosa* is rather low compared to organisms such as *H.pylori*[81]or *A.pleuropneumoniae*[82] which makes the introduction of mutations relying on homologous recombination rather difficult.

This method consists of several steps (for a schematic overview, see chapter 3.6):

1. Analysis of the gene to be replaced concerning DNA structure, naturally occurring restriction sites and compatibility with the antibiotic cassette to be used so that no restriction sites in the gene chosen for cloning procedures occur within the antibiotic cassette and the delivery vector. The results serve as a basis for construction of the deletion fragment.
2. Selecting a *sacB*-containing vector with suitable restriction sites from the generally used pEX100T and pEX18 derivatives developed by H.P. Schweizer[83].
3. PCR of the target sequence on the genomic DNA of the organism in question, if possible with gene-spanning primers to avoid polar effects.
4. PCR of the antibiotic cassette with primers that introduce a suitable restriction site to the ends of the gene. Here, it is crucial to allow for the amplification of a fragment that is larger than the resistance-conferring gene itself in order to avoid mutations in the gene due to the primer-generated restriction site which will render the cassette non-functional. Alternatively, if a suitable restriction site for a blunt-end cutting endonuclease is present close to or in the middle of the target sequence, the resistance cassette can be cut out of the vector pUC-Gm with the blunt-cutting endonuclease *Sma*I and ligated into the blunt-cut or filled-in target sequence.
5. Subcloning of both PCR fragments into linearized subcloning vectors that are commercially available. This greatly facilitates the excision of the PCR products and broadens the choice of restriction sites as most of the commercially available subcloning vectors contain convenient multi-cloning sites with rare-cutting endonucleases.
6. Excision of the resistance cassette and gel-purification, restriction digest of the vector containing the target DNA sequence with the restriction enzyme whose recognition sites have been introduced to the ends of the resistance gene. Thorough dephosphorylation of this vector should prevent vector religation and increase ligation efficiency.
7. The gel-purified PCR product of the resistance cassette is then ligated into the linearized vector containing the target DNA sequence and the ligation product transformed into a suitable *E.coli* strain, i.e. DH5α or its derivatives which is then grown on a medium containing the selection antibiotic.
8. Positive clones are selected for plasmid extraction and the plasmid structure is verified by restriction analysis.
9. The target DNA sequence is cut out and ligated into the *sacB*-containing vector which in turn is then transformed into *E.coli*, extracted and analysed.

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[80] Ohman D, personal communication, 2003, Schweizer HP, personal communication, 2002, Storey D, personal communication, 2003

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10. The vector containing the correct insert which consists of the recombination target DNA sequence separated in two parts by the antibiotic cassette is chosen. The two parts derived from the target gene serve as recombination sites and should consist of 400 base pairs minimum; the antibiotic cassette will serve as a positive selection marker.
11. The vector is then either electroporated into the recipient *P.aeruginosa* strain or transferred via triparental mating.
12. Positive transformants are resistant to the selection antibiotic and picked off the antibiotic-containing medium and are transferred to LB medium supplemented with 5% sucrose and the selection antibiotic to promote plasmid loss.
13. Sucrose and selection antibiotic resistant colonies are picked and transferred to a medium that contains an antibiotic for which a resistance-conferring gene is encoded on the plasmid. For the most common *sacB*-containing vectors, this is a beta-lactamase and the antibiotic of choice for *P.aeruginosa* in this case is carbenicillin. Colonies that grow on the antibiotic used for positive selection and not on carbenicillin (or other, depending on the employed *sacB*-plasmid) have integrated the selection antibiotic into the genome via homologous recombination and lost the rest of the plasmid. Those that still grow on the plasmid-encoded antibiotic are merodiploids and do not represent knock-out mutants.
14. The insertion site should be verified by Southern blotting and by PCR with one primer designed to bind on the targeted gene or upstream and one in the resistance cassette and the products should be sequenced or analyzed by informative restriction digest.

This technique has been employed in this or modified form for the analysis of several genes[84, 85] and has gained popularity in the *Pseudomonas* community. Nevertheless, the construction of mutants from isolates other than PAOI using this technique is a challenging task. During the course of this study, it was successfully adapted and refined for application in the clinical isolate TB and the environmental isolate SG17M.

1.2.3. DNA chip technology

DNA chip technology has found numerous applications in the last years and has proven a valuable tool for functional genome analysis.

1.2.3.1. Expression analyses using DNA microarrays

DNA microarrays[86] allow for the simultaneous expression and analysis of many thousand genes of a genome. This method makes the expression of a gene within a genome assessable in diverse contexts and is an indispensable tool for global expression analyses. Short oligonucleotides of all putative ORFs are generated and spotted onto a small glass slide by a roboter and serve as hybridization template. The whole mRNA of the organism of interest grown under specific conditions is isolated and transcribed into cDNA-fragments by randomly generated oligonucleotides and hybridized onto the chip. This way, an expression profile is generated that represents the gene expression of the organism in question under certain growth conditions, for example in the presence of chemicals.
The *in vivo* expression technology can be used to assess the expression of genes that are exclusively expressed in a specific habitat[87].

1.2.3.2. Mutational analysis applying DNA SNP-chips

SNP-chip analysis[88] is a technique that can be used to detect genomic variability within a species. It relies on the detection of single nucleotide polymorphisms that are characteristic of certain genes within the genome of the species to be analyzed. The snips have to be present in most of the genomes of the different strains to be tested and need to either generate or destroy a restriction site for detection. Together with certain strain-specific genomic properties such as gene islands or islets, the SNPs provide a reliable “fingerprint” of a strain and thus allow for its detection. SNP chips can be easily handled and the detection and evaluation process is straightforward, therefore, this method makes it possible to quickly identify genomic relationships between strains of a species.

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[87] Mahan MJ, Slauch JM, Mekalanos JJ: Selection of bacterial virulence genes that are specifically induced in host tissues. Science 259, 1993, 686-688
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1.3. Objectives

Functional genome analysis allows for the characterization and analysis of bacterial species and serves to broaden the understanding of their lifestyle, interactions with host organisms and in the case of pathogenic bacteria, sheds light on the pathogenicity mechanisms which the bacteria use to thrive and persist in a host.
Ultimately, in the post-antibiotic era, functional genome analysis of pathogenic bacteria could lead to the development of novel strategies for fighting microbial infections.
The study at hand was conducted using the environmental *P.aeruginosa* isolate SG17M as a model organism to apply novel tools of functional genome analysis and to unravel the determinants underlying the unexpected pathogenicity of this isolate.
A mutant library of about 3000 individual mutants was generated applying Signature Tagged Mutagenesis, and screening of the mutants in bioassays assessing their quorum sensing capabilities led to the identification of genes previously not linked to quorum sensing.

Furthermore, a universally applicable protocol for targeted mutagenesis in *P.aeruginosa* clinical isolates was to be established within this dissertation.
Genes that have been identified as essential for a specific phenotypic trait by screening a transposon mutant library need to be confirmed as responsible for the observed phenotype. Often, polar effects cause changes in gene expression downstream from the transposon insertion site and therefore, the correlation of the transposon insertion site with the phenotype needs to be verified. To complement a phenotype, an intact copy of the gene that was affected by the transposon insertion can be supplied *in trans* on a plasmid in order to complement the phenotype, but this method often yields instable phenotypes and therefore is inferior to the generation of an isogenic mutant. Targeted mutagenesis is an excellent method to construct isogenic mutants that can then be compared phenotypically and genotypically to the original transposon mutant. This task is not easily accomplished in *P.aeruginosa,* therefore, protocols that could be employed for this method had to be evaluated, modified and adapted for *P.aeruginosa* strains other than the genetic reference strain PAOI.
To prove the universal applicability of the protocol established for targeted mutagenesis, mutants of two different genes were constructed and a brief phenotypic and genotypic characterization provided.

2. Materials and Methods

2.1. Bacteria

Escherichia Coli
HB101:
F^-; *leuB6; proA2; recA13; thi-1; ara-14; lacY1; galK2; xyl-5; mtl-1; rpsL20; supE44*; *hsdS20*; (r_B^-; m_B^-)
The *E. coli* strain HB101 carries the helper plasmid pRK2013 which is necessary for conjugational transfer of plasmids.
DH5α (TOP 10, Invitrogen):
F^-; *φ80; m80lacZΔM1S; Δ(lacYZA-argF)*$_{U169}$ *; recA1; endA1; hsdR17*(r_k^-; m_k^+); *supE44;* λ^-; *thr*$^-$; *gyrA; relA1*
DH5α is the standard bacterium for maintenance of plasmids as it is easy to handle and its complete genetic background is known. In this work, it was used for all transformations unless stated otherwise.

The following *Pseudomonas* strains were used for this work:
P.aeruginosa
SG17M: Environmental isolate from a river in Mühlheim, Germany
Chromosome size: 6.8 Mb

TB: Clinical isolate from Hannover, Germany
Serotype: 4
Pyocine type: 1h
Phage lysotyping: F8; M4; PS2; PS 21; PS24; PS31; PS 73; 352; 46b/2; 1214; Col21; F7; F10;
TB does not carry a plasmid

CHA: Clinical isolate from Grenoble, France

892: Clinical isolate from Hannover, Germany
Serotype: 4
Pyocine type: 1h
Phage lysotyping: F8; M4; PS2; PS24; PS31
892 has not been examined for a plasmid

CSGB8: Clinical isolate from Hannover
Chromosome size 6.353 kb

Pseudomonas putida
Pseudomonas putida **KT 2440**[89] was used as a non-cytotoxic reference strain for calibration of the cytotoxicity assay

2.2. Vectors

p*Tn5*MOD-OGm
p*Tn5*MOD-OGm (pMOD) is a plasposon[90] and a derivative of the class of "self-cloning" transposons. These transposons contain genes that confer an antibiotic resistance and a replication origin that is functional under certain conditions. The inverted repeats are flanked by recognition sites of rare-cutting endonucleases which enable the localization of the transposon insertion in the genome either by restriction mapping or Southern blotting. Furthermore, plasposons can be used for the generation of a library of clones that contain large stretches of DNA flanking the insertion sites and allow for the construction of genomic libraries. This construct combines both characteristics of a transposon and a plasmid, as the origin of replication as a part of the plasmid vector is inserted into the genome alongside with the transposon while the transposase remains on the vector backbone and is

[89] Bagdasarian M, Lurz R, Ruckert B, Franklin FC, Bagdasarian MM, Frey J, Timmis KN: Specific purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF 1010-derived vectors, and a host vector system for gene cloning in Pseudomonas. Gene, 16, 1981, 237-247
Regenhardt, D, Heuer H, Heim S, Fernandez DU, Strömpl C, Moore ERB, Timmis KN: Pedigree and taxonomic credentials of Pseudomonas putida strain KT2440. Environ Microbiol. 4, 2002, 912-915
[90] Dennis JJ, Zylstra GJ: Plasposons: Modular Self-Cloning Minitransposon Derivatives for Rapid genetic Analyses of Gram-Negative Bacterial Genomes; Appl.Environ.Microbiology, 64 (7), 1998, 2710-2715

consecutively lost during replication of the genome. This way, only a single transposon insertion is achieved leading to a clearly defined mutation site.
The p*Tn5*MOD-OGm containing the inserted signature sequences is named p*Tn5*MOD-OGm-ST.

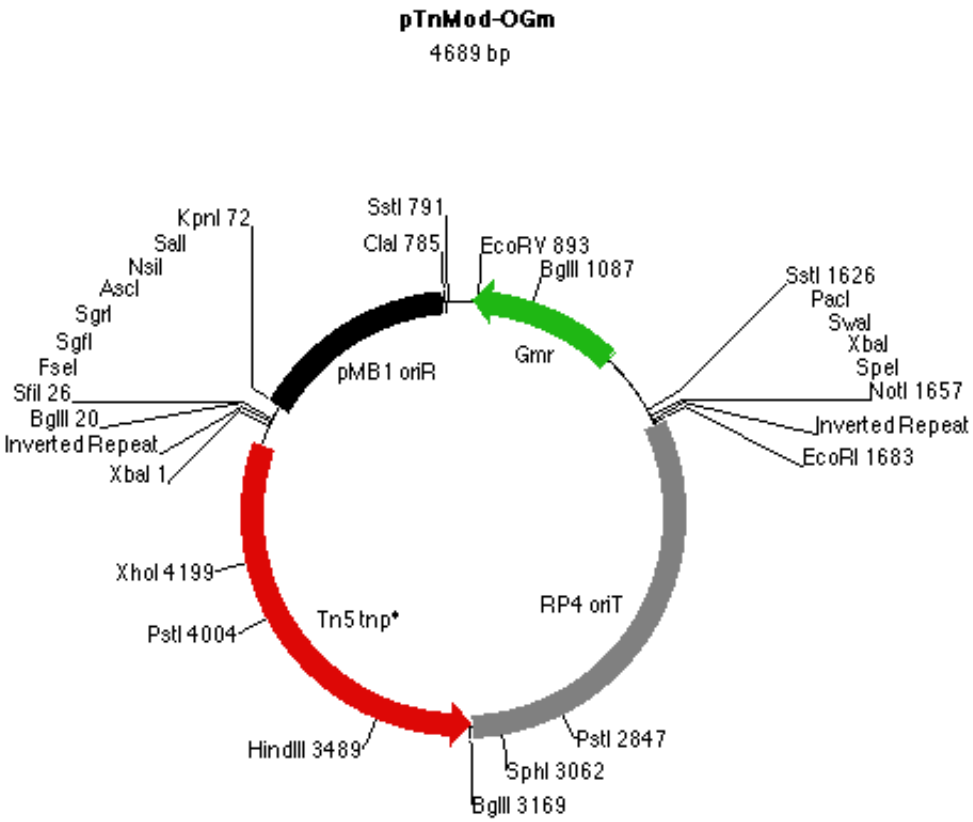

Figure 3: map of p*Tn5*MOD-OGm

pEX100T
pEX100T was designed for gene knock-out in *P.aeruginosa* PAO1[91] and features the counterselectable *sacB* gene, several recognition sites of rare-cutting endonucleases, a *lacZ*α-allele for blue-white screening, a *β*-lactamase that confers an ampicillin/carbenicillin resistance and an *oriT* for conjugal transfer of the plasmid. This plasmid can be used with an inserted target DNA sequence and a suitable selectable marker, i.e. an antibiotic resistance gene for the insertional inactivation of genes by homologous recombination. In contrast to a transposon insertion, the recombination is site-specific and the plasmid is lost by negative selection using the counterselectable *sacB* marker.

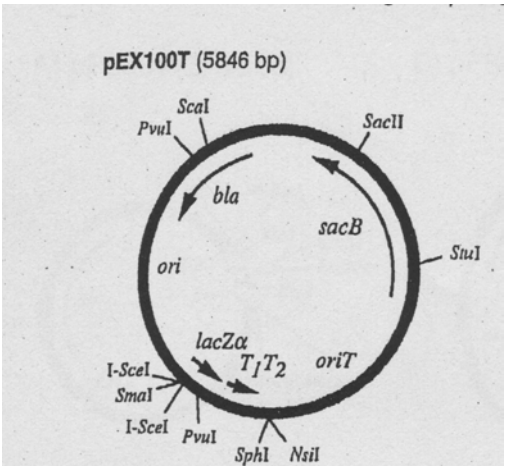

Figure 4: map of pEX100T

[91] Hoang TT, Schweizer H: An Improved System for Gene Replacement and xylE-Fusion Analysis in Pseudomonas aeruginosa; Gene158, 1995, 15-22

pEX18Ap
pEX18T[92] is a derivative of pEX100T and additionally to all pEX100T features, it contains the multicloning site from pUC18/19 which facilitates cloning procedures.

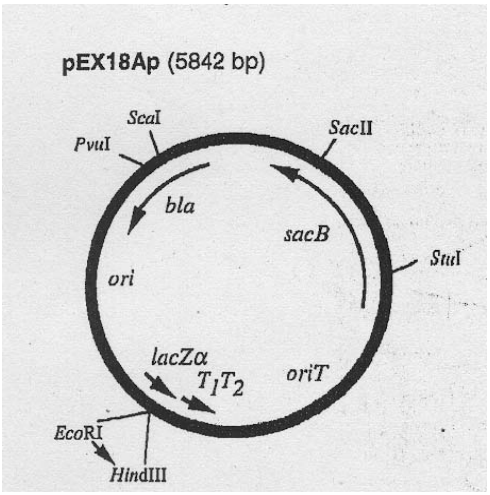

Figure 5: map of pEX18Ap

pGEM-T, pGEM-T*Easy*
These vectors are commercially available (Promega, Cat #A3600 and A1360, respectively) and were designed for the subcloning of PCR products. The vectors differ in their multicloning site (MCS, see vector map). The MCS of pGEM-T contains several single-cutting endonucleases in addition to a *Bst*ZI site flanking both insertion points. This way, only one endonuclease can be used to release the insert. pGEM-T Easy contains two restriction sites for *Eco*RI and *Not*I one flanking each insertion point, thus facilitating the release of the insert. Both vectors are linearized and have a single T overhang at both ends. Most thermostable polymerases add a terminal A to their products which in turn complements the T overhang of the vector and allows for easy ligation of the product into the vector. The insert generated this way can be multiplied with the vector and released after isolating the vector from a suitable bacterium and its restriction digest. The only drawback of this procedure is that ligation of the insert into the vector may, however, occur in any given orientation. If orientation is an issue for downstream applications, restriction mapping of the insert is essential so that suitable enzymes can be chosen for its release from the subcloning vector.
Depending on which gene replacement vector was finally to be utilized, either pGEM-T or pGEM-T Easy was chosen for subcloning in order to match the restriction sites of the gene replacement vector.

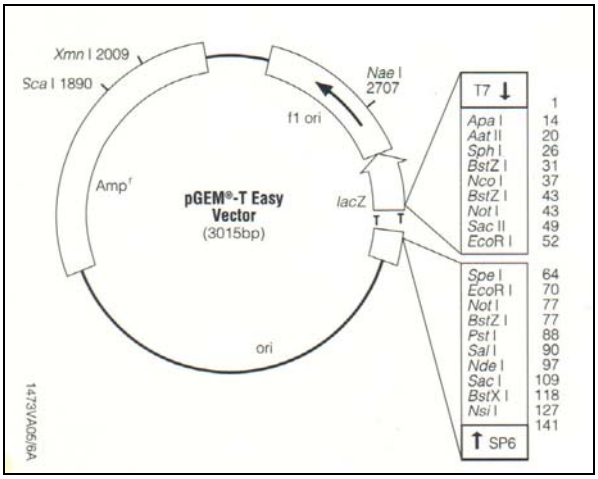

Figure 6: pGEM-T Easy

[92] Hoang TT, Karkhoff-Schweizer RR, Kutchma A, Schweizer HP: A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked Pseudomonas aeruginosa mutants; Gene 212, 1998, 77-86

pRK 2013
This plasmid[93] is a derivative of the ColE-type RK2 plasmid that contains the *RK2 tra* (transfer) function, which codes for genes necessary to enable the conjugation of a mobilizable plasmid. Vectors containing a transfer origin (RP4-*ori*-T) can be mobilized with the help of this plasmid even if the donor strain is not capable of conjugation itself. Relevant properties of the plasmid: Km^R, Mob^+, Tra^+, ColE1.

2.3. Equipment

Incubators: Heraeus
Dri- Block DB-3: Techne
eppendorf Centrifuge 5415C : Eppendorf
eppendorf Thermostat 5320 : Eppendorf
Hybridization oven: 400 HY : Bachofer
Labofuge I : Hereaus
Megafuge: Heraeus
Water bath: Model G76 : New Brunswick Scientific
Gel Shaker HS250 : Janke & Kukel Ika Labortechnik
Sorvall- Centrifuge RC5B Plus: DuPont
Rotors: GS-3 (SLA3000) , GSA (SLA1500), SS34
Tabletop centrifuge Hettich universal : Hettich
96 well plate centrifuge: Hermle
15 and 50 ml tube centrifuge: Heraeus Megafuge, Heraeus
Thermocycler : Landgraf
UV- Transilluminator: Bachofer
Vacuum concentrator: Bachofer
Waage BP3100S, BP210 S : Sartorius
Power Supply: Power pac 300, Bio-Rad
pH-Meter 761 : Calimatic Knich
Photometer: Hitachi
Electron microscope: Philipps

2.4. Chemicals and Enzymes

Restriction endonucleases were purchased from New England Biolabs,
Chemicals (purity grade: pro analysi) were purchased from Riedel de Haen, Sigma, Merck and Calbiochem.
The following kits were utilized:
Plasmid maxi preparation kit, Qiagen
Qiaprep Spin kit for gel elutions and purification of PCR products: Qiagen
Preparation of genomic DNA for y-linker ligation: Prestospin D “Bug”, Molzym
FailSafe PCR kit, Epicentre
FastPlasmid Kit, Eppendorf
Chemicals were purchased from Sigma-Aldrich, Calbiochem, Merck and Riedel de Haen, purity grade “pro analysi”

2.5. Consumables

Pipet tips for eppendorf multiwell pipettes were purchased from Eppendorf.
All other pipet tips, reaction tubes and plasticware were purchased from Sarstedt.
Blotmembran Hybond N^+: Amersham
Filter paper GB003 : Schleicher u. Schüll
Filter Celluloseester HA 0,45 µm: Millipore
Glass pasteur pipettes : Sarstedt

[93] Figurski DH, Helinski DR: Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci 76, 1979, 1648-1652

2.6. Buffers and Solutions

percentages:
Solids: weight/volume (g/100 ml)
Liquids: volume/volume (ml/100 ml)

(1) LB-Medium:

15 g/l Caseinhydrolysate
5 g/l Yeast Extract
10 g/l NaCl
pH 7.0

(2) LB-Gm: LB- Medium mit 25 (*E.coli*) or 50 (*Pseudomonas*) µg/ml Gentamicin

(3) LB- Agar, M9-Agar:

Solid medium was prepared by adding 15 g/l agar

(4) M9- Medium (5x):

64 g/l Na2HPO4 ·7 H2O
15 g/l KH2PO4
2,5 g/l NaCl
5 g/l NH4Cl

A carbon source was added before use

(5) TBE- Buffer (10x):

0.9 M Tris (108 g/l)
0.9 M Boric acid (55 g/l)
0.02 M EDTA (7,5 g/l) pH 8.3- 8.5

(6) Sample buffer for gel electrophoresis (6x):

15 % Ficoll 400
0.25% Bromophenolblue
0.25% Xylencyanol
0.5 M EDTA

pH 8.0

(7) SOB-Medium:

20 g/l Bactotrypton
5 g/l Yeast extract
mM NaCl (0.58 g/l)
2,5 mM KCl (0.185 g/l)
10 mM $MgCl_2$ (1 g/l) added before use
10 mM $MgSO_4$ (1.2 g/l) added before use
pH 7.0

(8) SOC-Medium:

SOB + 20 mM Glucose (3.6 g/l)

(9) PBS: 0.9% NaCl with 10mM H_2PO_4

(10) TE- Puffer:

10 mM Tris·Cl (1,2 g/l)
1 mM EDTA (0,38 g/l)
pH 7.5

(11) Lysis buffer :
40 mM Tris·Acetate (4.84 g/l)
20 mM Sodium acetate (1.64 g/l)
1 mM EDTA (0.38 g/l)
1 % SDS
pH 7.8

(12) NEB buffer 1:
10 mM Bis Tris Propane HCl
10 mM $MgCl_2$
1mM DTT
pH 7.0

(13) NEB buffer 2:
10 mM Tris·Cl (1.2 g/l)
10 mM MgCl2 (1 g/l)
50 mM NaCl (2.9 g/l)
1 mM DTT (0.15 g/l)
pH 7.9

(14) NEB 3:
50 mM Tris·Cl (6 g/l)
10 mM MgCl2 (1 g/l)
100 mM NaCl (5.8 g/l)
1mM DTT (0.15 g/l)
pH 7.9

(15) NEB *Eco*RI:
100 mM Tris·Cl (12 g/l)
50 mM NaCl (2.9 g/l)
10 mM MgCl2 (1 g/l)
0.025 % Triton X100
pH 7.5

(16) BSA 100x :(NEB)
10 g/l BSA

(17) Hexanucleotide-Solution (10x):
0.5 M Tris·Cl (60 g/l)
0.1 M MgCl2 (10 g/l)
1 mM DTE (0.15 g/l)
2 g/l BSA
pH 7.2

(18) DIG- DNA Labelling Mix (10x)
1 mM dATP, dCTP , dGTP :
0.65 mM dTTP
0.35 mM DIG- dUTP

(19) Sephadex G50- Dye mix:
0.8% dextrane blue (2·106 g/mol)
0.5% phenol red (376 g/mol)

(20) Blotting buffer:
0.4 M NaOH (20 g/l)

(21) Blot- wash buffer:
50 mM Sodium phosphate
(NaH2PO4·2H2O: 7.8g/l)
pH is adjusted with NaOH to 6.5

(22) Pre-hybridization buffer:
7% SDS
0.5 M Sodium phosphate (NaH_2PO_4·2 H_2O: 78 g/l)
1 mM EDTA (0.38 g/l)
0.5 % Blocking Reagent (Boehringer)
solubilized at 70°C
pH 7.2

(23) Hybridization- wash buffer:
40 mM Sodium phosphate (NaH_2PO_4·2 H2O: 6.25 g/l)
1 % SDS
1 mM EDTA (0.38 g/l)
pH 7.2

(24) Buffer I:
100 mM Tris ·Cl (12 g/l)
150 mM NaCl (8.7 g/l)
pH 7.5

(25) Buffer II:
Puffer I + 0.5 % Blocking- Reagent (Boehringer)

(26) Buffer III:
100 mM Tris·Cl (12 g/l)
100 mM NaCl (5.8 g/l)
mM $MgCl_2$ (4.8 g/l) pH 9.5

(27) Antibody solution:
1: 5000 dilution
Anti- Digoxigenin AP *Fab*- alkaline Phosphatase-conjugate (Boehringer)
in buffer II

(28) Wash buffer:
0.2 M NaOH (8 g/l)
0.1 % SDS

Ethidium bromide solution: 1 mg/ml ethidium bromide, Sigma, in H_2O,
stored in the dark at 4 °C.

(29) ABC-medium:
A-medium:
$(NH_4)_2SO_4$ 20g/l
Na_2HPO_4 60g/
KH_2PO_4 30g/l
NaCl 30g/l
Ad 1000 ml H_2O

B-medium:
1 M $MgCl_2$ x 6 H_2O 2 ml
0.5 M $CaCl_2$ x 2 H_2O 0.2 ml
0.01 M $FeCl_2$ x 6 H_2O 0.3 ml
Ad 900 ml H_2O

The solutions were autoclaved and stored in the dark.
C: carbon source: 15mM benzoate or 1M citrate
100 ml A-medium were added to 900 ml B-medium and 15mM benzoate were used as carbon source.
The medium was prepared freshly before use.

2.2.Microbiological Methods

2.2.1. Bacteria culture

For this dissertation, bacteria of the genus *Pseudomonas* and *E. coli* were used and cultured according to standard microbiological methods. Unless otherwise indicated, LB medium or LB-agar was used for bacteria culture at 37°C. Liquid cultures were shaken at 250 rpm to ensure optimal oxygen supply.

2.2.2. Determination of the bacterial number

1 OD at 600 nm corresponds to 1.7×10^9 cells/ml for *P.aeruginosa* and to
0.8×10^9 cells for *E.coli*

2.2.3. Longtime storage of bacteria

Glycerol stock cultures
Bacteria can be kept at -80°C for an extended period of time if suspended in medium containing 15% glycerol which protects the bacterial membrane during freezing.
For this purpose, bacteria from an overnight culture are pelleted at 6000 x g and 4°C for 5 minutes and resuspended in 1/5 vol. of fresh LB medium containing 15% glycerol.
As bacteria easily change their phenotype during subculturing, it has to be taken care that glycerol stock cultures are not subjected to frequent thawing and re-freezing.
Storing bacteria using cryobeads
Bacteria can be stored at -80°C without changes in phenotype when cryobeads are employed (Technical Service Consultants Ltd., Lancashire, UK). These beads are coated to allow for bacterial attachment and protection during freezing. However, they lack a carbon source for metabolic activities so that a change in phenotype is made nearly impossible. This is especially helpful if strains that have a very instable genome such as hypermutator strains[94] are to be frozen and frequently subcultured, thus subjected to numerous freeze-thaw-cycles.
An overnight culture was pelleted at 6000 x g and 4°C and resuspended in the cryobeads preservation fluid. The suspension was then pipetted onto the beads in the supplied cryotube, gently mixed and incubated standing up for 1-2 minutes.
The supernatant was suctioned off and the vial with the beads frozen at -80°C.

2.2.4. Antibiogram

Many *P.aeruginosa* strains are resistant to most of the antibiotics commonly used as selection markers which makes many common molecular biology applications difficult in these strains.
In order to determine selectable antibiotics for transposon mutagenesis in strain SG17M, several antibiotics for which gene cassettes conferring resistance are available were screened in different concentrations.

25 ml of hand-warm LB agar per plate was supplemented with the following antibiotics:
Gentamicin (Serva, Cat# 22185)
Kanamycin (Serva, Cat# 26899)
Tetracyclin (Sigma, Cat# T3383-25)
Carbenicillin (Serva, Cat# 15875)
These antibiotics were added to the medium in concentrations of 25µg/ml, 30 µg/ml, 40µg/ml, 50µg/ml, 75µg/ml, 100µg/ml, 125 µg/ml, 175 µg/ml and 200 µg/ml.

Streptomycin was supplemented in concentrations of 500 µg/ml, 750 µg/ml, 1 mg/ml, 1.5 mg/ml and 2 mg/ml. Higher concentrations were not tested because the solubility of Streptomycin decreased

[94] personal communication, Karen Larbig, 2001

drastically for concentrations higher than 2 mg/ml and an even distribution of the antibiotic in the medium could not be guaranteed.

2.3. Cell culture methods

For this dissertation, the human monocyte cell line THP1 was used. Furthermore, fresh polymorphonuclear neutrophils (PMN) from healthy donors were isolated and immediately utilized.

2.3.2. Culture of Monocytes and Differentiation into Macrophages

Macrophages are commonly used when assaying cytotoxicity of bacteria towards eukaryotic cells as they phagocyte the bacteria to be tested. One of the aims of this study is to analyze the interaction of human cells and *P. aeruginosa*, so the use of a human cell line to best mimic the *in vivo* host-pathogen situation *in vitro* was necessary. The human monocyte cell line THP-1 was cultured and differentiated into macrophages (monocyte-derived macrophages, MDM) using Phorbol-12-Myristate-13- Acetate (PMA, CAS# 16561-29-8, Calbiochem, Cat# 524400). PMA activates Protein Kinase C and via a mechanism most of which remains yet unknown induces the differentiation of monocytes to macrophages. These macrophages do not proliferate anymore and need about 72 hours to express all characteristic cell surface structures before they are ready to use.

Experimental procedure:

THP-1 monocytes were grown and maintained as suspension culture in Readymix RPMI 1640 with 10% FCS (PAA Laboratories, Austria, Cat# R15-802:) and 0.1% AMS. Preceding differentiation, 50 ml of cell suspension were centrifuged at 600 x g and room temperature for 10 minutes to sediment the cells while keeping vesicles and cell debris in the supernatant. The supernatant was then suctioned off and the cell pellet washed with 10 ml of pre-warmed sterile PBS, followed by centrifugation at 800 x g and room temperature/10 minutes.

The supernatant was suctioned off and the cells were resuspended by careful pipetting using a serological pipette in 5 ml of RPMI medium followed by 1:10 dilution and counting in a Neubauer chamber. The cell density was then adjusted to $3.5 \cdot 10^5$ cells per milliliter by diluting with medium. 16 nanomol of PMA were added to the cell suspension and 100 µl per well seeded into a 96 well plate and incubated for 72 hours. The course of differentiation was assessed by monitoring the phenotypic changes such as elongation of the rounded cells and the formation of plaspodia which go hand in hand with the transition to a sessile lifestyle and formation of epithelium-like structures. The change in phenotype was observed by microscopy.

2.4. Construction of an STM transposon library in *P.aeruginosa* SG17M

The construction of a large transposon mutant library relies on a transformation protocol for the parent strain that is easy to use and produces a large number of different mutants in few experiments.

The efficiency of the mutagenesis depends on the bacterial strain and its susceptibility to *Tn5*-insertion into its genome as well as a suitable shuttle vector. Generally, clinical isolates are difficult targets for mutagenesis and require thorough optimization of the protocol in order to yield an acceptable number of mutants. *P.aeruginosa* transposon mutants with stable phenotypes can be generated using an established protocol[95] that relies on the genomic insertion of a *Tn5*-derieved mini-transposon which is shuttled into *P.aeruginosa* by the plasposon p*Tn5*MOD-OGm (pMOD) via the donor strain *E.coli* DH5α aided by *E.coli* HB 101 carrying the p*RK*2013 plasmid to mobilize pMOD.

The plasmid source for the transposon can be introduced into the recipient strain either by electroporation, which is commonly applied to most bacterial species excluding *P. aeruginosa* clinical isolates, or by triparental mating which is generally the method of choice for constructing mutants in clinical isolates of *P.aeruginosa*.

The transposon library was constructed in several steps:

- Pretreatment of the acceptor strain
- Optimization of the triparental mating
- Triparental mating with DH5α-p*Tn5MOD*-OGm=donor,HB101carrying p*RK*2013=helper, and *P.aeruginosa* SG17M=acceptor strain
- Selection of the mutants and quality control
- Arraying the mutants in 96 well plates

[95] Wiehlmann L: Sequenzspezifische Transposonmutagenese in Pseudomonas aeruginosa, Dissertation, Universität Hannover, Deutschland, 2001

2.4.1. Conjugation- Triparental Mating

Conjugation is a common and naturally occurring method for horizontal gene transfer in bacteria and is most easily achieved via plasmids. For this study, conjugation between two different species was applied to shuttle the plasmid p*Tn5*MOD-*OGM* (pMOD) from *E.coli* to *P.aeruginosa*. pMOD is a mobilizable plasmid containing an *oriT*. It can not transfer autonomously but only with the auxiliary function of pBRK2013 which supplies the RK2*tra* function that pMOD does not possess. The RK2 function codes for the transfer machinery that interacts with the *oriT* of the mobilizable plasmid and enables its conjugation.

2.4.1.1. Pretreatment of the acceptor strain

It is a prerequisite for transposon mutagenesis by conjugation that the restriction system of the recipient bacterium is shut off in order to facilitate the uptake and maintenance of foreign DNA into and within the bacterial cell. This is achieved by pre-incubation of *P.aeruginosa* recipient strains on Columbia agar containing 5% sheep blood at 42°C for several days.
The pre-incubation time has to be carefully adjusted for every strain as its fitness decreases after a specific period of time that the bacterium is maintained at 42°C, but if the pre-incubation period is too short, the bacterium either will not take up the foreign DNA or will eliminate it quickly and no mutants are obtained.

2.4.1.2. Optimization of the triparental mating

To define the optimal pre-incubation period, the recipient strain SG17M was pre-incubated for 1-7 days and an aliquot of the bacterial culture was used for transformation by triparental mating each day. Reference strains with an already optimized pre-incubation period[98] were used as positive controls:
P.putida KT 2440 (environmental isolate) no preincubation time necessary[99]
P.aeruginosa PAO1 (clinical isolate from Australia): 1-2 days
P.aeruginosa 762 (clinical isolate from Tübingen, Germany): 3-4 days
P.aeruginosa TB (clinical isolate from Hannover, Germany): 5-7 days

The *P.aeruginosa* strains were streaked onto Columbia agar containing 5% sheep blood, incubated overnight at 42°C and an aliquot transferred onto a new plate each day. The rest of the culture was scraped off the plate, resuspended in 5 ml 10 mM $MgSO_4$ and its optical density measured.
The *E.coli* strains used as donors and helper strains were incubated overnight on LB agar supplemented with the appropriate antibiotic, 50 µg/ml kanamycin for *E.coli* HB 101-pRK2013 and 25 µg/ml gentamicin for *E.coli* DH5α-p*Tn5*-MOD-OGm.
The bacterial cultures were scraped off the plate, resuspended in 10 mM $MgSO_4$ and the cell density was determined by optical density measurement at 578 nm.
The bacterial suspensions were adjusted to an optical density of 1 and mixed in a ratio of donor:helper:acceptor of 10:10:1 so that each acceptor cell was surrounded by donor and helper from all sides[100].
400 µl of this mixture was plated onto LB agar and incubated overnight for at least 16 hours so that F-pili could form for the exchange of DNA from donor to acceptor strain.

2.4.2. Selection of the mutants

In order to obtain a higher ratio of different mutants, the mating plates were divided and each half was scraped off and separately resuspended in 10 mM $MgSO_4$. 150 µl of this suspension was plated onto M9 minimal medium agar plates that contained 15 mM sodium benzoate as sole carbon source and were supplemented with 50 µg/ml gentamicin. This allows for the exclusive selection of gentamicin-resistant *P.aeruginosa* mutants as *E.coli* is not genetically equipped to metabolize benzoate.

[98] Limpert AS: Einzelkopiespezifische Transposonmutagenese in Pseudomonas aeruginosa Klon C. Diplomarbeit, Universität Hannover, Deutschland 1999
[99] Weinel, C.:Personal communication, 2001
[100] Wiehlmann L: Sequenzspezifische Transposonmutagenese in Pseudomonas aeruginosa, Dissertation, Universität Hannover, Deutschland, 2001, see ref. 78

These selection plates were incubated for 24 to 48 hours depending on the formation of *P.aeruginosa* colonies. One mutant colony per 25 mutants/plate was picked and these mutants were asservated in 96-well plates. The statistical insertion of the transposon was verified by Southern blotting using randomly selected mutants.

2.4.3. Quality assessment of the mutant library

One major prerequisite for obtaining a high-quality transposon mutant library is to keep the number of identical mutants as low as possible as redundant mutants raise the amount of work and material to be spent on screening the mutant library. One conjugation experiment is conducted for 16 hours so that one single transposon mutant is able to divide several times and produce duplicates. Previous studies[104] have shown that numbers lower than 10 cfu equal one transposon mutation and with a result of 10-20 cfu, it can be assumed that two different transposon insertions have occurred. Based on this observation, the following limits for colony picking were established:

cfu	<20	20-50	51-80	81-120	121-170	170-230	> 230
Cfu picked	1	2	3	4	5	6	7

Table 1: Transformation efficiencies

The colonies were picked from the plate using a sterile toothpick or a pipette tip, suspended in 100 µl of LB medium in one well of a 96-well plate and incubated overnight. The following day, 50 µl of LB medium containing 40% glycerol were added, mixed well with the bacteria culture and the plate was frozen at -80°C.
The efficiency of the mutagenesis was monitored by Southern blotting using a probe specific for the gentamicin cassette on blotted *Pst*I-digested genomic DNA of transposon mutants.

2.4.4. Arraying the mutants

Transposon mutagenesis employing *Tn5*-minitransposons should produce statistical insertions into the genome of the targeted bacterium. However, it is always possible that identical mutants are picked from the same conjugation experiment although the cfu number was high enough to allow for the picking of several mutants (see quality assessment, above). To make their identification possible during bioassays, all mutants from one conjugation experiment are picked and arrayed according to their signature tag and a running number so that they are positioned at the same place in successive microwell plates. This way, if for example mutants 1A3 and 3A3 frequently have the same test results it can be assumed that they are identical and can be treated accordingly.

2.4.5. Southern Blotting to evaluate the mutagenesis efficiency

For Southern blotting, see molecular biology methods.

2.5. Bioassays

Once a mutant library of the desired size is generated, it can be subjected to assays that test for differences in phenotype compared to the parental strain.

2.5.1. Assaying Quorum Sensing Capabilities of the mutants

Many phenotypic characteristics of a bacterium are controlled by the quorum sensing regulatory network. This is especially true for the expression of virulence factors such as proteases. A mutant library can be easily pre-screened for mutants in quorum sensing-related genes by incubating them on M9 minimal agar containing 0.5% casein as a sole carbon source. Those mutants that are not able to degrade casein have a defect either in the type II secretion system, in the protease-encoding genes themselves or in the regulation of protease expression, the latter being controlled by quorum sensing.

[104] Wiehlmann L: 2001, see ref. 78

Experimental Procedure
The mutants were cultured in LB medium overnight to reach the stationary phase. Then, 5 µl of the bacteria culture were inoculated onto an agar plate containing M9 minimal medium and casein as a sole carbon source. A wild type control strain was also incubated on every plate.
The inoculated plate was incubated at 37°C for at least 16 hours until the formation of a slight white halo could be seen around casein degrading mutants and the wild type. Those mutants that could not degrade casein produced no halo and were subjected to the HSL production assay.

2.5.2. Homoserine lactone (HSL) production assay

The mutants that did not degrade casein when tested for protease secretion were subjected to the HSL production assay. This way, it could be determined whether the loss of protease secretion was really due to defects in the quorum sensing cascade.
Assessment of HSL-production of *P.aeruginosa* transposon mutants is based on measuring luciferase activity of an *E. coli* detector strain. This detector strain carries a plasmid with a luciferase gene that is only expressed in the presence of aliphatic HSLs.
Experimental Procedure
The transposon mutants were inoculated in 100 µl LB at 37°C for 4 hours. The detector strain was incubated to an OD of 0.3-0.4 and 100 µl of the bacteria suspension were added to the *P. aeruginosa* transposon mutants. After 4 hours, the luciferase activity was measured at 37°C with a photon camera. Mutants that did not activate luciferase expression were incubated with the detector strain in the middle on an agar plate containing modified LB medium with 0.4% (w/v) NaCl. HSLs could diffuse from the *P.aeruginosa* mutants to the detector strain and induce luciferase expression. After incubation for 16 hours at 37°C the induced luciferase activity was visualized with a photon camera at 37°C.
These experiments were done by Dr. Birgit Huber at the Technische Universität Freising, Germany.

2.5.3.Qualitative assessment of Siderophore Production

Many microorganisms produce and secrete molecules that serve to sequester iron, Fe (III)-specific high affinity ligands termed siderophores. *P.aeruginosa* produces two types of these fluorescent peptidic siderophores, pyochelin and pyoverdin; of the latter, three types exist that differ in their peptide chains[105]. A panel of *P.aeruginosa* strains isolated from patients with CF was analyzed for siderophore production and it was found that the isolates produced only type I or II pyoverdine, and strain SG17M as belonging to clone C produces type II pyoverdine.
The assay applied to test quorum-sensing deficient mutants for siderophore production relies on a medium that contains Fe(III) in a ternary complex with the dye chrome azurol S (CAS) and the detergent hexadecyl-trimethylammonium bromide (HDTMA). If the iron is removed by a strong chelator, the color of the complex changes to orange. Therefore, an orange halo can be detected around a siderophore-producing bacterial colony, its size depending on the amount of siderophores secreted by the bacterium. This assay does not allow for discrimination between the siderophores produced by *P.aeruginosa*, but the assessment whether a mutant colony is attenuated in siderophore production or secretion in comparison to the wildtype is possible employing the CAS-assay.
Experimental procedure:
CAS agar was prepared according to the protocol of Schwyn and Neilands[106] with a few modifications.
For one liter of CAS agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml of a solution prepared from 1 mM $FeCl_3$ x 6 H_2O and 10 mM HCl. The resulting blue dye solution was autoclaved. 72.9 mg of HDTMA in 40 ml H_2O were sterile- filtered and added to the still warm dye solution (CAS solution). A special M9 medium was prepared in which the phosphate buffer that would strongly bind the iron and make it unavailable for the bacterium was substituted with 1,4-piperazine-diethanesulfonic acid (PIPES), termed MM9. 100 ml MM9 medium in 750 ml H_2O were autoclaved with 15g agar, 30.24g of PIPES and 12 g of a 50% (w/v) NaOH solution were added to the still warm medium and mixed thoroughly. The CAS solution was added and after mixing, the CAS agar was poured into Petri dishes.

[105] De Vos D, De Chial M, Cochez C, Jansen S, Tümmler B, Meyer J-M, Cornelis P : Study of pyoverdine type and production by Pseudomonas aeruginosa isolated from cystic fibrosis patients : prevalence of type II pyoverdine isolates and accumulation of pyoverdine negative mutations. Arch. Microbiol. 175, 2001, 384-388
[106] Schwyn B, Neilands JB: Universal Chemical Assay for the Detection and Determination of Siderophores. Analytical Biochemistry 160, 1987, 47-56

Bacteria inoculation:

The transposon mutants were grown over night in LB medium. A dilution of this culture to an OD_{600} of 0.1 was made with PBS and 3 µl of this dilution were inoculated onto CAS agar plates. Siderophore production was assessed visually after 12h and 24h.

2.5.4. Assessment of Biofilm Production

The triphenylmethane dye crystal violet stains organic material such as carbohydrates and proteins and is ideal for a qualitative and quantitative assessment of biofilm formation.

Experimental procedure:

The cavities of a polystyrene microtiter plate were filled with 100 µl of ABC medium. Bacteria were pre-grown in LB medium over night prior to the experiment. The culture was diluted to an OD_{600} of 0.1 with PBS and 3 µl of the diluted cultures were inoculated into each cavity. Each mutant was inoculated in triplicate.

The bacteria were grown at 37°C for 2 days. After the incubation time, the medium was carefully removed and the cavities washed with H_2O, 100 µl of a 0.1% crystal violet solution was added and incubated within the cavities for 10 minutes. Afterwards, the crystal violet solution was removed and the plate washed with H_2O and dried at 37°C for 30 minutes. The crystal violet was then dissolved in ethanol and its absorbance measured at 570 nm against ethanol.

2.5.5. Establishing a Cytotoxicity Assay using THP-1 monocyte-derived macrophages

Cytotoxicity of bacteria towards eukaryotic cells is mostly associated with damage of the eukaryotic membrane. A cytotoxicity assay can therefore be based on the quantification of substances released from the cytosol into the medium such as enzymes or DNA, as the membrane of the nucleus is normally also affected by cytotoxic agents released from the bacterium. Two possibilities allow the quantification of bacterial cytotoxicity:

- Measuring the amount of ethidium bromide taken up into the infected cell which stains the DNA and can be visualized at 304 nm.
- Indirectly measuring the release of NADH from the eukaryotic cell by quantifying the conversion of MTT to its formazan as a means to assess the cell viability.

2.5.4.1. Ethidium Bromide Staining as an indicator of cell viability loss

The fluorescent dye ethidium bromide intercalates into the stacked bases of the DNA and thus can be used to stain and detect DNA under UV light. Therefore, it should be easy to visualize cells that suffer from a loss of cellular and nuclear membrane integrity using ethidium bromide that can permeate the cell and stain the DNA once the nuclear membrane has been compromised. The assay should provide an easy and quick qualitative assessment of the cytotoxicity of the tested *P.aeruginosa* strains.

Experimental procedure

THP-1 MDM were grown in 2 ml of RPMI medium containing 10% FCS and 1% AMS in 6-well plates at a density of $3.5 \cdot 10^5$ cells/ml. Prior to infection, the macrophages were washed twice with RPMI 1640 without FCS and AMS additives. The Infection was carried out with selected mutants as well as with the wildtype strains PAOI, TB, SG17M, CHA and 892. The bacteria were diluted to a MOI of 30 in 2 ml of HEPES-buffered RPMI 1640 medium, added and briefly centrifuged onto the cells at 800 rpm. The assay was incubated for 2 hours at 37°C. After the incubation period, 50µl of 1% ethidium bromide in PBS (w/v) was added to the wells, incubated for 10 minutes and the staining of the DNA visualized at 304 nm.

2.5.4.2. MTT assay to assess cell viability

Another way of analyzing cytotoxicity is the assessment of mitochondrial damage exerted by the bacteria. The enzyme succinate dehydrogenase, enzyme 7 of the citrate cycle, catalyzes the dehydrogenation of succinate to fumarate (formation of a C–C double bond and loss of 2 hydrogen). FAD is reduced to $FADH_2$ and is able to shuttle these two hydrogens to the tetrazolium dye MTT which is in turn reduced to its formazan. In a damaged mitochondrion, less redox equivalents are turned

over, thus less reduction of MTT to the corresponding formazan can occur. This reaction is described as very sensitive[109] and well-suited for gauging the loss of cell viability by cytotoxic agents[110] by absorbance measurement: A high absorbance is yielded by viable cells that produce physiological amounts of NAD and FAD, the latter can shuttle hydrogen to MTT and reduce it to its formazan. Low absorbances indicate low $FADH_2$ generation levels and impaired fitness of the eukaryotic cells and the presence of a cytotoxic agent.
In order to assess the reliability and reproducibility of the cytotoxicity assay, several experiments with different strains were carried out and the results compared. The MOI, amount of added MTT and incubation times were tested and adjusted until reproducible results were obtained. The assay was based on TB and CHA as two strains with maximal cytotoxicity, and CHA-*exsA*$^-$, TB *exsA*$^-$ and *P. putida* KT 2440 as strains with nearly no cytotoxicity. The safety strain E.coli K12 which is frequently used as a negative control in bioassays involving pathogenic bacteria was not utilized here as it is metabolically quite different from *P.aeruginosa* and does not yield comparable results when screening for MTT reduction activity.
The cytotoxicity assay was carried out as described above with MOIs of 10, 20, 30, 50, 100 and 200; with 2,3,4 and 5 µg of MTT in different experiments (one parameter variation per experiment), the results compared and the combination that delivered the most reproducible results was applied to future assays.

2.5.4.3. High-thoughput screening of the mutant library

To ensure reproducibility of high-throughput screening employing the MTT assay, all cell infection screenings were conducted in duplicates with sufficient controls. At least two wells of each microwell plate were left without bacteria and served as zero controls, and two or more wells were inoculated with wildtype strains for comparison with the mutants.
The STM mutants had been arrayed in 96-well plates which could conveniently be screened in duplicates. The cells were incubated with the bacteria at a MOI of 30 for 90 minutes. Then, MTT was added and the formazan formation could proceed during further incubation for 30 minutes. The reaction was stopped before the forming formazan crystals could destroy the cells by taking off the supernatant and two washing steps with PBS, followed by lysis of the cells and solubilization of the formazan crystals with DMSO. The absorption of the formazan solution was measured against DMSO at 570 nm.

2.6. Molecular Biology Methods

2.6.1. Preparation of Genomic DNA from *P.aeruginosa*

Genomic DNA from *P. aeruginosa* was prepared according to the following protocol:
4 ml of an overnight culture were sedimented at 13000 x g and 4°C for 2 minutes and thoroughly resuspended in 500 µl lysis buffer and incubated at room temperature for 5 minutes. 200 µl of a 5 M NaCl solution was added, the lysate mixed thoroughly and centrifuged at 14000 x g and 4°C for 30 minutes to pellet cell debris and proteins. The supernatant was transferred into a clean microcentrifuge tube and 1 µl of RNAse A (Qiagen, Cat# 10913897) was added followed by an incubation at 37 °C for 30 minutes. Phenol, phenol chloroform (1:1) and chloroform:isoamylalcohol (24:1) extractions followed before an equal volume of isopropanol was added to the aqueous phase. Slight shaking mixed the two phases until the formation of a cloudy precipitate indicated the sedimentation of the DNA. The solution was centrifuged at 13000 x g at room temperature for 15 minutes. The pellet was first washed with 70 % ethanol, followed by a wash with absolute ethanol, air-dried at room temperature and then solubilized in TE buffer.

2.6.2. Preparation of Genomic DNA from *P. aeruginosa* for Direct Genomic Sequencing

Direct genomic sequencing is a technically demanding task that requires high quality and purity of the DNA especially if it originates from an organism that has a rather large and G+C rich genome like *P. aeruginosa*. Therefore, the general protocol for the preparation of genomic DNA had to be adjusted to these requirements. A digestion step with proteinase K was added after the lysis of the bacterial cell, so that all remaining protein and cell debris could be completely removed from the aqueous, DNA-

[109] Denizot F, Lange R: Rapid colorimetric assay for cell growth and survival: modification of the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Meth. 89: 271-277, 1986
[110] Mosmann T: Rapid colorometric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65: 55-63, 1983
-Sieuwerts AM, Klijn JGM, Peters HA, Foekens JA: The MTT tetrazolium salt assay scrutinized: how to use this assay reliably to measure metabolic activity of cell cultures in vitro for the assessment of growth characteristics, IC50 values and cell survival. Eur. J.Clin.Chem. Clin Biochem. 33:813-823, 1995

containing phase by centrifugation. Also, a polyethylene glycol extraction step was added in order to remove polysaccharides which also interfere with the sequencing process if present in the DNA preparation.

Experimental procedure:
A 500 ml bacteria culture in LB medium was grown at 37°C at 250 rpm overnight. The cells were pelleted at 6000 x g for 10 minutes at room temperature. The pellet was washed with a solution of 0.5 % EDTA and 0.1% SDS in H_2O and then resuspended in 10 ml DNA lysis buffer until the suspension was clear and slightly viscous. 40µl of a 100 mg/ml Proteinase K (Roche) solution were added and the mixture incubated overnight at 56°C. The following day, 6 ml of 5M NaCl were added and mixed thoroughly so that the biphasic mixture became homogenous. Centrifugation at 18000 x g and 4°C for 60 minutes served for the separation of proteins and cell debris from the DNA in the supernatant which was then carefully taken off. RNA contaminations were digested by adding 50 µl RNAse A (Qiagen) and incubating the mixture for 30 minutes at 37°C. The DNA-suspension was purified by extractions with equal volumes of phenol, phenol:chloroform (1:1) and chloroform : isoamylalcohol (24:1) which were followed by an extraction step with a solution of 30% PEG 6000 in 1.6 M NaCl. The supernatant was taken off carefully and added to an equal volume of isopropanol at room temperature. The DNA precipitated upon mixing and centrifugation at 10000 x g yielded a translucent to white DNA pellet which was washed twice in 70% ethanol and once in abs. ethanol and air-dried. The DNA was either solubilized in water for downstream applications such as sequencing or in TE for storage at -20°C.
Direct genomic sequencing was carried out by Qiagen and GATC using the Sanger procedure. The sequencing reaction was primed by an oligonucleotide specific for the transposon inserted into the genome of the mutants (sequence see appendix).

2.6.3. Y-linker PCR product generation and sequencing

As genomic sequencing of transposon mutants is not always applicable, for example if the transposon insertion occurred in a genomic region with highly repetitive sequences, a PCR product of the transposon insertion can nevertheless be generated and sequenced. Ligating a linker of a 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 will generate a PCR product that can subsequently be sequenced.[111]
The linker is constructed as follows: 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 which prevents annealing to the linker itself. This way, a second primer is necessary for logarithmic amplification of the targeted sequence. If this primer is constructed to lie within the transposon sequence, a PCR product can be generated from Y-linker primer to transposon primer. DNA fragments that were not ligated to the Y-linker will not give any product. For a schematic overview of this method, see chapter 3.3
Y-linker strand 1:
5' TTT CTG CTC GAG CTC AAG CTT CGA ACG ATG TAC GGG GAC ACA TG 3'
Y-linker strand 2:
5' TGT CCC CGT ACA TCG TTC GAA CTA CTC GTA CCA TCC ACA T 3'
Y-primer: 5' CTG CTC GAG CTC AAG CTT CG

Experimental procedure:
Y-linker preparation:
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°C for 15 minutes and then heated to 95°C for 20 minutes. 4 µl of linker strand 2 (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.
Digestion of the DNA
Genomic DNA of transposon mutants prepared according to the protocol described in 2.6.1. was digested with *Sph*I as follows:

[111] Kwon YM, Ricke SC: Efficient amplification of multiple transposon-flanking sequences. J. Microbiol. Methods, 41, 2000, 195-199

1µg of DNA was mixed with 2 µl of NEB buffer 2 and 0.5 µl of *Sph*I. ddH_2O was added to a final volume of 20 µl. The reaction was incubated at 37°C for 3 hours and the enzyme subsequently heat-inactivated at 65°C for 20 minutes.

Ligation of the digested DNA to the Y-linker:
40 ng of *Sph*I-digested genomic DNA was mixed with 5 µl of the Y-linker, 2 µl of 10x ligase buffer concentrate, 2 µl 10 mM ATP, 0.5 µl T4 DNA ligase (NEB), and ddH_2O was added to a final volume of 10 µl. The reaction was incubated at room temperature for 2 hours and afterwards purified by phenol, phenol-chloroform and chloroform-isoamylalcohol extraction and precipitated with isopropanol. The pellet was washed twice with ice-cold abs. ethanol and air-dried.
PCR of the ligation product:
PCR was carried out using the *FailSafe*™ PCR kit (Epicentre, see 2.2.6.4.). For transposon primer sequences, see appendix.

PCR REACTION COMPONENT	VOLUME (µL)
genomic DNA, 20-50 ng in 1 µl	1 µl
Transposon Primer	2.5 µl
Y-linker primer	2.5 µl
FailSafe™ Enzyme and nucleotide mix	0.5 µl
FailSafe™ polymerase buffer	12.5 µl
ddH_2O	6µl

Table 2: Y-linker PCR

PCR program:
Initial denaturation: 300 s at 95°C
35 cycles of:
Denaturation: 60 s at 95°C
Ramp: in 30 s from 95°C to 60°C
Annealing: 75 s at 60°C - 63°C
Ramp: in 30 s from 60°C - 63°C to 72°C
Elongation: 180 s at 72°C
Terminal elongation: 600 s at 72°C.
PCR products were stored at -20°C.

The PCR products were purified by gel electrophoresis and elution from the gel as described in 2.11 utilizing the Qiaquick gel extraction kit as directed by the manufacturer (Qiagen).
PCR products were air-dried and dispatched for sequencing.

2.6.4. Establishing an easy to use-protocol for directed mutagenesis employing gene replacement

Directed mutagenesis is a powerful tool for the analysis of gene function and the impact of gene function in the genomic context and complements transposon mutagenesis. This method relies on homologous recombination of plasmid-borne DNA derived from the targeted gene which is disrupted by a gene conferring antibiotic resistance. This way, the plasmid-borne DNA replaces the targeted gene or parts of it in the genome and hence renders it non-functional.
Whole-plasmid-integration is selected against by a *sacB*-gene cassette on the plasmid that, if expressed, will result in the bacterium's death. It encodes an enzyme complex that allows the bacterium to take up sucrose which leads to the uptake of more and more water in order to maintain the cellular osmotic equilibrium until the bacterial cell bursts.
Illustrations of all the steps described here can be found in chapter 3.6.

2.6.4.1. Construction of the deletion sequence

DNA analysis was performed using NEB cutter (www.neb.com). The sequence of the target gene plus up to 600 base pairs of flanking region on both sides was obtained from the genome database (www.pseudomonas.com) and entered into the web-interface of NEB cutter. The generated restriction maps for single, double and triple cutters as well as a list of non-cutting enzymes were obtained and compared to those of the *sacB*-containing vectors to be used. If the target DNA sequence showed restriction sites that matched those of either vector, they were utilized for cloning. If not, either the *SphI/NsiI* sites from subcloning vectors pGEM-T were used for ligation into the corresponding sites of pEX100T or the *Eco*RI sites as well as a combination of sites from pGEM-T Easy were used to correspond to those in pEX18Ap. The target sequence was then amplified on genomic DNA and the product subcloned into the subcloning vector.
To construct a deletion sequence that is disrupted by a suitable antibiotic cassette, it is best if this cassette is ligated into a naturally occurring unique restriction site close to or directly in the middle of the target sequence. The resistance cassette can be amplified by PCR with primers that generate the suitable restriction site, subcloned and released from the subcloning vector with the specific endonuclease whose recognition sites have been artificially introduced.
The subcloning vector that contains the target sequence is cut with the respective endonuclease, dephosphorylated and the antibiotic cassette ligated into the insert.
Another possibility is the use of a site for a blunt-cutting endonuclease and cut out a suitable resistance cassette from its vector with the respective endonuclease. The obtained cassette can then be blunt-end ligated into the cut target sequence. The advantage of this method is that generally, the respective restriction site is lost and does not interfere with cloning procedures afterwards.
The deletion sequence generated this way was released from the subcloning vector with restriction endonucleases that were also present in the *sacB*-containing vector. Before the complete deletion sequence was ligated into the *sacB*-containing vector, this vector was also cut with the suitable restriction enzyme(s) and dephosphorylated twice.
The resulting suicide vector-insert construct was then electroporated into *P.aeruginosa* SG17M or TB. Transformants were selected on LB medium supplied with 50 µg/ml gentamicin, and resistant colonies were transferred to LB medium containing 5% sucrose and 50µg/ml gentamicin. Subcultures of colonies that grew on this selection medium were restreaked onto LB agar containing 200 µg/ml carbenicillin. If they proved to be sensitive to carbenicillin, they were selected as correct mutants and analyzed further to determine the genomic integration site of the gentamicin cassette.

2.6.4.2. Polymerase Chain Reaction (PCR)

Stretches of DNA that are flanked by known DNA sequences can be amplified from very small amounts of DNA employing thermostable DNA-Polymerases and sequence-specific primers[113].
The primers are small oligonucleotides that are constructed to be complementary to the flanking sequences, bind to them and serve as starters for the synthesis of new DNA that includes the stretch of interest.
This study relied on PCR-based cloning as well as the identification of unknown stretches of sequence from the *P.aeruginosa* genome by PCR followed by sequencing of the product.

2.6.4.2.1. Primer Design

Primers for the target sequences were picked manually and designed for a G+C content of about 60-65 %, a melting temperature of 60-64°C and a length of 20-22 base pairs. (Primers and DNA sequences, see appendix). Where necessary, recognition sites for restriction endonucleases were added to the primer sequences.
There are several guidelines for primer design. Optimally designed primers are absolutely complementary to the targeted sequence; however, few mismatch nucleotides are tolerated if they are not directly adjacent to one another. The optimal length for primers depends on the target sequence; generally, primers of 20-30 bp length yield good results in standard PCR reactions. Primer dimers can be avoided if the primers have neither intra- nor intermolecularly complementary sequences. The PCR yield can be optimized with primers that have the same melting temperatures, which can be calculated accordingly:
T_m = n(A+T) · 2°C + n(G+C) ·4°C at a salt concentration of 1 M.

[113] Saiki RK, Gelfand DN, Stoffel S, Scharf SJ, Higuchi R, Hon GT, Mullis KB, Erlich HA: Primer directed enzymatic amplification of DNA with a thermostable DNA-Polymerase, Science 238, 1988, 487-491

Experimental Procdures
All PCRs for allelic replacement procedures were carried out using the FailSafe-PCR™ system (Epicentre, Cat.# FS 99060, FS 99100) that allows for easy optimization and optimal reproducibility following the manufacturer's specifications.

2.6.4.2.2. PCR Protocols

Standard PCRs were carried out according to the following protocol:

PCR REACTION COMPONENT	VOLUME (µL)
Plasmid or genomic DNA, 1:10 dilution	1-3 µl
Primer 1	2.5 µl
Primer 2	2.5 µl
dNTP-mix (2 mM for each nucleotide)	2.5 µl
Taq-polymerase buffer, 10 x concentrate	2.5 µl
DMSO	1.25 µl
$MgCl_2$ (5 mM)	2-4 µl
Taq-polymerase	0.2-0.5 µl
ddH_2O	Ad 25 µl

Table 3: PCR reaction components

This reaction could be scaled up proportionally; nevertheless, reaction sizes of more than 200 µl do not yield sufficient product due to irregular temperature conditions in the sample.
The reaction mix was overlaid with paraffin oil to prevent sample evaporation and treated according to the following standard PCR program:
Denaturation: 300 sec at 95°C
30-40 cycles of:
Annealing: 60 sec at 58-64°C, depending on primers and template
Elongation: 60 sec at 72°C
Denaturation: 90 sec at 95°C
Terminal annealing: 60 sec at 58-64 °C
Terminal elongation: 300 sec at 72°C.
The samples were cooled to 10°C and stored at -20°C.
FailSafe™ PCR protocols:

PCR REACTION COMPONENT	VOLUME (µL)
genomic DNA, 20-50 ng in 1 µl	1 µl
Primer 1	2.5 µl
Primer 2	2.5 µl
FailSafe™ Enzyme and nucleotide mix	0.5 µl
FailSafe™ polymerase buffer	12.5 µl
ddH_2O	6µl

Table 4: FailSafe PCR reaction

PCR program for *FailSafe™* reactions:
Denaturation: 180 sec at 96°C
30-40 cycles of:
Annealing: 45 sec at 62°C, depending on primers and template
Elongation: 90 sec at 72°C
Denaturation: 60 sec at 94°C
Terminal annealing: 45 sec at 62°C
Terminal elongation: 150 sec at 72°C.

2.6.4.2.3. Purification of PCR products

PCR products need to be purified from excess nucleotides, primers and reagents for downstream applications. PCR cleanup kits are commercially available but usually not very practical as the yield is very low.

PCR products for this study were purified by filtration in centricon filtration devices (Amicon, Millipore, Cat# 42410). The exclusion size of the filter membrane has to be chosen in a way that primers can pass through but the PCR products are retained on the membrane. The PCR product was recovered by inverting the device and elution with water or TE.

Experimental procedure
The membrane of the centricon device was rinsed twice with ddH_2O to avoid contamination of the sample with fabrication residues. The PCR product was pipetted onto the membrane and the device was centrifuged at 6000 x g until the sample liquid had passed through the membrane. The DNA was washed with 100-200 µl ddH_2O before the device was inverted and placed into a fresh reaction tube. The DNA was eluted by pipetting water or TE directly into the middle of the device and centrifuging at a maximum of 3000 x g for 5 minutes at room temperature. The yield was checked by agarose gel electrophoresis and usually, 95 % of the DNA was recovered if compared with the PCR control gel.

If the PCR control gel showed by-bands that accompanied the product, the band that corresponded to the expected product size was cut out of the gel and purified according to the methods described in chapter 2.11

2.6.5. Ligation

Ligations can be performed with DNA that was eluted from an agarose gel, precipitated and solubilized or with restriction fragments after heat-inactivation of the enzyme. However, it is important that vectors and inserts to be ligated are incubated at 45°C for 10 minutes to separate cohesive ends. The insert/vector ratio should be as high as possible especially for small inserts, and as insert/vector ligations are intermolecular reactions; they are facilitated by an overall high DNA concentration.

2.6.6. Subcloning procedures

The vectors used for subcloning of PCR products were pGEM-T and pGEM-T Easy (Promega, Cat.# A3600; Cat.# A1360).
Ligations into the linearized subcloning vectors were performed according to the specifications of the manufacturer (Promega) with components of the vector kit.

LIGATION COMPONENT	PCR-PRODUCT LIGATION	POSITIVE CONTROL	BACKGROUND CONTROL
2x rapid ligation buffer	5µl	5µl	5µl
pGEM-T vector (50 ng)	1 µl	1µl	1µl
PCR product	10-200 ng in 1-3 µl	-----	----
Control insert DNA	----	2 µl (4 ng/µl)	----
T4 DNA ligase,	1 µl (3 Weiss units/µl)	1 µl (3 Weiss units/µl)	1 µl (3 Weiss units/µl)
Deionized water	Final volume of 10 µl	Final volume of 10 µl	Final volume of 10 µl

Table 5: Ligation reactions for subcloning procedures using pGEM-T or pGEM-T Easy

Ligation was either carried out at room temperature for one hour or overnight at 4°C for a maximum number of transformants.

LIGATION COMPONENT	PCR-PRODUCT LIGATION	POSITIVE CONTROL	BACKGROUND CONTROL
2x rapid ligation buffer	5µl	5µl	5µl
vector (50 ng)	3-4 µl	1µl	1µl
PCR product	10-200 ng in 2-6 µl	-----	----
Control insert DNA	----	2 µl (4 ng/µl)	----
T4 Quick DNA ligase,	1 µl (3 Weiss units/µl)	1 µl (3 Weiss units/µl)	1 µl (3 Weiss units/µl)
Deionized water	Final volume of 20 µl	Final volume of 10 µl	Final volume of 10 µl

Table 6: Ligation reactions for general cloning procedures

2.7. Transformation of chemically competent cells

The ligation reactions were transformed into chemically competent *E.coli* TOP 10 cells (Invitrogen, Cat,#: 44-0301).

Transformation protocol:

The competent cells were thawed on ice and 17 µl aliquots were prepared. 3 µl of the ligation reaction were added to the cells and the mixture incubated on ice for 10 minutes. DNA uptake was mediated by heat shock for 60 seconds at 42°C; immediately after the heat shock, pre-warmed SOC-medium was added to the bacteria which were then incubated at 37°C and 500 rpm for 60 minutes. Afterwards, the mixture was plated onto LB agar plates containing a suitable selection antibiotic and incubated for 16 hours at 37°C. Transformants were picked and cultured overnight in liquid LB containing a suitable selection antibiotic so that a plasmid preparation could subsequently be carried out.

2.8. Preparation of Plasmid DNA

Recombinant DNA can easily be obtained if it is cloned into a plasmid that is preferably maintained in a high copy number in a suitable host organism and replicated with this plasmid. The bacteria transformed with the plasmid are cultured, the cells harvested and lysed and the plasmid DNA is subsequently purified.

The different properties of plasmid and genomic DNA are used to separate both DNA types. While a genomic DNA preparation always contains the whole DNA of the cell, a plasmid preparation is supposed to give only plasmid DNA which is free of genomic DNA. The crucial step in this procedure is the cell lysis: Anchor proteins attach the genomic DNA to the cell membrane while the plasmid DNA is located in the cytoplasm. It can be obtained in pure form if the cell lysis is done for 5 minutes using a strongly alkaline buffer which contains SDS. During this time, the cell wall is lysed and the plasmid DNA can be obtained while the genomic DNA is still attached to the anchor proteins of the cell wall and is subsequently centrifuged and sedimented with the cell debris pellet while pure plasmid DNA can be found in the supernatant.

Experimental procedure for a *mini*-scale plasmid preparation:

5 ml of an overnight culture of *E. coli* in LB medium supplemented with the suitable selection antibiotic were sedimented at 13000 x g at 4° for 2 minutes. The pellet was resuspended in 330 µl resuspension buffer (buffer 1) and incubated at room temperature for 5 minutes.

Lysis of the bacterial cells was achieved by adding 330 µl of freshly prepared lysis buffer (buffer 2), gentle mixing and incubation at room temperature for 5 minutes.

The mixture was neutralized by adding 330 µl of neutralization buffer (buffer 3), thorough mixing and 10 minutes incubation on ice. The liquid phase containing the plasmid DNA was separated from cell debris and proteins by centrifugation at 13000 x g and 4°C for 30 minutes. The lysate was suctioned off and transferred into a clean microcentrifuge tube. For the digestion of ribosomal RNA that often contaminates plasmid DNA, 1 µl of RNAse A (Qiagen, 100 mg/ml, Cat# 10913897) was added and the mixture incubated at 37°C for 30 minutes.

Extractions with phenol, phenol/chloroform and chloroform/isoamyl alcohol yielded pure plasmid DNA that was subsequently precipitated in isopropanol at room temperature to prevent co-precipitation of salts. After centrifugation at 13000 x g and room temperature for 30 minutes, the supernatant was suctioned off, the pellet washed with 70 % ethanol followed by a wash with absolute ethanol and air-dried at 37°C. For immediate use, the DNA was solubilized in water, for storage in TE buffer to prevent the degrading of the DNA. The quality of the DNA was assayed by gel electrophoresis or spectrometry.

High-purity plasmid DNA was obtained using the FastPlasmid Kit (Eppendorf, Cat.# 0032.007.655) which allows for the preparation of extremely pure plasmid DNA in a very short time. The kit was used according to manufacturer's specifications.

Plasmid-*maxi* Preparation

Vectors that were frequently used for this dissertation, either as source for an antibiotic cassette, vector backbone or for obtaining inserts of cloned DNA in large amounts were isolated from transformed *E.coli* strains using the Quiagen MaxiPrep kit following the supplier's protocol.

2.9. Restriction analysis of plasmid- and genomic DNA

Restriction enzymes recognize specific palindromic target sequences on a DNA molecule and precisely cut the DNA at that recognition site. Nowadays, there is a great variety of commercially available restriction endonucleases and good software which allows for the detection of restriction sites in virtually every DNA sequence so that restriction maps and cloning strategies can be comfortably planned and carried out. If a DNA molecule is cut with several suitable restriction

endonucleases, it is cleaved into fragments of distinct size which can be separated using agarose or, in the case of very small fragments, polyacrylamide gel electrophoresis. The migrated distance then corresponds to the logarithm of the fragment length.
Restriction digests can vary in volume, DNA or enzyme concentration; to avoid unspecific cleavage, the concentration of the enzyme suspended in glycerol should not exceed 10 % of the reaction volume. An analytic restriction digestion normally consists of about 20-25 microliters total volume with an incubation time of a few hours, while a preparative digestion with subsequent elution of the DNA fragments from a gel can have a volume of about 100-120 microliters and an incubation time from 16-24 hours. The enzyme concentration depends on the number of recognition sites the enzyme has in the DNA to be cleaved, on the enzyme's stability and the potential for star activity (unspecific cleavage).

General analytic restriction digestion:
5 µl of 10-50 ng of plasmid DNA in TE
1 µl (1-10 U) of restriction enzyme
0.2 µl 100x BSA
2 µl of the supplied buffer for the enzyme
11.8 µl ddH_2O

General preparative restriction digestion:
50 µl of 1-2 µg of plasmid DNA in H_2O
5-10 µl of restriction enzyme (10-30 U)
1 % BSA
10% supplied buffer for the specific enzyme
ddH_2O ad 100-120 µl

2.10. Analysis of DNA fragments by agarose gel electrophoresis

Agarose gel electrophoresis is the standard method to separate and purify nucleic acids. The migration distance of a DNA molecule depends on the pore size of the gel which itself is determined by the agarose concentration. The agarose concentration and thus the resulting pore size of the gel matrix can be chosen according to the DNA to be separated. Agarose concentrations from 0.5% to 4% can be used to separate fragments of several megabases to those of only 50-100 base pairs. For genomic DNA from *P.aeruginosa* that was digested with *XhoI* or *PstI* for Southern blotting, gels with an agarose concentration of 1.2% were chosen; plasmid DNA was usually separated in 0.8-1% agarose gels. The gels were prepared with TBE buffer which also served as electrolyte. Gel runs were conducted with a field strength of 8.5 V/cm for 2 hours at room temperature or with 0.5 V/cm overnight at 4°C. Visualization of the separated DNA was achieved by post-run ethidium bromide staining. A gel of 15 cm length and 8 cm width was stained in a glass tray with 100 ml of a 0.1% ethidium bromide solution for 20 minutes and de-stained twice with 200 ml of de-ionized water. Photographs of the gels were taken on a UV transilluminator table (304 nm).

2.11. Recovery of DNA fragments from agarose gels

DNA purification via gel electrophoresis and subsequent elution from the gel is a standard method in order to separate restriction fragments or DNA of other origin and use them for cloning procedures afterwards. Large fragments can be cut out, frozen at -70°C so that the structure of the agarose gel is destroyed and the DNA can be eluted by centrifugation. Then, the DNA can be pelleted by ethanol or isopropanol precipitation. Washing with 70% ethanol and air-drying yields the pure DNA ("Freeze-Squeeze" method[114]). For fragments smaller than 1000 base pairs, the yields are very low so that the elution of small fragments requires a different method.
Experimental procedure:
The band was cut out from the gel using a sharp scalpel, cut into small pieces and stored at -80°C for at least 2 hours. The sample was thawed and centrifuged through a piece of gauze at 13000 x g for 15 minutes at 4°C. The agarose was held back by the gauze while the DNA was eluted in TBE. The eluate was adjusted to 0.3 M sodium acetate and the DNA precipitated with ethanol. The pellet was washed with 70% ethanol and air dried.

A more convenient method for DNA elution which is also suitable for very small fragments has been developed by Qiagen. This method relies on the solubilization of the agarose in a suitable buffer and binding the DNA to a column with a silica matrix. The DNA is then washed with an ethanol-containing

[114] Walker JM: Methods in Molecular Biology, Vol.2, 1984: Nucleic Acids Humana Press, Clifton, New Jersey, USA

wash buffer and eluted with either an elution buffer supplied by the manufacturer, ddH_2O or TE, depending on the downstream applications. This method allows for high yields of very pure DNA that can be used for the various downstream applications such as cloning, direct sequencing or transfections.
Experimental procedure: The Qiaquick DNA purification kit was utilized according to manufacturer's specifications.

2.12. Vector dephosphorylation

Dephosphorylation is essential whenever a vector is cut with a single restriction enzyme or with two restriction endonucleases that generate compatible overhangs. Removal of the 5'-terminal phosphate groups by alkaline phosphatase minimizes but rarely ever completely prevents recircularization of the vector.
Dephosphorylation was carried out directly in restriction digests by adding 1 µl of Antarctic phosphatase (NEB) and 1/10 vol. of 10x Antarctic phosphatase buffer concentrate at 37°C for a minimum of 12 hours. The procedure was repeated by adding new Antarctic phosphatase to the reaction and incubating for 1-2 hours to ensure maximal dephosphorylation of the vector.
Inactivation of the phosphatase as well as the restriction enzymes still present in the reaction mixture was achieved by 20 minutes incubation at 65°C for enzymes that allowed heat inactivation. For other enzymes, phenol-chloroform extraction of the reaction mixture was necessary.

2.13. Electroporation

Electroporation is the method of choice for introducing foreign DNA into most bacterial strains. It is a physical method of transformation that uses a strong electrical field for the transient formation of holes in the bacterial cell membrane through which foreign DNA can enter into the bacterial cell. This method does not require donor strains such as *E.coli* and thus eliminates one major source of contamination of the desired mutants as opposed to the conventional three-parental mating. However, with clinical isolates of *P.aeruginosa,* electroporation has proven to be difficult due to the thick layer of exopolysaccharides that surround the bacterial cell.
However, it was found during the course of this work that mucoid strains can be electroporated after thorough removal of the exopolysaccharide layer.
To evaluate the transformation efficiency, the bacteria were pelleted at 13000 x g and 4°C, and prior to resuspension; the thick layer of polysaccharides was carefully removed by gently pipetting the liquid onto the pellet. This way, the exopolysaccharide layer lifted off slightly and could be suctioned off before resuspension of the pellet in 0.5 % EDTA.
Electroporation was carried out in a BioRad GenePulser at 1.25 kV and 25 µFd/400Ω using BioRad 1 mm electroporation cuvettes.
The cells were then transformed using the vector pUCP20 which was designed for cloning and expression in *P.aeruginosa*[115] and confers ampicillin (*E.coli*) / carbenicillin (*P.aeruginosa*) resistance. Transformation efficiency was monitored by counting the carbenicillin resistant colonies on LB agar containing 200 µg/ml carbenicillin after incubation for 18 hours at 37°C.

AMOUNT OF DNA	COLONY FORMING UNITS
10 ng	88
50 ng	259
100 ng	186
500 ng	77
1 µg	0-2

Table 7: electroporation efficiencies

Other vectors yielded similar results so that this protocol was used for the electrotransformation of *P.aeruginosa* with 10-100 ng of vector DNA.

[115] Schweizer,HP, personal communication 2003
[117] Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis; J. Mol.Biol. Vol.98, 1975, 503-517

2.14. DNA Analysis by Southern Hybridization

Southern Hybridization[117] is a technique that allows for the conservation of DNA from an agarose gel by its transfer onto a nylon membrane and subsequent UV fixation.

2.14.1.DNA -Transfer onto Nylon Membranes

The gel containing the DNA fragments is treated with 0.4 M sodium hydroxide for alkaline denaturation of the DNA which is then transferred onto the nylon membrane using a classical Southern transfer pyramid[118]: The gel is placed upside down onto filter paper covering the blot table and reaching into both buffer reservoirs of the capillary blot apparatus. The nylon membrane is smoothed onto the gel to avoid the formation of air bubbles which interfere with the DNA transfer, and overlaid with several layers of filter paper and kitchen towels and a weight of about 500 g was placed on top of the pyramid. The transfer was conducted over 16-24 hours; afterwards, the membrane was washed and neutralized twice in wash buffer, air dried and the DNA was then covalently cross linked with the membrane surface for 45 seconds (UV-Crosslinker, Stratagene).

2.14.2. Generation of DIG-Labeled Hybridization Probes

DNA fragments that have been fixed onto a nylon membrane can be detected either with radioactive hybridization employing radioactively labeled probes or by non-radioactively labeled probes fused to a chemiluminescent marker.
Non-radioactive DNA probes are easily obtained employing the same “Random Primed Labeling”-reaction that was first established by Feinberg and Vogelstein[119] for the radiolabeling of DNA. Heat denaturation of the DNA is followed by incubation with Klenow-Polymerase that synthesizes the complementary strands. This reaction is primed by hexanucleotides with statistical base sequences obtained from DNAseI-digested calf thymus DNA. These hexanucleotides bind to the template DNA approximately every 100 base pairs and the complimentary strand is synthesized with a mixture of nucleotides in which 1/3 of the dTTP was replaced by DIG-dUTP. This marker molecule contains Digoxygenin and dUTP separated by a 2-Propenamide-Lys-ε-2-oxamide spacer that is coupled to the steroid moiety of the Digoxygenin via an ester bond to C(7) and via a *trans* double bond to dUTP. The spacer ensures that the Klenow-Polymerase recognizes the modified DIG-dUTP as its substrate and incorporates the marker molecule approximately every 20-25 base pairs. This way, DIG-labelled probes for both strands can be obtained.

2.14.3. Random-Primed Labeling

The labeling reactions were carried out using the DNA labeling kit from Roche
(Cat# 1277065) according to manufacturer’s specificities:
15µl of DNA were heat-denatured at 100°C for 10 minutes, placed on ice and centrifuged briefly. 2µl of 10x hexanucleotide solution, 2µl 10xDIG-DNA-labeling mix and 5U Klenow-Polymerase were added and the reaction mix was incubated overnight at 37°C. Excess hexanucleotides were separated from the labelled DNA by Sephadex G50 column chromatography, and the DNA was eluted with TE buffer by centrifugation and stored at -20°C.

2.14.4. DNA Fixation and Hybridization of specific DNA probes to a Southern blot

Short single-stranded DNA molecules that have been labelled can be hybridized to and form double strands with blotted DNA.
The membrane was rolled up with the DNA-covered side pointing inwards and placed into a capped glass tube. The tube was filled with 20 ml of pre-hybridization buffer and incubated in a rotation oven at 68°C for at least 2 hours. The labeled hybridization probe was heat-denaturated, placed on ice and mixed with 5 ml of pre-hybridization buffer so that the content of probe DNA was approximately 0.5-50 ng. The pre-hybridization buffer was decanted, the probe solution filled into the glass tube and the membrane incubated for 24 hours at 68°C in a rotation oven.

2.14.5. Washing and Labeling of the Southern Blot

Detection of DNA fragments on a Southern blot was preceded by decanting the probe solution, washing, and incubation with blocking reagent to prevent non-specific hybridization.

[118] Ausubel F et al: Current Protocols in Molecular Biology, 1987-1996
[119] Feinberg AP, Vogelstein B: Technique for Radiolabelling DNA Restriction Endonuclease Fragments to high Specificity; Anal.Biochem. 132, 1983, 6-13

The membrane was rinsed in 20 ml of washing solution at room temperature and then incubated twice with 25-30 ml of washing solution at 68°C in a rotation oven for 45 minutes to wash off non-specifically bound hybridization probe. Afterwards, the pre-treated membrane was equilibrated in buffer I for 5 minutes, the buffer decanted, and buffer II added to the membrane. Incubation for 30 minutes prevented unspecific labeling as this buffer contains blocking reagent. 30 ml of antibody solution was added to the membrane for an incubation time of 30 minutes. The membrane was then rinsed with 200 ml of buffer I three times for 15 minutes and afterwards incubated with 200 ml of freshly filtered buffer III for two minutes. This buffer contains Mg^{2+} which is needed as a cofactor for the alkaline phosphatase that is employed in the following labeling reaction.

2.14.6. Labeling Reaction

Labeling of the membrane for the immunological detection was carried out in 40 ml of anti-digoxygenin-alkaline phosphatase conjugate diluted 1:5000 in buffer II for 30 minutes. Afterwards, the membrane was washed three times in buffer I and equilibrated in 250 ml of freshly filtered buffer III for 2 minutes and then incubated with 10 ml of CDP-Star™ (Tropix, CAS#:160081-62-9) diluted 1:1000 in buffer III.

2.14.7. Immunological Detection of Digoxygenin-Labeled DNA

The detection of DNA blotted onto a nylon membrane depends on signal amplification by chemoluminescence reactions. An antibody that specifically recognizes digoxygenin was coupled to alkaline phosphatase which is able to cleave CDP-Star™ into 3-methoxy-4-chlorophenolate and an adamantone derivative under light emission of 480 nm. The maximal chemoluminescence is reached after 30-45 minutes of incubation time at 37°C after the beginning of the reaction, and the light emission signal can be captured using an X-ray film (Kodak X-OMAT AR).

2.14.7. Washing and Stripping of Hybridized Membranes

Southern blots can be washed and stripped of the hybridization probes to be reused. This technique relies on the denaturation of DNA double strand bonds between complementary strands of blotted DNA and hybridization probe by a 3M sodium hydroxide solution. However, the covalent bonds between blotted DNA and nylon membrane are left intact and the hybridization probe can be washed off the blot. This procedure ensures that the entire blotted DNA is accessible for other hybridization probes.
The membrane was washed twice with 3M NaOH for 10 minutes, neutralized in wash buffer and afterwards equilibrated in TE buffer to prevent degradation of the DNA by DNAses. The moist membrane was then wrapped in cling film and stored at -20°C.

A different and gentler method is the regeneration of the Southern blot membrane by removal of the alkaline-sensitive DIG-labeling; however, DNA fragments that have been hybridized to DNA probes will not be accessible for further hybridization.
The membrane was washed twice for 30 minutes with 0.2 N NaOH solution containing 0.1% (w/v) SDS, neutralized, equilibrated in TE, wrapped in cling film and stored at -20°C.

3. Results and Discussion

The aim of this study was to discover mechanisms and determinants of virulence in *P.aeruginosa* by functional genome analysis; furthermore, the focus of this work was on development and integration of new tools to verify the virulence determinants elucidated during the course of the study.
As a prototype of a commonly occurring *P.aeruginosa* clone, an environmental isolate was chosen for analysis that proved to be more virulent than initially expected.
Strain SG17M was isolated from a riverine ecosystem in the Ruhr area, Germany and belongs to clone C, which represents the predominant clone found in CF patients as well as in aquatic habitats all over Europe. Strain SG17M is highly mucoid and produces a significant amount of elastase, proteases and siderophores and is fully capable of quorum sensing (QS). Furthermore, it shows an invasive phenotype if incubated with Chang epithelial cells, but does not survive within polymorphonuclear neutrophils[120]. The isolate exhibits significant toxicity towards the nematode *C.elegans* but shows limited cytotoxicity towards macrophages.
Two other strains used for this study were the clinical isolates CHA and TB, which represent two of the most virulent *P.aeruginosa* isolates known to date, their respective phenotypes have been extensively analyzed and the results reported[121].
In parallel to the studies for this thesis, the author was involved in a coordinated collaborative project with other European participants. The author was entrusted with the outline of tasks and coordination of the tasks to be executed in Hannover and the communication between the partners. Furthermore, the author contributed experimental results and took part in the evaluation of data generated for the project. The highlights of this project are presented in the first chapter of this thesis; however, the expertise and contribution for this chapter was recruited from the whole research group as well as the collaborators.

3.1. Genotypic and phenotypic comparison of two highly virulent P.aeruginosa isolates

In order to shed light onto the molecular mechanisms underlying their significant pathogenicity, an extensive phenotypic and genotypic comparison of two unrelated *Pseudomonas aeruginosa* clinical CF isolates has been carried out by the Hannover/Grenoble team employing comparative sequencing (A.S.Limpert, A.Meyer), SNP chip genotyping (B.Siebert, L.Wiehlmann), electron microscopy (G.Brandes), *in vivo* and *in vitro* pathogenicity assays (A.S.Limpert, A.Strüßmann) and Affymetrix GeneChip transcriptome analysis (P.Salunkhe) as well as a comprehensive survey of the TTSS-dependent secretome of both strains and the assessment of pack-swarming (E.Faudry, I.Attree).

Pseudomonas aeruginosa is an opportunistic gram-negative pathogen associated with infections of immunocompromised or critically ill patients. Furthermore, infection with *P.aeruginosa* is the major cause of morbidity and mortality in patients suffering from Cystic Fibrosis (CF). The pluripotency of its virulence determinants together with its metabolic versatility allow this bacterium to survive and persist in virtually every habitat and enable it to establish chronic infections which are extremely difficult if not impossible to eradicate. Some of the virulence factors of *P.aeruginosa* that contribute to pathogenesis have been intensively examined and well-characterized, among them the Type III Secretion System (TTSS) which upon contact with the host cell actively secretes effectors into the eukaryotic cell, interfering with multiple steps of its signal transduction[122]. In addition to actively exerted virulence, *P.aeruginosa* also employs passive means of evading the host immune response such as conversion to a mucoid phenotype, loss of surface antigenicity and the formation of biofilms with high intrinsic resistance against antibiotics. It has been reported that clinical isolates of *P.aeruginosa* obtained from CF patients were able to circumvent the bactericidal effects of PMN which constitute the first line of defense against microbial pathogens. Two clinical isolates have been tested in co-incubation with PMN and compared to the genetic reference strain *P.aeruginosa* PAOI: while PAOI was effectively eliminated by the PMN after the first hour of co-incubation, the *cfu* count of both clinical isolates assessed on culture plates after plating of serial dilutions remained stable after the first hour and

[120] Wiehlmann L: Personal communication, 2001
[121] Miethke N: Das Exopolysaccharid von Pseudomonas aeruginosa: Zusammensetzung und Bedeutung für die Pathophysiologie des Lungenbefalls bei Mukoviszidose. Dissertation, Medizinische Hochschule Hannover, 1985
Tümmler B: Unusual mechanism of pathogenicity of Pseudomonas aeruginosa isolates from patients with cystic fibrosis. Infection 15, 1987, 311
[122] see ref. 38, 42, 23, 44, 45

increased continually with the time of co-incubation[123]. Both clinical isolates originate from entirely different regions in Europe. Strain CHA was isolated from a patient in the University Hospital, CHU Grenoble, France, while strain TBCF 10839 (TB) was obtained from a patient in the CF care unit of the Medical School in Hannover, Germany. Both strains share the phenotype of extreme virulence, difficult eradication from the lung of the CF patient and TTSS-mediated cytotoxicity towards macrophages.

3.1.1. Description of the respective phenotypes:

Strain CHA and PMN from healthy donors were co-incubated for up to three hours with samples taken and plated onto *Pseudomonas* isolation agar each hour[2]. After incubation for 24 hours, the plates were examined for *cfu* count, showing that CHA was not killed by the PMN but had evaded their response to the bacterial threat. Further analyses directed at unravelling the mechanism by which strain CHA was able to do so revealed that it might be a concerted action of alginate protection against the toxic mediators released by the PMN as well as actively exerted TTSS–mediated cytotoxicity resulting in the killing of the eukaryotic cells rather than their eliminating the bacteria. It has been shown that the cytotoxicity of CHA is ExoU–independent and requires the TTSS–secreted proteins PopB, PopD, and PcrV[124]. A rather striking phenotypical characteristic is the ability of strain CHA to react to chemoattractants leaking out of damaged phagocytes, a phenomenon termed “pack swarming”, which describes an unusual accumulation of a vast number of bacteria around a dying phagocyte and has as yet never been observed with *P. aeruginosa*. A combination of the described phenotypic traits might play a role in conferring the extreme virulence to strain CHA, therefore, strain TB was also examined for pack-swarming.
Strain TBCF 10839 (TB) was first isolated in 1983 from a CF patient suffering from severe acute hypoxemia. It was unusually virulent and persisted in the lung of the patient even under stringent antibiotic therapy. Finally, it was eradicated from the patient with a combined antibiotic therapy followed by replacement with a different *P.aeruginosa* clone. Further analysis of the isolate revealed an unusual phenotype for *P.aeruginosa*: Upon incubation with PMN, the bacteria were phagocyted but persisted in the PMN[125]. Furthermore, the bacteria replicated within the phagolysosome, escaped into the cytosol and effectively killed the PMN after an incubation period of 2 hours. This phenotype has never before been reported for *P.aeruginosa*, although electron micrographs of an unspecified strain in context with the immune status of CF patients infected with *P.aeruginosa* clearly show bacteria that survive, persist and replicate intracellularly in PMN of healthy donors after an incubation time of 6 hours[126]. By this time, the PMN examined in that study showed a rounded shape but the phagolysosomal membranes were still intact and the bacteria mainly confined to that particular compartment although a few extracellular as well as cytosolic bacteria could be seen. In the study at hand, the PMN were dead in the case of CHA after co-incubation with the bacteria for 120 minutes, the same is true for PMN incubated with strain TB. However, the mechanism of eukaryotic cell death seems to be different for both isolates.

3.1.2. Establishing the genotypic relationships between CHA and TB by SpeI restriction mapping and SNP chip analysis

CHA and a panel of 71 *P.aeruginosa* strains were tested and evaluated employing a SNP-Chip to detect genotypic relationships. At the same time, conventional macrorestriction analysis utilizing PFGE was performed. The results revealed that two other strains out of the selected panel shared the CHA genotype. Strains ATCC 14886, a North American soil isolate, and PT22, an environmental isolate from Mülheim/Ruhr, Germany, proved to be distant genotypic variants of CHA.
Strains TB and CHA have completely different *SpeI* genotypes indicating that they belong to unrelated clones, moreover, the two strains are unrelated in their SNP profile and their repertoire of genome islands and genome islets representing the accessory genome.

[123] Dacheux D, Attree I, Schneider C, Touissant B: Cell Death of Human Polymorphonuclear Neutrophils Induced by a Pseudomonas aeruginosa Cystic Fibrosis Isolate Requires a Functional Type III Secretion System. Infect Immun 67 (11), 1999,6164-6167
[124] Gouré J, Pastor A, Faudry E, Chabert J, Dessen A, Attree I: The V antigen of Pseudomonas aeruginosa is required for assembly of the functional PopB/D translocation pore in host cell membranes. Infect Immun, 2004, in press.
[125] Tümmler B: Unusual mechanism of pathogenicity of Pseudomonas aeruginosa isolates from patients with cystic fibrosis. Infection 15, 1987, 311,
[126] Doggett RG and Harrison GM: Pseudomonas aeruginosa: Immune Status in Patients with Cystic Fibrosis. Infect Immun 6 (4), 1972, 628-635

3.1.3. Comparative sequencing of selected genes of CHA and PAOI

The genetic reference and laboratory strain *P.aeruginosa* PAOI (DSM 1707) is relatively non-pathogenic, non-mucoid and lacks several other phenotypic traits that are commonly linked to pathogenicity. Loci that have been found to be pathogenicity-related by an STM screen of mutants generated from strain TB have already been compared to the PAOI sequence. Among the loci that are present in TB as well as PAOI, only a few differences have been found such as minor amino acid exchanges. However, strain TB possesses several genes that are not present in the PAOI genome, termed "non-PAOI" genes, which could also be identified in other clinical isolates. These genes are TB 47D7, TB25A12, TB45A12 and TBD8A6, in accordance with the numbers of the transposon mutants.

As these genes were found in the STM screen for intracellular survival, they are supposedly important for pathogenicity. Therefore, a PCR approach was applied to find out whether these genes are present in the highly virulent isolate CHA as well. Surprisingly, none of the hitherto identified non-PAOI genes were detected in CHA.

In addition, several genes from the PAOI genome that have either previously been associated with virulence or suspected to play a role in pathogenicity were tested for their presence in the CHA genome by PCR. The PCR products were then dispatched for sequencing to detect possible variations in the genes under examination.

The results are summarized in table 9:

PA NUMBER (NAME) OF SEQUENCED GENE	GENE RANGE	SEQUENCE DATA RANGE	PAO / CHA NT EXCHANGES	PAO / CHA AA EXCHANGES
PA 0852 (Chitin binding protein precursor *CbpD*)	931822-930653	930585-932047	none	none
PA 1441 (hypothetical protein)	1570496–1571779	1570385–1571859	4 nt exchanges Pos. 1571232 C–>T Pos. 1571234 C–>G Pos. 1571240 G–>A Pos. 1571243 C–>T	One AA exchange at pos. 448 of coding sequence: I–>M, no structural significance.
PA 1572 (conserved hypothetical protein)	1712908–1714053	1712819–1714260	13 nt exchanges Pos. 1712955 G–>T Pos. 1712985 T–>C Pos. 1713046 A–>G Pos. 1713052 G–>C Pos. 1713117 T–>G Pos. 1713213 T–>C Pos. 1713214 T–>C Pos. 1713426 G–>A Pos. 1713693 C–>T Pos. 1713915 C–>G Pos. 1713967 A–>G	3 AA exchanges within coding sequence : Pos. 47 I –> V Pos. 49 V –> L Pos. 354 T–> A, no significance for protein structure or function could be attributed to the AA exchanges.

PA 1572 cont.			Pos. 1714063 G–>A Pos.1714192 G–>T	
PA 2300 (Chitinase)		2530301–2531902 and 2531154–2531083, sequence gap of 62 bp	5 nt exchanges: Pos. 2530968 A–>G Pos. 2531206 T–>C Pos. 2531207 G–>T Pos. 2531717 C–>T Pos. 2532071 G–>T	1 AA exchange at pos. 42 D–>N, two nominal exchanges resulting from sequencing errors : pos. 212 Q–>R and pos. 220 Q–>R
PA 2591 (virulence and quorum sensing regulator *vqs*R)	2934388–2933528	2934636–2933192	2 nt exchanges Pos. 2933570 T–>C Pos. 2933340 A–>G	1 AA exchange at pos.1 of coding sequence M –> V corresponding to nt exchange at pos. 2933340
PA 3724 (*lasB*, elastase)		4168865–4169765 and 4170795–4170038, sequence gap of 318 bp	4 nt exchanges Pos. 4168970 G–>A Pos. 4169362 A–>G Pos. 4169374 A–>T Pos. 4169511 T–>C, 3 sequencing errors.	A significant stretch of AA is nominally exchanged in correspondence with 3 sequencing errors at nt pos. 4169684, 4169722 and 4169730. No AA difference resulting from nt exchanges
PA 3841 (*exoS,* Exoenzyme S)		4303360–4303921 and 4304966 and 4304405 sequence gap of 105 bp	1 nt exchange at pos. 4303904 G–>A	One AA exchange in correspondence with the nt exchange. P –> L at pos. 80 within the coding sequence. No significance for protein structure or function could be attributed.
PA 5349 (probable Rubredoxin reductase)	6018778–6017624	6018816–6017559	none	none

Table 9: Results of comparative sequencing of CHA and TB.

As in strain TB, there are no major differences between CHA and PAOI if the nominal amino acid exchanges due to sequencing errors in gene LasB are left out of consideration, except for gene PA 1572. Here, an exchange of 13 nucleotides leads to 3 amino acid exchanges; an impact on protein structure or hydrophobicity could nevertheless not be observed. Sequencing of large genes with their promoter region as has been attempted here is difficult due to technical reasons. Often, gaps of several hundred nucleotides remain as the average sequencing reaction only allows for the sequencing of about 700 bp per read. More accurate sequencing has to be carried out with a second primer pair that is located inside the gene to be analyzed. This could be of interest for the gene *lasB* as this coding sequence shows the most differences. Furthermore, it is a proven virulence factor, and to confirm that the several amino acid exchanges found in this comparison are just due to sequencing errors, the sequencing of this gene should be repeated with two primer pairs.

3.1.4. Electron microscopy monitoring intracellular survival of CHA and TB in fresh human PMN

The isolates CHA and TB share several phenotypic characteristics: Both of them are extremely mucoid and have been isolated from an acute infection of the respiratory system of CF patients. Both isolates have proven to be particularly difficult to eradicate from the patient's respiratory system due to their intrinsic multiresistency against antibiotics commonly used for treating this kind of infection in CF patients. However, the most striking characteristic of the isolate TB is its ability to survive and persist within PMNs of healthy donors, a phenotype that is normally not seen in this species and has so far been the only reported case of this kind for *P. aeruginosa.*

For the phenotypic comparison undertaken in this study, it is of great interest to examine the isolate CHA for this phenotype as well. Therefore, TB and CHA have been incubated with freshly isolated PMN of healthy, non–CF donors for 15, 30, 60, 90 and 120 minutes and bacteria-host cell interaction was examined by electron microscopy.

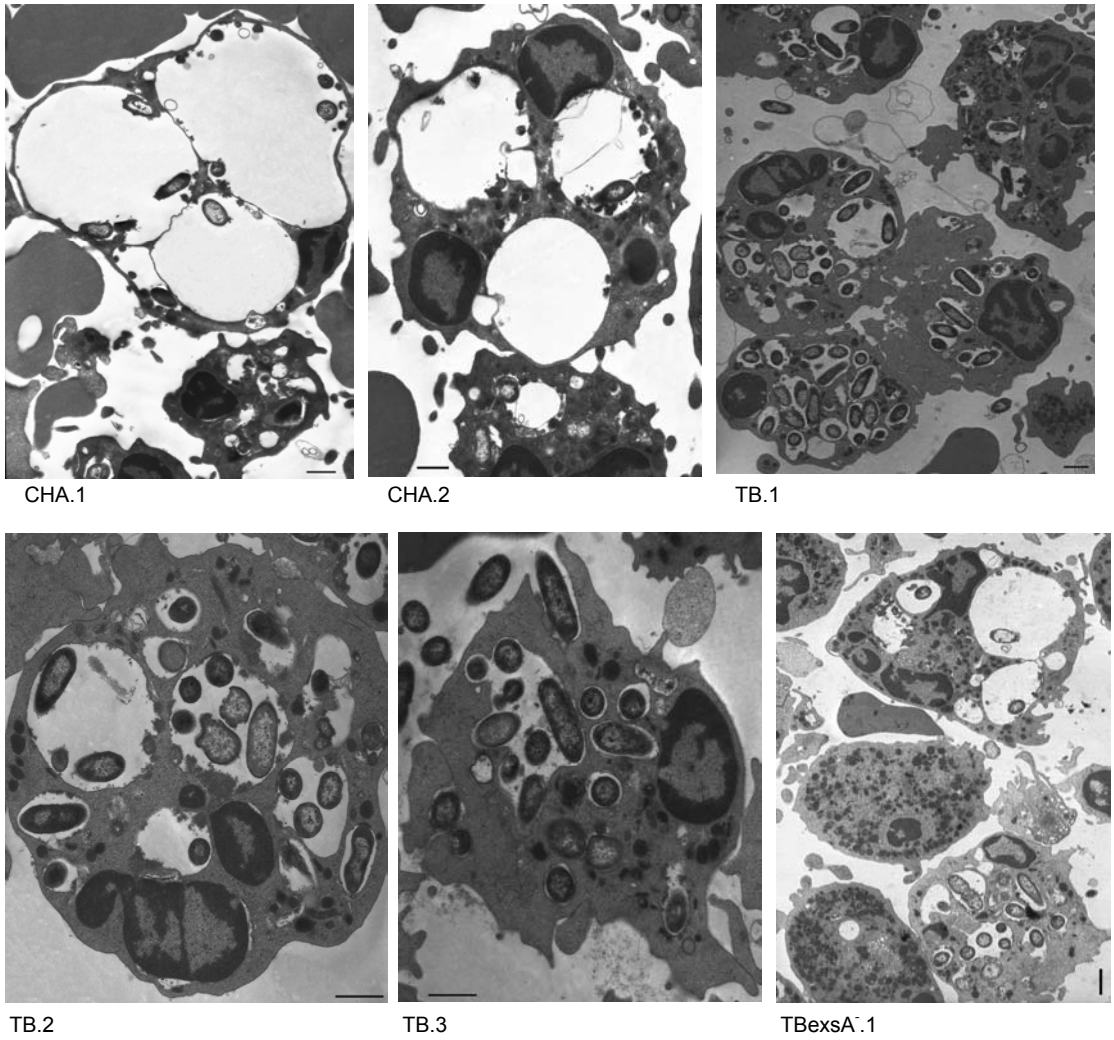

Figure 7: CHA, TB and TBexsA⁻ incubated with PMN for 15 minutes.

After an incubation time of 15 minutes, PMNs incubated with the *P.aeruginosa* isolates already harbour a large number of intracellular bacteria which have been taken up into phagosomes. In the case of the TB strains, the shape of the PMN already shows slight alteration indicating cell damage but still remains rather intact. Membrane blebbing can rarely be detected yet and only few vesicles are shed. PMN that have taken up strain CHA present an entirely different picture. The main distinction in the shape of PMN that have taken up strain TB to those harbouring strain CHA is the formation of unusually large, "giant" vacuoles with enclosed bacteria. The PMN that phagocyted strain TB have enclosed the bacteria in numerous smaller vacuoles. In their overall numbers, the volume within the cell that is made up of phagosomes might be the same for both strains, but the distinct shape

difference of vacuoles with strain TB and CHA is remarkable. Virtually no intact bacteria can be seen in the “giant” vacuoles any more, indicating that the PMN fulfil their function in effectively clearing away the pathogen. High lysosomal activity is represented by small black oval shapes within the cells, but a peculiarity can be detected: In the case of the TB strains, the lysosomal contents do leave the lysosome upon fusion with a phagosomal membrane, but they are not evenly distributed within the vacuole. They stay located around the fusion area at the phagosomal membrane instead and are recognizable as small black balls. This effect is observed less in the case of CHA, several bacterial cells engulfed by lysosomal contents can be seen (picture CHA.1), and furthermore, the rather effective clearance of the pathogen is especially visible in picture CHA.2. These facts indicate that the lysosomal response is functional or -if at all- only slightly impaired in PMN harboring strain CHA.
The electron micrograph of strain TBexsA$^-$ shows the cell division of a bacterium within a phagosome (indicated by the arrow in picture TBexsA$^-$.1). Survival within the phagosome is already a rather unusual trait in *P.aeruginosa*, even more so is the persistence and replication. This indicates that the bacterial cell is intact and thriving, most likely it has either evaded the early onset of oxidative stress and lytic enzymes exerted by the PMN.
After 30 minutes of PMN incubation with bacteria, the cell morphology is beginning to change considerably.

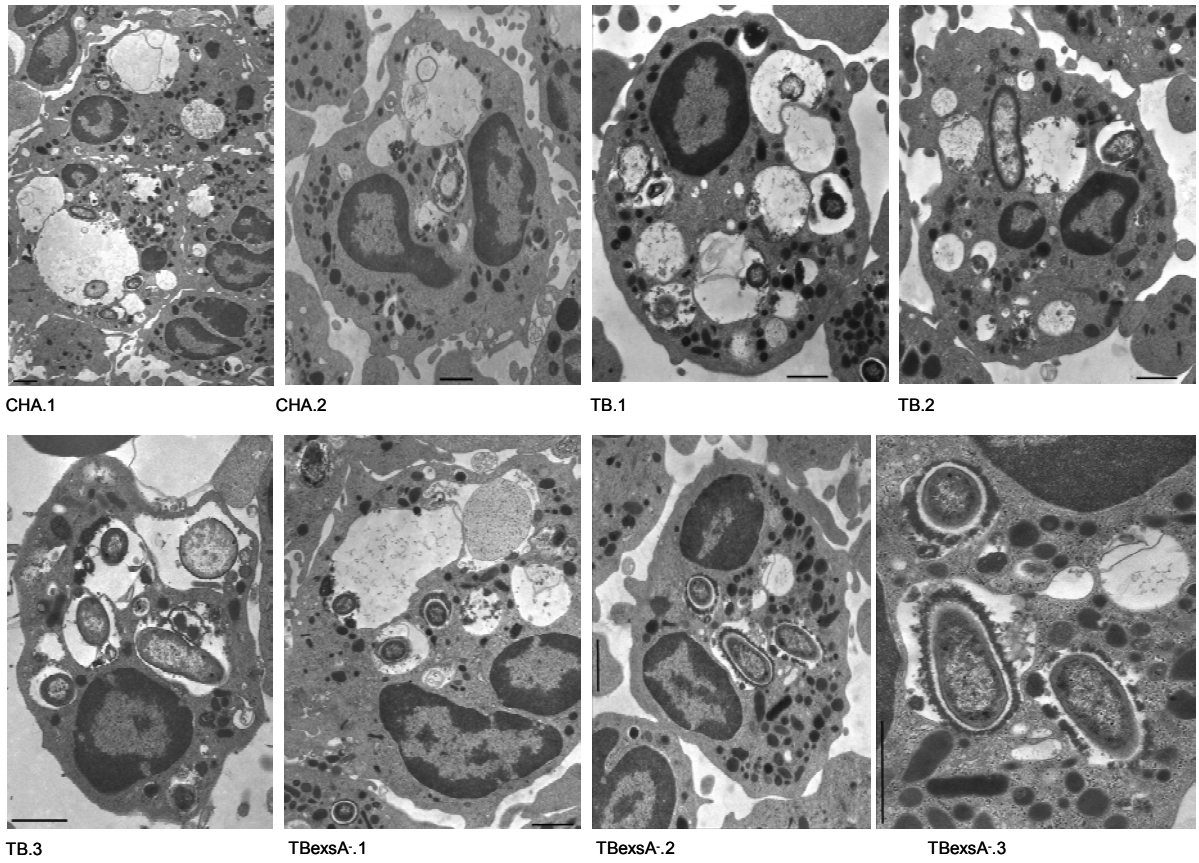

Figure 8: CHA. TB and TBexsA$^-$ incubated with PMN for 30 minutes

After an incubation period of 30 minutes, the vacuoles have started swelling and the cell membrane begins to form blebs, an indication for the loss of membrane integrity and the onset of oncosis. As already seen in pictures taken after an incubation time of 15 minutes, the lytic substances released by lysosomes are not distributed within the phagosome but stay balled-up near the phagosomal membrane, not reaching the bacterial cell wall. This can be best observed with the TB mutant strain (pictures TBexsA$^-$.3 and its close-up, picture TBexsA$^-$.2). A reasonable explanation for this phenomenon might be the strongly mucoid phenotype of the isolate: the bacterial cell is covered by a thick layer of exopolysaccharides which is not contrasted by uranyl acetate staining, visible as white space around the bacterium. This layer could effectively inhibit the bacterial cell lysis by protecting the bacterium from the lytic enzymes like a shield while the lysosomal contents in turn are pressed onto the phagosomal membrane. This could be a major contribution to the virulence of the isolate TB because the destruction of the pathogen is effectively hindered.

The PMN that have been incubated with strain CHA have mainly cleared the pathogen from the phagosomes, remnants of lysed bacteria as well as bacteria engulfed by lysosomal contents and undergoing lysis can be detected within the vacuoles. The natural cell shape has changed to a more rounded morphology.

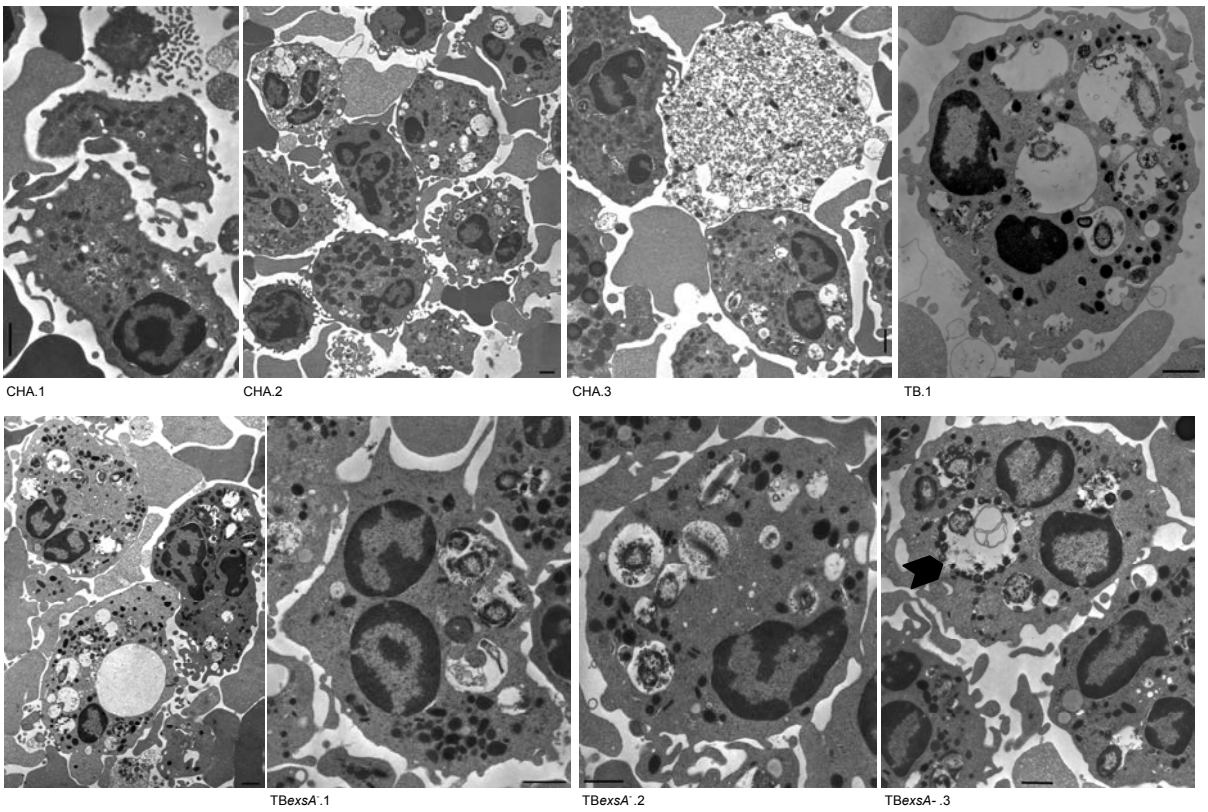

Figure 9: CHA, TB and TBexsA$^-$ incubated with PMN for 60 minutes

After an incubation time of 60 minutes, all PMN are rounded and those infected with CHA show severe deformation ranging from strong membrane damage accompanied by vesicle shedding (pictures CHA.1 and 2) to the complete destruction of the cell (picture CHA.3). No intact bacterial cells can be found within the phagosomes anymore, only their remnants. This fact points to complete lysis and clearance of the pathogen by this time with the PMN undergoing oncotic cell death. The PMN that have taken up strain TB look less damaged than the ones which were infected with CHA. But so do the bacterial cells- numerous still more or less intact bacterial cells can be observed within phagosomes. In general, many bacterial cells of strain TB still look rather viable and only slightly affected by the hostile environment present in PMN, suggesting that this particular strain has a mechanism that renders it immune to the oxidative stress and lytic enzymes that PMN use as weapons against microbial pathogens. The cells that harbour the exsA$^-$ mutant of strain TB also exhibit an only slightly changed morphology. The phagosomes contain bacteria engulfed by lytic substances; however, the signs of progressive bacterial cell lysis that should be observed at this time point can not be detected in most cases.

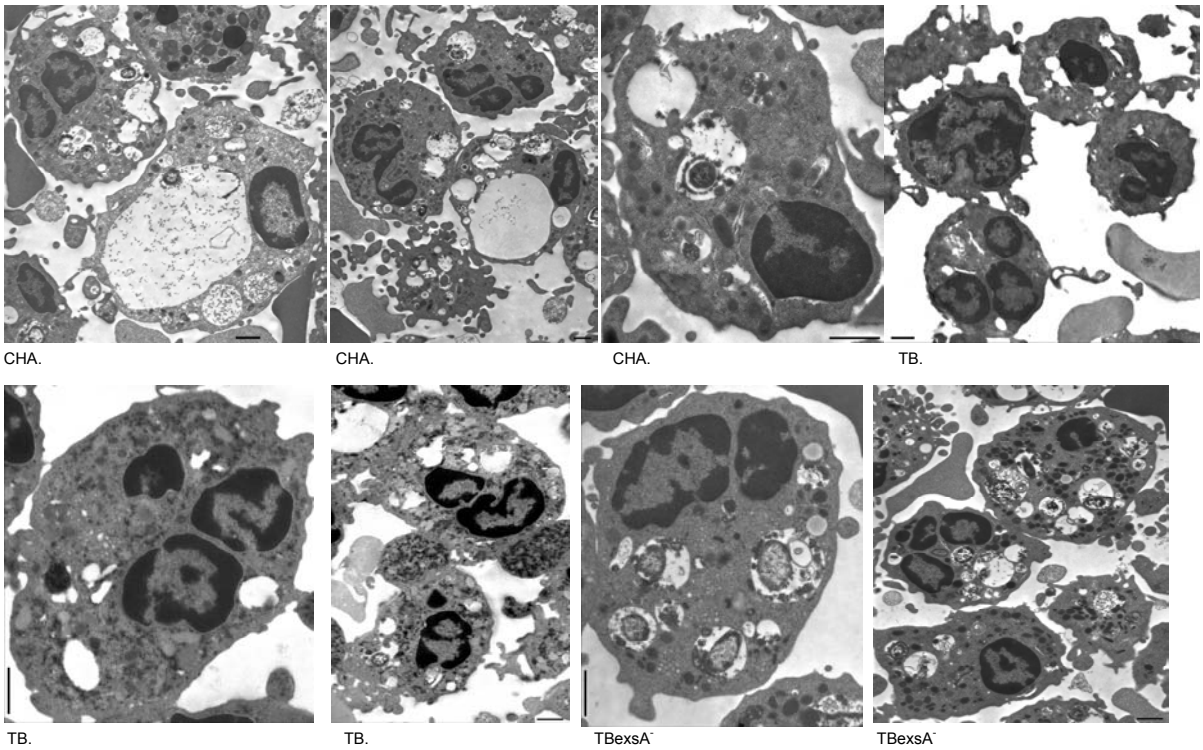

Figure 10: PMN incubated with CHA, TB and TBexsA⁻ for 90 minutes

After 90 minutes of incubation time, the differences between the interactions of both strains with the PMN are most evident. PMNs that have taken up CHA have lysed the bacteria rather efficiently and are undergoing oncosis themselves, this can be seen as a progression of the events that have already set on after 15 minutes of incubation time. The shape of the eukaryotic cell in picture CHA.1 is typical for PMN incubated with strain CHA; giant phagosomes contain residues of bacterial cells, the cell membrane structure is highly compromised and the PMN are severely rounded up and about to burst. Remnants of burst PMN are everywhere and intact bacteria can rarely be detected anymore. PMN incubated with the strain TB show also a complete change in cell morphology after 90 minutes of incubation time. The cells have rounded, but unlike necrotic cells that swell; these cells have shrunk, some of them to nearly half of the size compared to PMN that have taken up CHA. The cytoplasm is highly condensed, visible by a darker color of the cells as well as a highly granular structure which cannot be observed in earlier pictures.

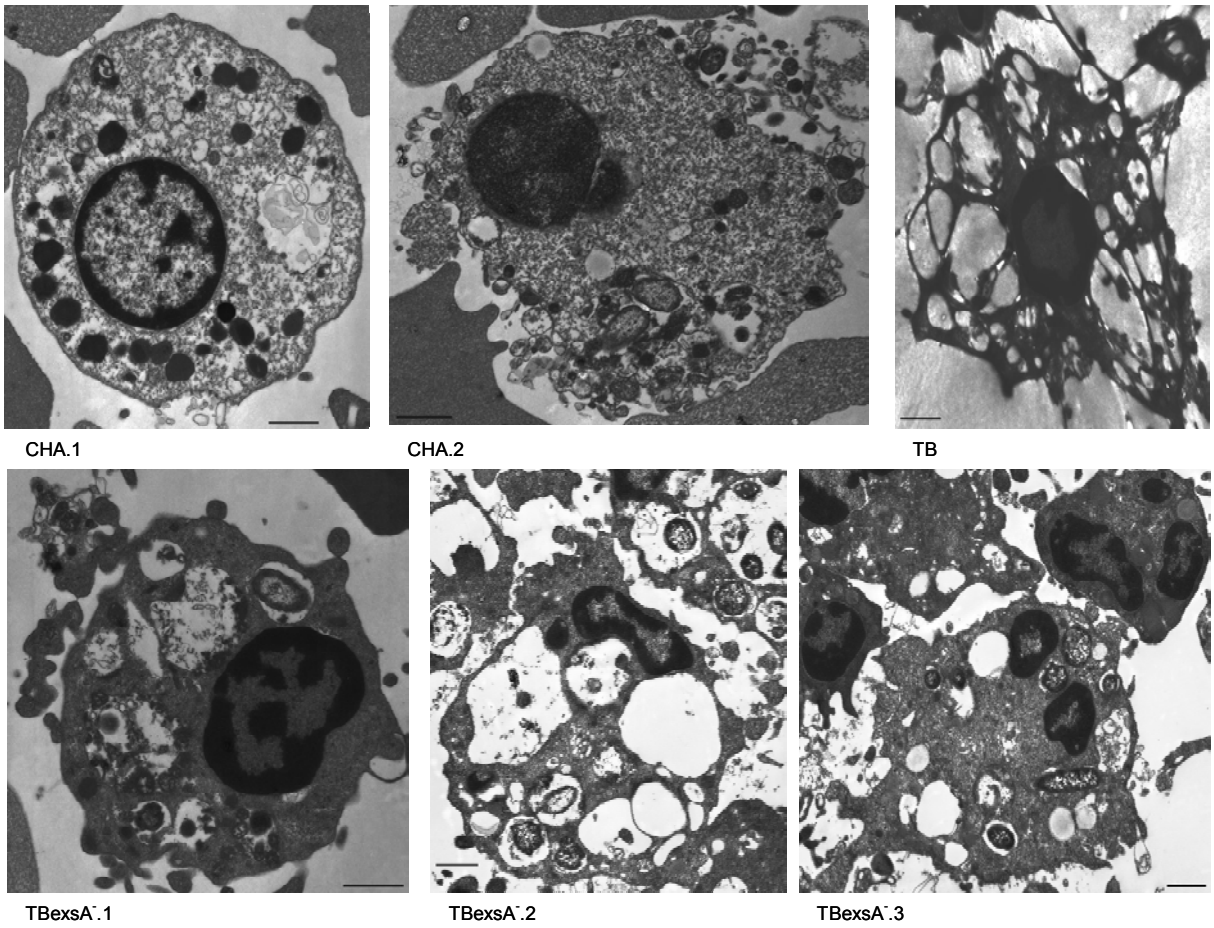

Figure 11: PMN incubated with strains CHA, TB and TBexsA⁻ for 120 minutes

After 120 minutes of incubation time, nearly all cells are destroyed. PMNs that took up TB have shrunk drastically and their cytoplasm is completely condensed and clotted. Picture TB shows a completely disintegrated cell, no intracellular bacteria can be seen any more. An interesting detail is that uptake of CHA does not lead to a condensation of the PMN cytoplasm. Strain CHA is definitely less capable of surviving in PMN, if at all, compared to strain TB. This strain is mostly unaffected by the PMNs and not only does it survive in PMNs but as seen in pictures taken at earlier time points, TB as well as its exsA⁻ mutant is also capable of replicating in this hostile environment which should be a major factor to contribute to this isolate's virulence.
When strains CHA and TB are compared, the PMN that have taken up the bacteria die in both cases, but the way of dying is significantly different: While CHA mediates oncosis with cellular and nuclear swelling followed by the „explosion" of the cell, uptake of the isolate TB causes the cell to shrink, the vacuolar membranes and the cell membranes disintegrate, the cytoplasm condenses and the cell „implodes".
The study at hand aimed at the elucidation of the role the TTSS plays in the interactions between human PMN and two clinical CF isolates of *P.aeruginosa* and one TTSS-deficient mutant. This mutant carries a mutation in the exsA locus which encodes the main transcriptional activator of all genes necessary for the TTSS machinery. Several effectors are secreted via the TTSS pathway, and their expression is globally abrogated by a knock-out of exsA, but as there has been no difference observed in intracellular survival of the TBexsA⁻ mutant in comparison of the parental strain, it can be suggested that intracellular survival of strain TB is TTSS-independent. The damage that is inflicted upon the eukaryotic cell however is clearly a result of TTSS activity as the PMN that have taken up the TBexsA⁻ strain show significantly less damage than those that took up either CHA or TB.
The main task of PMN is to take up pathogens by phagocytosis, engulf them in a phagosome which upon fusion with lysosomes becomes a phagolysosome, characterized by a low pH imperative for the function of lytic enzymes and a massive accumulation of reactive oxygen species (ROS). For a bacterium to persist in this highly hostile environment, the first step towards survival could be to upregulate genes that are essential for detoxifying ROS and to protect itself from the damage. Transcriptome data gained on strain CHA when grown in the presence of H_2O_2 do indeed point at

such a response of this strain: If strain CHA is confronted with oxidative stress, it downregulates the core metabolism and upregulates oxidative stress-response genes as a defence strategy, such as superoxide dismutase (SodM), or the organic hydroperoxidase resistance gene (Ohr). In addition to defence by detoxifying reactive oxygen compounds generated by the host, strain CHA "powers up the weapons": Also upregulated if CHA is grown in the presence of H_2O_2 are the genes of the TTSS machinery as well as stress-related transcriptional activators such as PhoP and Sigma 70. The drawback of this strategy is obvious: it is metabolically expensive for the bacterium to try and defend itself in this manner; it has to downregulate its central metabolism which decreases and compromises its fitness so that in the end, the bacterium does kill the phagocyte but is not able to survive itself either. The evidence obtained by electron microscopy points to a battle between host and pathogen that lasts for about 60 minutes and ends with the deaths of both the bacterium and the PMN. Strain TB however employs another strategy in order to survive. Once this isolate is grown in the presence of H_2O_2, the transcriptome data reveal a completely different picture: Upregulated are genes of stress adaptation like cold shock and starvation proteins, furthermore the DNR transcription regulator, genes of nucleotide biosynthesis and RNA processing. Also upregulated are genes essential for protein biosynthesis such as ribosomal proteins, elongation factors, aminoacyl-tRNA-synthetases, moreover genes belonging to post-translational processing pathways (LepA, SecA, SecB). Interestingly, also among the significantly upregulated genes are those of central metabolic pathways: fatty acid biosynthesis, respiratory chain, and ATP synthases. Flagella biogenesis is upregulated as well as are genes essential for cell division. All this evidence leads to the conclusion that strain TB rather adapts to the stress exerted by the reactive oxygen compound as well as strongly activates its core metabolism. In a concerted action, that strategy should help the bacterium to thrive, grow and divide and overwhelm the PMN by the sheer number of the bacteria. Transcriptome data of both strains grown in rich LB medium to late exponential phase had also been generated to assess the general fitness of the strains: Strain TB strongly expressed genes of the intermediary metabolism as well as genes related to pilin and flagella biosynthesis and quorum sensing regulated genes. The interpretation of these data would be that strain TB is intent on persisting and thriving, not primarily on causing damage in this favorable environment whereas strain CHA is primed on attack even here: Growth in LB medium triggers upregulation of carbohydrate (alginate) metabolism and the TTSS machinery, furthermore, protein biosynthesis, transporters and pumps as well as various transcriptional regulators are also strongly expressed. The considerable virulence of isolate CHA seen *in vitro* and *in vivo* correlates well with the transcriptome data, so does that of strain TB. In the nematode model of virulence as well as in the MTT cytotoxicity tests, strains CHA and TB proved to be of similar virulence. Although an assessment of virulence is always dependent on the model system employed, the evidence gathered during the course of this study may allow the interpretation that both strains are extremely virulent, but strain TB in addition to its virulence is also more persistent. This might lead to longer-term effects in the interaction with the mammalian host that are seemingly in the favor of strain TB: survival in PMN could be this isolate's means of effectively dodging the host immune response and to establish a persistent infection.

3.1.5. Cytotoxicity of CHA and PMN towards human THP-1 macrophages

The cytotoxicity of strain CHA towards human PMN and the mouse macrophage cell line J774 has been measured and calculated according to LDH release as described previously in the Grenoble laboratory. Strain TB was evaluated the same way along with its exsA$^-$ mutant and a CHA exsA$^-$ mutant. Figure 4 illustrates the results:

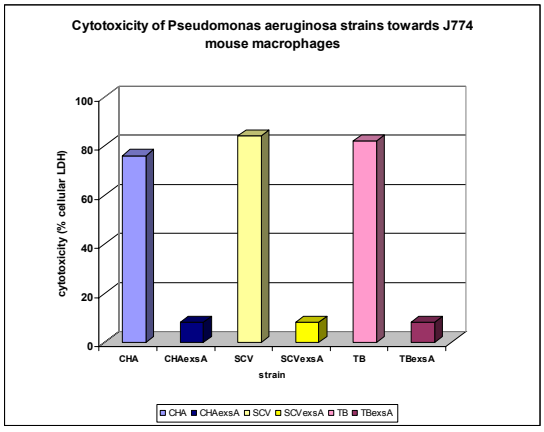

Figure 12: Cytotoxicity of CHA, TB, respective exsA$^-$ mutants.

As can be seen, a mutation in the exsA gene that renders it unfunctional resulted in a complete loss of cytotoxicity. LDH is a cytosolic enzyme that reduces lactate to pyruvate, the hydrogen from lactate is transferred to NAD^+ resulting in $NADH+H^+$. The redox equivalent reacts with the diaphorase-FAD enzyme complex and transfers hydrogen to the FAD which in turn transfers it to a tetrazolium dye. Hydrogen addition to the tetrazolium system reduces the dye molecule and changes its chromophor, resulting in a change of color and absorption. The dye is non-membrane permeable, so that LDH activity, however indirectly measured by this method, allows for assessing the cytotoxicity of the bacteria by their induced membrane damage of the eukaryotic cell. This method is very sensitive even if low multiplicities of infection are used[127].

Another way of analyzing cytotoxicity is the assessment of mitochondrial damage exerted by the bacteria. The enzyme succinate dehydrogenase, enzyme 7 of the citrate cycle, catalyzes the dehydrogenation of succinate to fumarate (formation of a C–C double bond and loss of 2 hydrogen). FAD is in turn reduced to $FADH_2$ and is able to shuttle these two hydrogens directly to the tetrazolium dye MTT which is then reduced to its formazan. In a damaged mitochondrion, redox equivalents are not as efficiently generated and reduced, thus less subsequent reduction of MTT to the corresponding formazan can occur. This reaction is described as very sensitive[128] and well-suited for measuring the loss of cell viability by cytotoxic agents[129].

As in the LDH assay, the cytotoxicity of strains TB and CHA was detected with the MTT assay as well. This method allows for the detection of bacterial interference with the host cell metabolism as it directly measures the quantity of redox equivalents generated by the citrate cycle. This central metabolic pathway links catabolism with energy generation and is a good indicator for the vitality of a cell. The results obtained from the MTT test are comparable with those gained by LDH measurement; the cytotoxicity of both strains is almost the same, and the exsA mutant showed no cytotoxicity in this assay either.

Figure 13 illustrates the results obtained with the MTT test. The maximum absorption was determined by adding MTT plus assay medium to the cells without any bacteria. Cells treated this way should be optimally viable and produce physiological amounts of redox equivalents. Therefore, this reaction was used as a positive control in order to determine the impact of the different *P.aeruginosa* strains on cell viability.

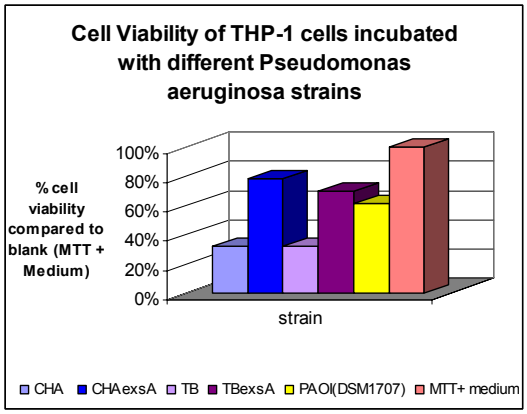

Figure 13 : Strains CHA and TB as well as their respective $exsA^-$ mutants tested for their interference with cell viability of human THP1 monocyte–derived macrophages.

A library of transposon mutants of strain TB had been generated for a previous study[130] and was screened for mutants attenuated in cytotoxicity towards human macrophages employing the MTT test. A total of 8 mutants were found to exhibit either a loss of virulence or a gain of virulence compared to the wildtype. The mutants that showed decreased virulence towards macrophages were either

[127] Attree, Ina: Personal communication, 2003

[128] Denizot F, Lange R: Rapid colorimetric assay for cell growth and survival:modification of the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Meth. 89,1986: 271

[129] Mosmann T: Rapid colorometric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65: 1983, 55-63

-Sieuwerts AM, Klijn JGM, Peters HA, Foekens JA: The MTT tetrazolium salt assay scrutinized: how to use this assay reliably to measure metabolic activity of cell cultures in vitro for the assessment of growth characteristics, IC50 values and cell survival. Eur. J.Clin.Chem. Clin Biochem. 33,1995:813

[130] see ref. 78

affected by transposon insertion in genes related to central metabolism (PA 3366, *amiE*, aliphatic amidase; PA 4829, a dedydrolipoamide dehydrogenase; PA 4401, a glutathione S-transferase) or in the gene Phenazine B, *phnB*, PA 1002. This gene codes for an anthranilate synthase and is important for the synthesis of the phenazine poison pyocyanin which has been recognized as a major virulence factor[131].
Mutants that exhibited decreased virulence towards human macrophages were affected by transposon insertion in genes that were annotated to code for hypothetical proteins (PA 0086, PA 4827) as well as genes ascribed regulatory functions: PA 4982 has been annotated as part of a probable two-component system and PA 5342 as a transcriptional regulator. As the screening for cytotoxicity was carried out as a part of this thesis, the different mutants are described in more detail in chapter 3.4.

3.1.6. TTSS dependent killing of *C.elegans*

Strains CHA and TB were examined for their virulence *in vivo* using a previously established *C. elegans* model[132]. Calcium depletion of the medium induces TTSS activity and its impact on the nematode viability was monitored by the count of dead worms during the course of 6 hours. Figure 14 shows the results of these experiments and corroborates the importance of the TTSS for virulence towards the nematode *C.elegans*.

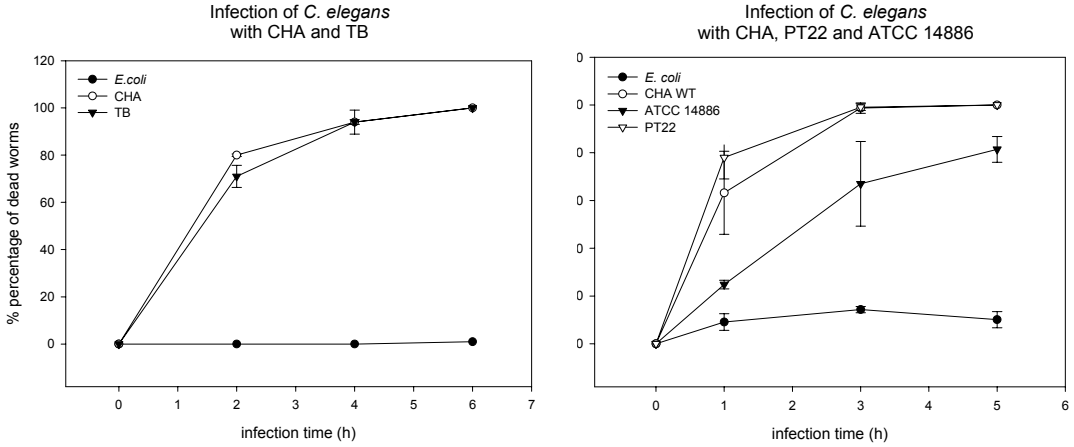

Figure 14: *C.elegans* infected with CHA and TB

The results obtained from this *in vivo* virulence model suggest that both strains are comparably virulent towards the nematode. Furthermore, it shows that virulence is not a clone-dependent trait: Both clonal variants of strain CHA are compared wh CHA in picture b; it can clearly be seen that strain PT22 kills the nematode almost as quickly as strain CHA while ATCC 14886 seems to be not as virulent as its two related clones.

3.1.7. Summary and Conclusion

The study at hand aimed at the genotypic and phenotypic comparison of two highly virulent *P.aeruginosa* isolates originating from CF patients.
Both strains have proven to be genetically unrelated, although their secretome under TTSS inducing and non-inducing conditions is similar, as is their cytotoxicity towards professional phagocytes. One phenotypic dissimilarity between both isolates is the different chemotactical behaviour: Strain CHA pack swarms as a chemotactical response to contents leaking out of damaged macrophages, strain TB does not show this behaviour although both strains express a fully functional TTSS. This leads to the conclusion that the pack swarming phenotype is not solely TTSS dependent; other factors might come into play as well. On the other hand, TB is capable of surviving and persisting in PMN, a phenotype that was not seen with strain CHA. Transcriptome data showed that strains CHA and TB employ different strategies for survival within a hostile environment simulated by culturing the bacteria in the presence of hydrogen peroxide: Strain CHA upregulates genes important for the detoxification of antioxidants and virulence and downregulates the core metabolic activity whereas strain TB upregulates genes necessary for stress adaptation as well as central metabolic pathways, therefore is more primed on persistence and multiplication than defending itself and exerting active damage to the host. In the end, this strategy seems to be the more successful one as strain TB manages to persist in PMN much longer than strain CHA. It has also been shown that intracellular survival is independent of

[131] Ran H, Hassett DJ, Lau GW: Human targets of Pseudomonas aeruginosa pyocyanin. Proc. Natl. Acad. Sci. 100 (24), 2003, 14315-14320
[132] Jordan D: *Dissertation*, Tierärztliche Hochschule Hannover, *2004*

TTSS activity while the other examined virulence traits such as cytotoxicity towards macrophages and *C.elegans* prove the significance of this system for the virulence of the two compared strains. This study shows once more the wide range of phenotypical diversity of *P.aeruginosa* which together with its excellent environmental and metabolic adaptability contributes to the virtually ubiquitous distribution of this facultative pathogen.

3.2. Construction of a Signature Tagged Mutant library in P.aeruginosa SG17M

Signature Tagged Mutagenesis (STM) has become an increasingly popular method for functional genome analysis in bacteria, represented by the vast numbers of publications of the last years. First published by D. Holden and M. Hensel in 1995[133] as a means of identifying virulence genes in *S.typhimurium*, it allows for the large-scale identification of new virulence determinants and is applicable, with a few adaptations, for most bacterial species susceptible to transposon mutagenesis[134].

Mutants generated applying STM are uniquely tagged with a signature sequence and can be identified according to this sequence all the way through experimental procedures. The tagged mutants are pooled, subjected to the experiment, recovered and the signal sequences are amplified from their genomic DNA using primers that are specific for the conserved part of the signal sequence. The specific parts of the signature tags are then hybridized onto DNA blots of the complete set of signature sequences which were generated as reference. Signature sequences that are missing in the recovered pool of mutants represent mutants that did not survive the experimental conditions. This way, STM is an ideal method for animal models of virulence as it greatly minimizes the number of animals necessary for screening of a large mutant library. However, the detection, especially if done non-radioactively, is rather expensive and if experiments can be performed in 96- or 384 well format, it is preferable to do so. Nevertheless, a transposon mutant library of an organism that was generated applying STM has a broader spectrum of applicability and therefore constitutes a valuable tool for functional genome analysis.

The STM mutant library generated from *P.aeruginosa* SG17M was constructed by transforming the recipient strain with a plasposon containing a mini-Tn5 transposon that is mobilized and integrated into the genome by a transposase located outside its IS elements. This should guarantee that the transposition is a unique event and mutants with multiple transposon insertions in their genome should not be generated. This is still a problem with many transposon constructs[135] and makes it impossible to attribute a mutant phenotype to a single gene. The pMOD plasposon construct seems to be ideal at least for application in *P.aeruginosa* as this problem has not been observed when constructing and examinating the TB[136] -and SG17M mutant library.

3.2.1. Optimization of acceptor strain pre-treatment

The first step towards the construction of the STM library in SG17M was the optimization of the pre-incubation at 42°C of the acceptor strain. This treatment increases conjugation efficiency as it shuts off the endogenous restriction system of *P.aeruginosa*[137] but also decreases the fitness of the bacterium, especially if the incubation is carried out without supplying humidity. Hence, a water bath was placed in the incubator to generate a slightly humid climate which is necessary for *P.aeruginosa* to thrive, especially at high temperatures. The optimal pre-incubation time was determined to be 6 days on Columbia agar containing 5% sheep blood during which the bacteria culture was transferred to a new plate each day.

Table 8 illustrates the conjugation efficiency after different pre-incubation times and on blood agar and LB medium:

DAYS OF PRE-INCUBATION	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
average *cfu* count per 10 plates (LB medium)	0	0	0	3	15	17	1
average *cfu* count per 10 plates (blood agar)	0	0	0	9	26	112	16

Table 8: optimization of acceptor strain pre-incubation

[133] Hensel M, Shea JE, Gleeson C, Jones MD, Dalton E, Holden DW: Simultaneous Identification of Bacterial Virulence Genes by Negative Selection. Science, 269, 1995, 400-403

[134] Lehoux DE, Sanschagrin F, Kukavica-Ibrulj I, Potvin E, Levesque RC: Identification of novel pathogenicity genes by PCR signature-tagged mutagenesis and related techniques. Methods Mol. Biol. 266, 2004, 289-304

[135] Harper M, Boyce JD, Wilkie IW, Adler B: Signature-Tagged Mutagenesis of Pasteurella multocida Identifies Mitants Displaying Differential Virulence Characteristics in Mice and Chickens. Infect. Immun., 71 (9), 2004, 5440-5446

[136] Wiehlmann L, personal communication, 2000

[137] De Lorezo V, Timmis KN: Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods in Enzymology 235, 1994, 386-405

This assessment was of quantitative nature only, the resulting colonies were not checked for a transposon insertion, and conjugation efficiency was solely based on the number of gentamicin-resistant mutants obtained.
At a later stage of the study, a second pre-treatment of the acceptor strain was included in the procedure: Strain SG17M produces significant amounts of exopolysaccharides which lower conjugation efficiency, most likely due to hindering cell-to-cell contact necessary for DNA transfer. This was probably the reason why no mutants were obtained when the procedure optimized for strain SG17M was applied to the clinical isolate CHA for the generation of STM mutants. This isolate is extremely mucoid, a phenotype which renders the application of all sorts of techniques involving DNA handling difficult. Three washing steps of the bacteria culture that was scraped off the blood agar plate were added: The bacteria were not resuspended in 10 mM $MgSO_4$ but instead with 5mM EDTA solution containing 0.1% SDS, followed by centrifugation at 13,000 x g and 4°C. The resulting pellet was resuspended in 5 mM EDTA, centrifuged again and then, the bacteria were resuspended in 10 mM $MgSO_4$.
The addition of these washing steps greatly increased the number of *cfu* obtained per plate. Pre-treatment of mucoid bacteria with 5 mM EDTA in combination with 0.05-0.1 % SDS improved all DNA handling techniques during this study, especially DNA preparation for direct genomic sequencing.

3.2.2. Optimization of the triparental mating

The major optimization of the triparental mating procedure definitely was the adjusted pre-treatment of strain SG17M. Initially, strain CSGB8 was used for this study, but conjugation of this strain with pMOD-bearing *E.coli* donor strains yielded no transposon mutants. As the adjustments to the pre-treatment were made at a later stage during this work, a few mating experiments with strain CSGB8 were repeated to check whether the added washing steps or different incubation times at 42°C would make a difference. Unexpectedly, no mutants were obtained even with these changed parameters, suggesting that strain CSGB8 possesses some sort of mechanism that either prevents the transformation with the pMOD construct, or the uptake of this construct is such an unfavorable process for the bacterium that cells that took up the foreign DNA either die or grow so slowly that they can not be selected. Using the pre-treated strain SG17M, a generous number of *cfu* were obtained per plate, stably ranging between 110 and 150 mutants so that 4 colonies per plate were initially picked. A valuable further optimization of the mating was the selection of the transposon mutants on M9 minimal medium containing benzoate as a sole carbon source. *P.aeruginosa*, like *P.putida*, is equipped with genes for catabolism of aromatic compounds which *E.coli* lacks; therefore, the donor strains can not metabolize benzoate. Furthermore, benzoate is an efficient preservative; many bacteria as *E.coli* do not even survive if the medium is supplied with benzoate. This way, the change from glycerol to benzoate as carbon source made it possible to avoid *E.coli* contamination when selecting and arraying the *P.aeruginosa* transposon mutants. To illustrate this fact, “mutant” 1A2 which was the second mutant to be arrayed in the library seemed to have a defect in protease and homoserine lactone secretion, but when the transposon insertion was sequenced, the BLAST alignment of the insertion site with the NCBI Nucleotide database revealed that this “mutant” was in fact a donor *E.coli* that had found its way into the mutant library. This contaminant was so far the only one found in the SG17M mutant library whereas in the TB mutant library which was comprised of mutants selected with glycerol as carbon source, *E.coli* contamination constituted a serious problem and was only remedied by restreaking the mutants on M9 benzoate agar.

3.2.3. Arraying the mutants

The obtained transposon mutants were arrayed in 96-well plates to allow for easy access to and transfer of the mutant library. Mutants of the same signature tag were always arrayed consecutively and in the same position of each consecutive plate. This format was applied to the selection assays.

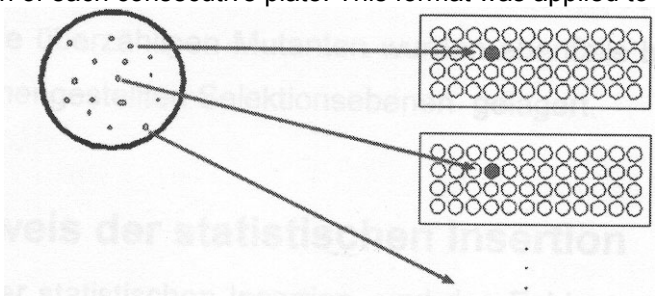

Figure15: arraying STM mutants

figure courtesy of L.Wiehlmann and reprinted with permission

3.2.4. Quality control of the mutant library

A major problem with large transposon mutant libraries is their redundancy[138]. Although new publications prove the near-randomness of Tn5 insertion[139], most mutant librarys are constructed in a way that a large excess of mutants is picked and each one is verified for transposon insertion before being arrayed. In this study, a selection like that was not applicable as it is very time-consuming and expensive, therefore, random mutants were selected and verified for their uniqueness. In addition, several mutants picked from one selection plate and mutants picked from consecutive plates were tested. This revealed a disadvantage of strain SG17M in comparison to strain TB: While the mutants generated from strain TB which were picked according to the guidelines presented in 2.4.3. proved to be different individual mutants, all of the SG17M mutants that were picked off one plate proved to be identical as can be seen on the Southern blot depicted in figure 16, even though the plates were separated in two halves and the bacterial lawn taken off of both halves leaving a zone in the middle untouched as not to mix the two halves. This was supposed to ensure a maximum number of individual mutants per plate but did not prove to be of any advantage.

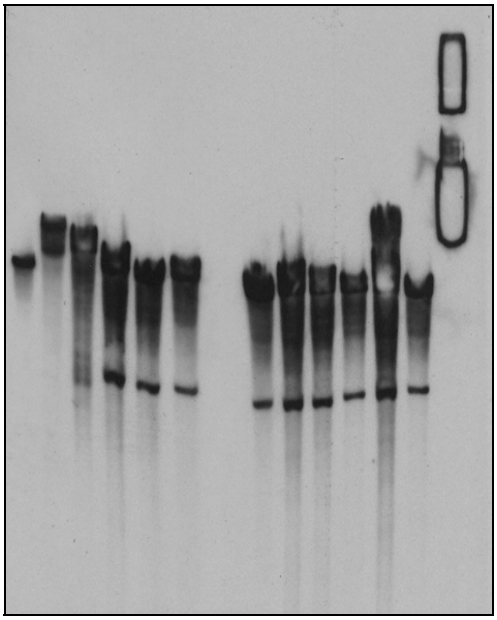

Figure 16: Southern blot of *Xho*I-digested genomic DNA of STM mutants hybridized with a probe specific for the gentamicin cassette. From left to right: 2 mutants picked off different plates in lanes 1 and 2, 4 mutants picked off the same plate containing more than 120 cfu in lanes 3-6, 2 empty lanes, lanes 9-14: mutants picked from off a plate with more than 200 cfu.

This figure shows the major problem during the generation of the SG17M mutant library. The strain did take up foreign DNA and integration of the transposon into the genome did occur, but apparently the mating procedure yielded a very low number of different mutants per conjugation event. This problem had to be circumvented by picking only one colony per plate which greatly increased the amount of mating experiments needed for a mutant library of roughly 3000 mutants. The mutants were picked and arrayed according to this prerequisite and this way, a library of individual mutants was generated from isolate SG17M comprising 2976 mutants ordered in 96-well plates according to consecutively numbered signature tags. As plasmids carrying some signature tags proved to be transferred with greater efficiency than those with other tags, 786 mutants could not be arrayed this consecutive fashion and were ordered in 96-well plates according to their tags: All mutants carrying for example the tag A1 were arrayed next to each other, followed by all mutants of the tag A2, and so forth. This way, a mutant library with optimal distribution of tags for STM experiments is available as well as mutants arrayed in a way that they can still be used in a 96 well format instead of being surplus, or a different signature tag reference can be generated to make this format STM-compatible as well.

[138] Levesque R: Personal communication, 2003
[139] Kang Y, Durfee T, Glasner JD, Qiu Y, Frisch D, Winterberg KM, Blattner FR : Systematic Mutagenesis of the Escherichia coli Genome.

Random mutants were selected and examined for their individuality. Figure 17 shows a Southern blot of arbitrarily picked mutants with the signature tag A1, each mutant originates from a consecutive plate and therefore was generated in a single conjugation event. This evidence indicates that the STM transposon mutant library generated in this way should indeed comprise 2976 individual mutants.

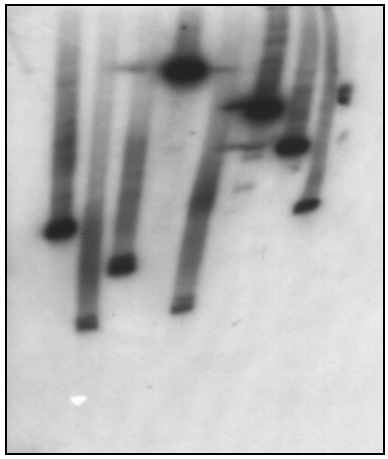

Figure 17: Southern blot of *Xho*I-digested genomic DNA of 9 STM mutants bearing signature tag A1, hybridized with a probe specific for the gentamicin cassette.

A non-redundant STM transposon mutant library of *P.aeruginosa* isolate SG17M was generated applying an optimized procedure for triparental mating employing *E.coli* donor strains carrying the signature tag-bearing plasposon pMOD and an *E.coli* strain carrying a helper plasmid to mobilize the plasposon. However laborious the mating procedure proved to be, a library of non-auxotroph mutants representative of the *P.aeruginosa* genome was constructed and subjected to screening for genes involved in cytotoxicity and quorum sensing.

3.3. Identifying and sequencing of genes with a transposon insertion

If a transposon mutant library of an organism is subjected to various bioassays in order to identify genes that are important for either this organism's survival or for a different phenotype in comparison to the wildtype, the identification of the mutants with altered behavior is just the first step. The second step is the identification of the gene in which the transposon insertion has occurred. This can be done by a plasmid rescue; a method described by Zylstra[140] and modified and adapted for *P.aeruginosa* by Wiehlmann[141]. This method is based on the presence of the pMB1 *ori* and the gentamicin resistance cassette in a fragment of genomic DNA that was digested with a frequent cutting restriction enzyme. The fragments which should have a size of 1-10 kb can be religated and transformed into an *E.coli* strain, and only those fragments that contain both the ori and the resistance cassette can be replicated as a plasmid and extracted from the *E.coli* strain. The plasmid can then be dispatched for sequencing with transposon-specific primers that are designed to amplify fragments outward from the transposon and therefore allow for obtaining sequence information of the transposon-flanking region and thus the gene of interest. This method has been successfully applied to identify several genes from an STM screen carried out previously[142], but has failed with many genes of great interest from that screen. In some cases, no religated plasmids were obtained, in other cases, a religated plasmid could be detected when the ligation mix was analyzed on an agarose gel, but transformation into an *E.coli* strain to replicate the plasmid failed or plasmid rearrangement occurred[143]. In one case, a relegation product of a large plasmid was observed when the ligation mix was checked on an agarose gel, but transformation in *E.coli* yielded no transformants. The gene and its flanking region was later obtained as a long-range PCR product[144] and when the ORF itself was cloned into an expression vector and the construct transformed into *E.coli*, again, no transformants were obtained[145]. An obvious reason for that has not been found yet, but it can be assumed that the gene codes for a product which is toxic for the *E.coli* strain that hosted the plasmid.

[140] Dennis JJ, Zylstra GJ, 1998, see ref. 90
[141] See ref. 78
[142] See ref. 78
[143] Salunkhe, personal communication 2002
[144] Charizopoulou and Wiehlmann, personal communication, 2003
[145] Salunkhe, personal communication, 2004

To circumvent these difficulties, the identification of the genes which had a transposon insertion and found to be essential for either quorum sensing or cytotoxicity was achieved applying two different methods: Direct genomic sequencing and the y-linker method.

3.3.1. Direct Genomic Sequencing

Direct genomic sequencing has not yet been applied to *P.aeruginosa* as its large genome gives rise to a lot of sequencing "background noise" due to unspecific binding of the sequencing primers. Furthermore, common preparation methods for genomic DNA always yield the DNA as well as fragmented DNA and rRNA and thus even more material that can interfere with specific primer binding.
In order to apply genomic sequencing to the *P.aeruginosa* genome, a modification of the established methods for the preparation of genomic DNA had to be found that would yield genomic DNA fragments of high purity and a length of 1-10 kb at minimum in order to obtain enough sequence information with primers leading from the transposon outwards. A key step of the modified procedure was the removal of the extracellular polysaccharide layer of the mutant cultures by washing the bacterial pellet with 5 mM EDTA in combination with 0.05-0.1 % SDS as well as a digestion step with proteinase K. The proteinase digestion step was carried out over night so that all proteins could be removed from the DNA. Several phenol/chloroform extractions as well as a washing step with PEG 100 greatly improved the quality of the obtained genomic DNA. Absorption values were generally between 1.7 and 1.8, indicating extremely pure DNA, and yields were typically from 2.5-4 µg/µl DNA in TE. Stretches from 75 to 387 nucleotides were sequenced using genomic DNA purified with this method, and all of the obtained sequences were diagnostic and specific for the respective genes. Therefore, this method is a valuable tool for the direct analysis of insertion sequences on genomic DNA without the necessity of generating plasmids including the insertion site. The sequences were analyzed by similarity searches using blastall 2.0[146] versus the complete and annotated genome of *P.aeruginosa* (www.pseudomonas.com) with a gap opening penalty set at -3 as default and lowered to -1 if necessary. All sequencing results obtained led to the identification of the respective gene with the transposon insertion.
Figure 18 shows an analytic agarose gel of genomic DNA obtained from a 100 ml culture of transposon mutants, digested with *Nru*I.

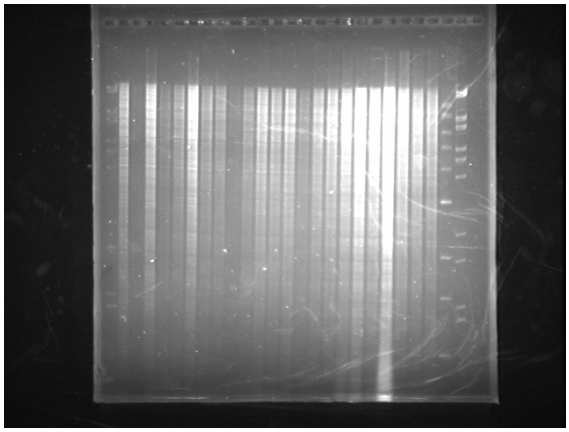

Figure 18: Genomic DNA prepared for direct genomic sequencing

3.3.2 Y-linker method

The y-linker method[147] is a PCR based method for the identification of insertion sites and is independent of the composition of the insertion sequence. The genomic DNA extracted from a transposon mutant is digested with a frequent cutting restriction enzyme that yields DNA fragments from 100-1000 bp. A 40 bp oligonucleotide linker is specifically designed to consist of one half where one strand is not complementary to the other, thus forming a "Y" (see chapter 2.6.3., Materials and Methods). The complementary half of the linker contains the recognition site for a chosen restriction enzyme, in the case of this study *Sph*I, which can be ligated to fragments of the genomic DNA digested with this enzyme. The ligation products are then used as templates for a PCR reaction that is primed with one primer specific for the non-complementary part of the linker while the other primer is

[146] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic Local Alignment Search Tool. J.Mol. Biol. 215, 1990, 403-4010,
[147] See ref. 111

specific for the inserted transposon. Therefore, only fragments that contain the transposon and the linker will give a product that can be sequenced (Figure 19):

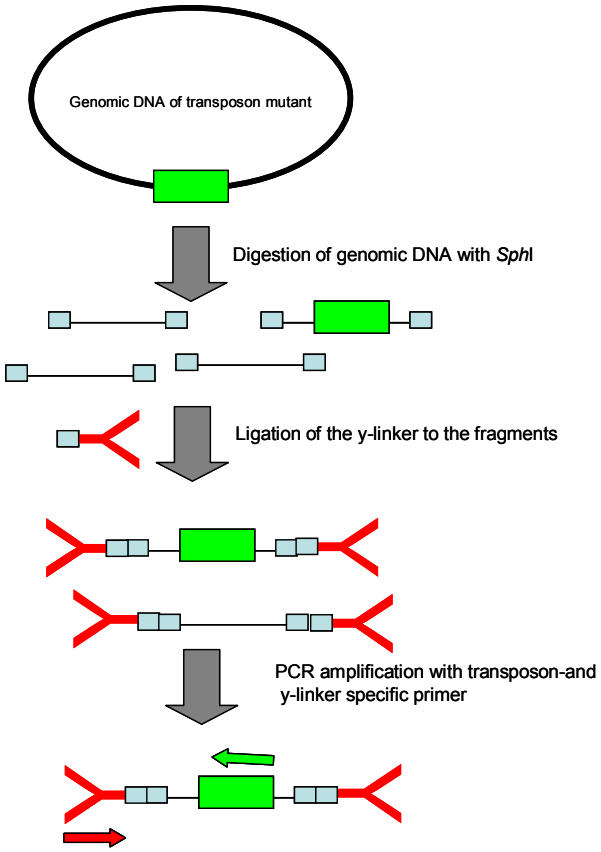

Figure 19: A schematic overview of the y-linker procedure. The green box represents the transposon insertion and the blue boxes the overhang left by *Sph*I digestion.

This method has proven to be very valuable as the DNA preparation procedure necessary for direct genomic sequencing is rather laborious and genomic sequencing is more expensive than the sequencing of PCR products. The lengths of the PCR products obtained with this method varied greatly from approximately 300-1000 bp, but all obtained sequences except for one could be matched to genes from the PAOI genome. This one sequence was obtained from the quorum-sensing deficient mutant 10A3 and revealed homology to an integrase and a transposon region on a plasmid known to be integrated into the SG17M genome. In this case, the transposon insertion has occurred in a gene that might not play an obvious role in quorum sensing, and the mutant genome should be searched for a second insertion that might have occurred by re-mobilization of the transposon.
Both direct genomic sequencing and the y-linker method have proven to be valuable tools for the quick identification and sequencing of transposon insertion sites of mutants generated with the STM method. The y-linker method has been adapted to and used for the identification of transposon insertion sites in *P.putida* as well[148], and direct genomic sequencing as established in this study should be applicable to other *Pseudomonads* and possibly to other bacterial species as well.

3.4 Screening of the TB STM library for genes involved in cytotoxicity towards human monocyte-derived macrophages

Cytotoxicity of *P.aeruginosa* depends on multiple factors, of which the TTSS plays a major role and its impact on host-pathogen interactions of *P.aeruginosa* with diverse human cell types has been intensely investigated. The objective for this part of the study at hand was to apply a fast and inexpensive screen to identify mutants that show significant differences in their phenotype when compared to the parental strain. Therefore, an assay had to be found that allowed for the detection of global virulence determinants, not only TTSS-dependent effectors, and a prerequisite for the screening of a large STM library was that the assay be reproducible, easy to handle, fast and cheap.

[148] Weinel C: Comparative and functional genome analysis of *Pseudomonas putida* KT2440. Universität Hannover, 2003.

At first, DNA staining with ethidium bromide was considered as a reaction that could allow for measurement of cell damage, but as ethidium bromide is a highly cancerogenic chemical and the results obtained by this method were not promising, this approach was not followed any further.
The MTT assay was first described by Mosmann[149] and in a modified way by Denizot and Lang[150]. In this work, the assay was performed according to the protocol of Plotkowski et al.[151] with several modifications.
The assay relies on the colorimetric detection of the conversion of the tetrazolium dye MTT to its corresponding formazan. The enzyme succinate dehydrogenase, enzyme 7 of the citrate cycle, catalyzes the dehydrogenation of succinate to fumarate. In this reaction, a C–C double bond is formed and two hydrogen are removed from the carbon skeleton of succinate and shuttled to FAD, which is in turn reduced to $FADH_2$. The $FADH_2$ is able to add these two hydrogens to the tetrazolium dye MTT which is reduced to its formazan this way. In a damaged mitochondrion, the turnover of redox equivalents is reduced, thus less subsequent reduction of MTT to the corresponding formazan can occur and no or very little absorption is measured. If cells are fully viable, MTT reduction can be visually assessed by the change of the cell medium to a deep purple color. Absorption of this formazan can be quantitated at 570nm.
This reaction is described as very sensitive and well-suited for measuring the loss of cell viability by cytotoxic agents[152].
The bacteria/eukaryotic cell mixture was incubated with MTT for 30 minutes instead of 60 minutes as described by Plotkowski as this shortened incubation period proved to enhance the signal-noise ratio and thus sensitivity. Furthermore, DMSO instead of isopropanol was used to dissolve formazan crystals due to its superior solvent properties towards aromatic quaternary nitrogen compounds, and the cells were washed to rinse off remaining bacteria before measuring the formazan absorption as to avoid interference of bacterial metabolic activity.
These experimental conditions proved to yield the best signal/noise ratio and reproducibility; wells with bacteria as well as cells only served as negative and positive control.
The absorption values were evaluated as follows:
Each plate was assessed in duplicate and the absorption values were measured three times every fifteen minutes, starting immediately after the 30 minute incubation with MTT solution. The three obtained values for each mutant were added and their mean was calculated. The overall mean absorption value for the whole plate was also calculated and the mean for each mutant divided by the plate mean. The resulting value was termed “mean absorption value”. This way, outliers could easily be detected as absorption values that were either significantly lower or higher than the average. In general, it was observed that the mutants caused less damage to the cells than the wildtype strain represented by generally higher absorption values. Figure 20 shows the distribution of the calculated means for all mutants and illustrates this finding.

[149] Mosmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J.Immunol. Methods 65, 1983, 55-63
[150] Denizot F, Lang R: Rapid colorimetric assay for cell growth and survival. Modification to the tetrazolium dye procedure giving improved sensitivity and reliability. J.Immun. Methods 89, 1986, 271-277
[151] Plotkowski M-C, Helvécio CC, Zahm, J-M, Lizard G, Pereira GM, Tournier J-M, Puchelle E: Early Mitochondrial Dysfunction, Superoxide Anion Production, and DNA Degradation Are Associated with Non-Apoptotic Death of Human Airway Epithelial Cells Induced by Pseudomonas aeruginosa Exotoxin A. Am. J. Respir. Cell Mol. Biol. 26, 2002, 617-626,
[152] Denizot F, Lange R: Rapid colorimetric assay for cell growth and survival:modification of the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Meth. 89,1986: 271

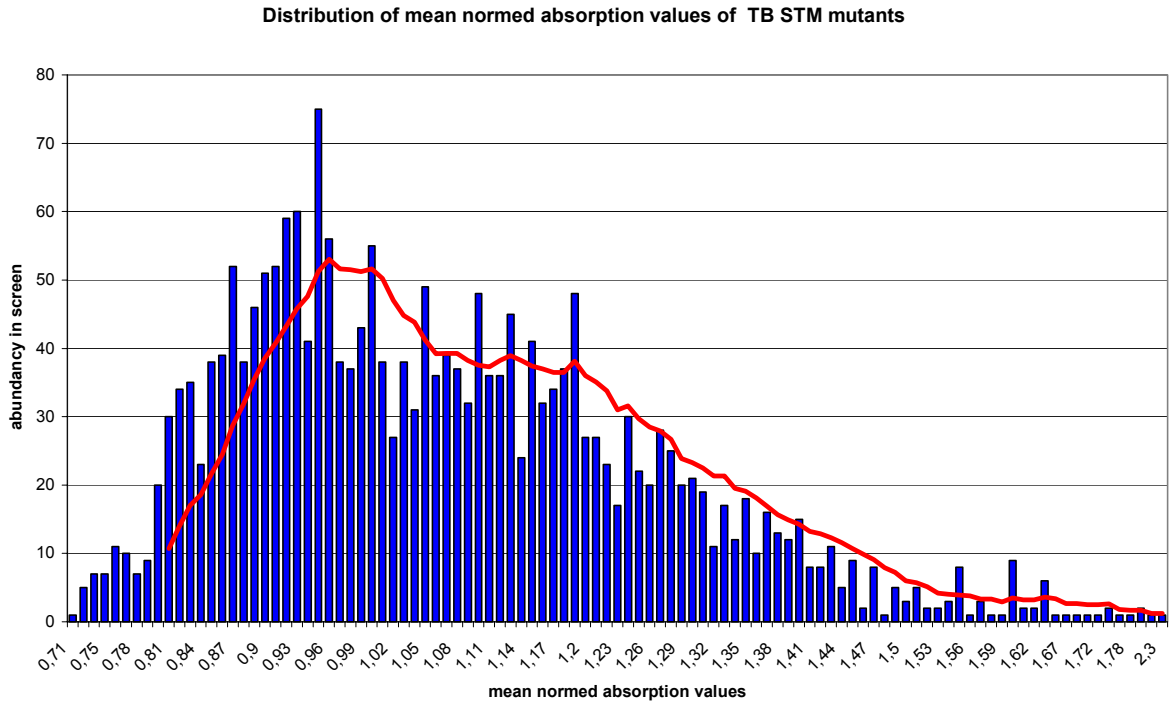

Figure 20: distribution of mean normed absorption values obtained by screening the STM library generated from strain TB (4512 mutants).

Values that were represented in the 95 % confidence interval of either the lowest 5% or highest 5% absorption range in three separately conducted screens were attributed to their respective mutants and the genes with the transposon insertions were sequenced.
A total of 8 mutants were found to show a distinctively different phenotype in comparison to the parental strain, which stably yielded normed absorption means of 0.73-0.76 during all screens. The gene which was disrupted by the transposon insertion was determined by sequencing and information about the sequenced genes was obtained from publicly accessible databases[153]. Table 10 summarizes the screening results:

PA gene number	Gene name	mutant number	screening values	Virulence	Gene function	Category
PA 0086	---	40A3	1,2 1,2 1,61 1,45 1,53 1,22	loss of virulence	unknown	unknown hypothetical
PA 1002	phnB	47A8	1,61 1,63 1,3 1,24 1,33 1,33	loss of virulence	anthranilate synthase component II	phenazine biosynthesis, production of virulence factors
PA 3366	amiE	32A3	1,7 1,32 1,34 1,59 1,59 1,34	loss of virulence	aliphatic amidase, carbon compound metabolism	central metabolism

[153] NCBI, TIGR; SwissProt expasy.com , genome.jp/KEGG databases

PA 4401	---	45A12	0,91 1,9 1,66 1,5 1,66 1,5	loss of virulence	glutathione-S-transferase	detoxification, maintaining redox status
PA 4829	lpd3	8D11	2,27 1,18 1,52 1,58 1,53 1,53	loss of virulence	dihydrolipoamide dehydrogenase	central metabolism
PA 4911	---	30B6	0,71 0,79 0,69 0,59 0,67 0,67	possibly gain of virulence	probable permease of branched-chain amino acid transporter	transporters, membrane proteins, central metabolic pathways
PA 5342	---	48C11	1,21 1,7 1,58 1,88 2,15 2,09	loss of virulence	probable transcriptional regulator	regulators

Table 10: Results of the MTT screen

Gene **PA 0086** is a hypothetical protein which does not seem to have any close homologs in the *P.aeruginosa* genome or in other microbial genomes[154]. The three closest homologs are hypothetical proteins from *X.axonopodis* (XAC 4144) with 67% similarity and 49% identity, from *B.parapertussis* (BPP0717) with 59% similarity and 45% identity, and from *B.bronchiseptica* (BB 0803) with 59% similarity and 45% identity. The two genes of the *Bordatellae* show a high similarity to each other, but no function has been assigned to theses genes yet. Searches in terms of function, structure and the identification of domains did not yield any results, so it has to be concluded that this gene is heretofore completely non-described, except that, if knocked out, *P.aeruginosa* TB loses its virulence towards human macrophages.

Gene **PA 1002** is well-known, it has been attributed a function in the synthesis of phenazine compounds and codes for an anthranilate synthase component II. This enzyme catalyzes the formation of anthranilate from chorismate by transferring an amino group from glutamine to the *ortho* position of the aromatic ring, removes the *meta*-substituted side chain from chorismate and mediates the formation of an aromatic system. In *P.aeruginosa*, this step is essential for the production of quinolones by the *phnAB* genes, which in turn regulate the structural genes for phenazine biosynthesis[155]. To rule out a potentially diminished fitness of the mutant, it was grown overnight under assay conditions and the OD_{578} compared with the TB wildtype grown under the same conditions. No difference in growth between wildtype and mutant could be detected.

The toxicity of pyocyanin is widespread, it has been reported that it has a function in nematode killing, is required for toxicity towards the wax moth *G. mellonella*, and has been attributed to interfere with numerous mammalian cell functions such as cell respiration, ciliary beating, epidermal cell growth or calcium homeostasis[156]. Furthermore, it has been attributed a function in the imbalance of protease-

[154] www.tigr.org/tigr-scripts/CMR2

[155] Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, Tomashow LS: Functional analysis of Pyocyanin and Phenazine-1-Carboxamide from Pseudomonas aeruginosa PAOI. J.Bacteriol. 183 (21), 2001, 6454-6465
- Ran H, Hassett DJ, Lau GW: Human targets of Pseudomonas aeruginosa pyocyanin. Proc. Natl. Acad. Sci. USA 100 (24), 2003, 14315-14320

[156] Wilson R, Sykes DA, Watson D, Rutman A, Taylor GW, Cole PJ. Infect. Immun. 56, 1988, 2515-2517
Sorensen RU, Klinger JD. Antibiot. Chemother. 39, 1987, 113-124

antiprotease activity in the airways of CF patients[157], the mechanisms of pyocyanin pathogenicity are however only poorly understood.
It should be reasonable that a mutant in a gene that is involved either in regulation or synthesis of this virulence factor displays less virulence than the wildtype strain that is fully capable of synthesizing pyocyanin. The exact impact of this gene on *P.aeruginosa* TB virulence towards human macrophages should be verified by the construction of an isogenic mutant or by complementation of the gene *in trans*, so that further analyses could shed light on the regulatory functions of the *phnAB* genes for pyocyanin production and pyocyanin pathogenicity.

Gene **PA 3366**, *amiE*, is an aliphatic amidase belonging to the *ami* operon (PA 3362-3366) and is involved in nitrogen compound catabolism. This mutant was further tested for growth in RPMI medium which is a medium devoid of proteins. It just contains carbohydrates and the essential amino acids and is therefore a good medium to check whether the reduced virulence might be due to impaired fitness of the mutant. The MTT test was carried out in this medium as to minimize interference of proteins with the MTT reaction, therefore, it could be possible that the mutant was simply not fit enough in order to produce virulence factors. It was found that this mutant indeed grows less well in RPMI medium compared to LB and DME medium. The latter is a rich medium commonly used for eukaryotic cells, and the mutant reached an OD_{578} of 6.8 after 12 hours of growth in LB and DME medium whereas if grown in RPMI medium the maximum OD_{578} was measured to be 2.2. No significant increase of OD of the mutant was observed in all mediums after 24 hours, suggesting that the mutant grown in rich medium had reached its stationary phase after 12 hours and growth in RPMI medium would not exceed the OD of 2.2 in general. This evidence leads to the interpretation that not a loss of virulence but a general loss of fitness accounts for the phenotype of the mutant, and although the bacteria were grown in LB overnight prior to the experiment and incubated with macrophages at an MOI of 30, the explanation for this mutant's different phenotype might just be that the bacteria, once added to the cells, did not thrive and multiply and therefore, the maximal multiplicity of infection in comparison to the other bacteria was not reached and thus the phenotype of the mutant mistakenly interpreted as a loss of virulence. This example shows that it is absolutely necessary to check for impaired fitness of putative loss-of-virulence mutants, and this is presumably true for most virulence models. Therefore, the PA 3366 mutant might not be a loss-of-virulence-, but instead an auxotrophic mutant.
The same is true for the knock-out mutant of gene **PA 4829**. This gene codes for a dihydrolipoamide-deydrogenase and is one of three lipoamide dehydrogenases in *P.aeruginosa*. It shows stronger homology to eukaryotic lipoamide dehydrogenases than to the other two enzymes of *P.aeruginosa*, and it is the only one in this bacterium not to be organized in an operon. The closest bacterial relatives are PP 5366 of *P.putida* KT2440, CC0342 of *C. crescentus* as well as PSPTO 2201 from *P.syringae* , all of these genes are assumed to be involved in lipid and intermediary metabolic pathways.
This enzyme is generally involved in several metabolic pathways as the citrate cycle, glycine, serine and threonine metabolism as well as glycolysis, gluconeogenesis and pyruvate metabolism. As *P.aeruginosa* is not equipped with all enzymes for glycolysis, this pathway might be of lesser importance in this context, whereas the citrate cycle is one of the main metabolic pathways of this organism. Although a lipoamide dehydrogenase is not one of the key enzymes directly involved in the citrate cycle, it seems to have an impact on the fitness of the knock-out mutant of PA 4829 as this mutant is also impaired in growth in RPMI medium. Therefore, the loss-of-virulence mutant 8D11 should also be renamed loss-of-fitness mutant.

Gene **PA 4401** encodes a glutathione-S-transferase (GST). This enzyme plays a role in the detoxification of reactive oxygen species and helps maintain intracellular proteins in the correct redox status. A decreased fitness of the mutant could not be observed when it was grown in the assay medium, but database search for functions of GST that are obviously related to virulence did not yield any results.
Gene **PA 4911** seems to be a similar case, only that a mutant with a transposon insertion in this gene proved to be more virulent than the wildtype, if only slightly. This mutant could be identified as a false positive because the MTT assay is subject to strong biological variations as the interaction of two different living systems is assessed. This assay is normally used with drugs interfering with metabolism and proliferation of eukaryotic cells, and although it does yield stable results in the high absorption ranges, those gained from low absorption values representing gain of virulence in this case could be regarded at least partly as circumstantial.
The gene codes for a branched-chain amino acid transporter and shares 89% similarity and 79% identity with PP 4865 from *P.putida* KT2440. This gene has also been annotated as a branched chain

[157] Britigan BE, Railsback MA, Cox CD. Infect. Immun. 67, 1999, 1207-1212

amino acid transporter and its substrates have been identified as amino acids, peptides and amines, most likely due to structural interaction studies. In *P.syringae*, gene PSPTO 4917, *braE,* has been attributed the function of a high affinity branched chain amino acid transporter and has 91% similarity and 79% identity with PA4911. Gene PA 1072 (*P.aeruginosa* PAOI) shares 71% similarity and 53% identity with PA 4911 and has been annotated as branched chain amino acid transporter *braE*. 12 transmembrane domains have been identified by structural analyses and hydrophobicity plot so that its function as a transporter should be fairly certain. However, its role in virulence remains elusive, especially as no difference in fitness could be observed between mutant and wildtype under assay conditions.

Gene **PA 4872** is an unknown hypothetical and as with PA0086, no hints in terms of structure or function could be found in the databases. The closest relatives are PP1389 from *P.putida*, a putative carboxyphosphoenolpyruvate phosphonomutase (CPEP, 88% similarity, 78% identity), the same attributed function was found for the gene PSPTO 1443 from *P.syringae* (85% similarity, 74% identity). Also, a slight similarity to PA 0796 was found, this gene has also been annotated as a CPEP. Interestingly, a knock-out mutant of exactly this gene was found to be deficient in protease and elastase as well as in homoserine lactone production by screening for quorum-sensing deficient mutants in the study at hand. A CPEP has heretofore not been linked to either virulence or quorum sensing in *P.aeruginosa*, but verification and further characterization of both knock-out mutants could be of interest.

PA 5342 encodes a transcriptional regulator framed by two hypothetical proteins on each side. An operon structure could be proposed for genes PA 5342 and 5343, and also no terminator structures could be detected between genes PA5342 and 5341. The gene PA 5341 shares homologies with PP 3021 from *P.putida*, a transporter with LysE family signature which binds and transports amino acids, peptides and amines. The closest relatives of PA 5342 are SAV 4003, a putative araC family transcriptional regulator from *S.avermitilis* (37% identity; 55% similarity) and to PA 3269 which has also been annotated as a transcriptional regulator of the *araC* family (53% similarity, 35% identity). Transcriptional regulators of the *araC* family mainly act as activators[158] of their target gene and bind the DNA sequence by two conserved helix-turn-helix motifs that represent the signature of the family. A recent study with *M.tuberculosis* STM mutants[159] has shown that a mutation in the gene Rv1395, encoding an araC family transcriptional regulator was attenuated in the lungs of mice. In the case of Rv1395, the target gene of the regulator, Rv 1394c, is a member of the cytochrome P450 family, as in the case of PA 5324 a completely different gene. Its function for virulence has not yet been determined but analyses addressing this topic as well as the nature of the regulatory interaction of Rv 1394c and Rv 1395 are under way. It might be interesting to verify the role of the gene PA 5342 by testing an isogenic mutant or the original knock-out mutant complemented with the intact gene in the MTT assay. Furthermore, a knock-out mutant of both flanking genes of PA 5324 could be generated and also examined for their virulence. Should the experiments confirm a function of the regulator or its flanking genes, the mutant strains could be studied other virulence models as well which would make it possible to attribute a function to the genes PA5341, PA 5342 and PA 5343.

A profound characterization of the genes found to be possibly related to virulence of the isolate *P.aeruginosa* TB towards human macrophages is well beyond the scope of the study at hand. The MTT test has yielded 8 mutants which differed in their impact on macrophage viability from the wildtype strain; however, the results should be regarded at least partly as circumstantial for two reasons:

First of all, although this test has been described being specific for eukaryotic cells and bacterial levels of MTT have always been low in the screening experiments, there are nevertheless two living and therefore metabolically active compartments involved that both contribute to the production of redox equivalents. Hence, it is difficult to exactly discriminate between the MTT reduction and formazan formation activity of the eukaryotic cells and that of the bacteria.

Second, this test apparently allows for a selection of auxotrophic mutants as well, as a minimal medium without added proteins has to be used for the screening procedure. Mutants that have defects in metabolic pathways relying on these nutrients are impaired in fitness and will therefore appear less virulent in the assay because they thrive less well as other mutants.

As a conclusion, it can be stated that the MTT assay is more suitable for measuring a decrease in cell viability caused by cytotoxic chemicals, but for the application with two types of organisms that are metabolically active, it is not an ideal method to measure cytotoxicity.

[158] Martin RG, Rosner JL: Curr. Opin. Microbiol. 4, 2001, 132-137

[159] Recchi C, Sclavi B, Rauzier J, Gicquel B, Reyrat JM: Mycobacterium tuberculosis Rv 1395 is a Class III Transcriptional Regulator of the AraC Family Involved in Cytochrome P450 Regulation. J. Biol. Chem., 278 (36) 2003, 33763-33773

3.5. Screening of the SG17M STM library for genes involved in Quorum Sensing

As a pre-screening for mutants that are attenuated in quorum sensing, a simple assay for protease degradation was used. Proteases are quorum-sensing-regulated virulence factors that *P.aeruginosa* secretes at a high bacterial density. Therefore, protease secretion can be used as an indicator for quorum sensing activity.
On agar containing 0.5-0.8% casein, bacteria that secrete proteases are surrounded by a halo indicating casein degradation. All STM mutants of the STM library were inoculated on casein agar and the 192 mutants that did not show a halo around the colony were singled out and subjected to the HSL production assay. Those mutants that did not degrade protease and did not produce HSL were identified as quorum-sensing deficient.

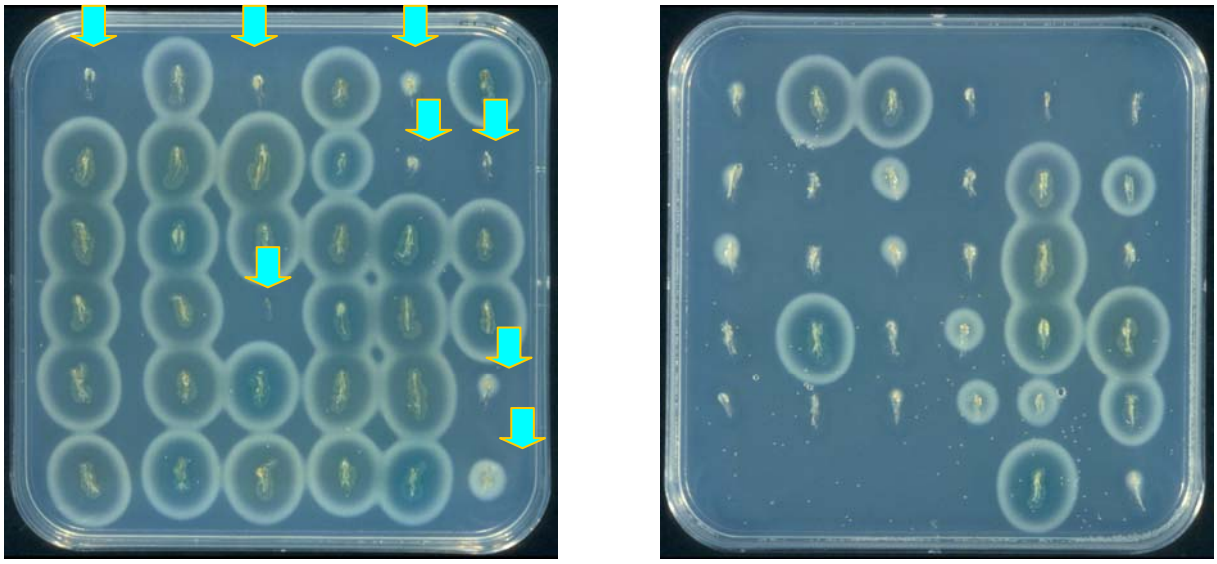

Figure 21: Screening of the SG17M mutant library for mutants deficient in protease secretion on M9 agar containing 0.5% casein as sole carbon source. The white halo around the colonies indicates casein degradation; mutants that do not secrete proteases show no halo. Arrows: Mutants deficient in protease secretion.

The screening of the STM mutant library generated from isolate SG17M for genes related to quorum sensing yielded 23 individual mutants that were neither capable of protease secretion nor homoserine lactone production. Furthermore, elastase production was reduced in most of the mutants, and several mutants showed decreased siderophore production:

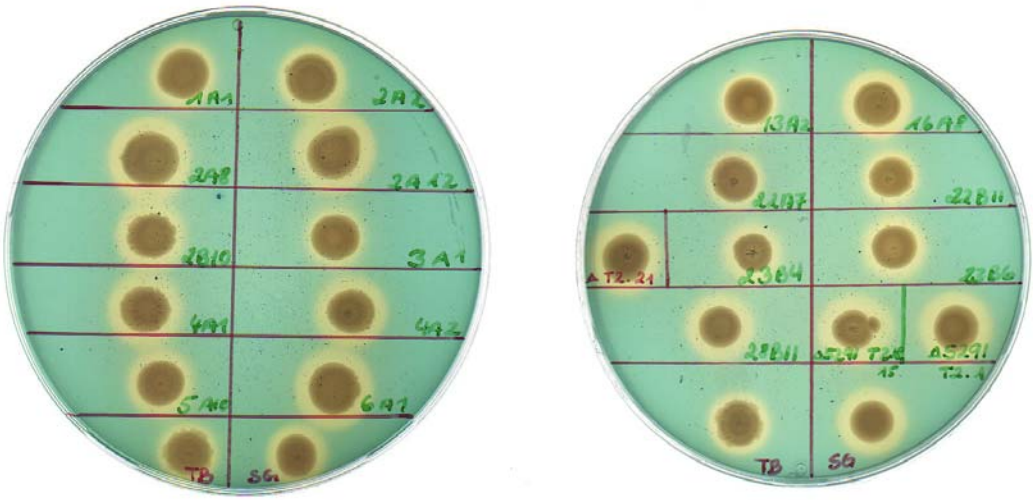

Figure 22: An example of siderophore production of mutants deficient in HSL production. This assay can be used to pre-screen for mutants that might have altered siderophore production.

Table 11 presents a short overview of the genes, their genomic context, homologies and similarities found in other bacterial genomes as well as function and structural features, if applicable. Transcriptome data as well as a preliminary interpretation of the phenotype shall serve for a short characterization of the examined mutants.

3.5.1. Sequenced genes found in the protease- and homoserine lactone production assay

GENE NUMBER	MUTANT NUMBER	GENE NAME	ATTRIBUTED FUNCTION	GENOMIC CONTEXT	HOMOLOGIES/SIMILARITIES
PA0104	4 A 1	----	Hypothetical, unclassified, unknown	Next to *coxB*	gene 0103 is a probable sulfate transporter, genes PA 0105-0109 are quorum-activated (Schuster et al.)
PA0796	3A1,8A 1	*prpB*, alt: *bcpA*	CPEP phosphomutase	operon with 0797 and 0798, conserved also in *S.typhimurium*, adjacent to propionate degrading enzymes *prpCD*	89% similar to prpB gene *product of S.typhimurium*, 56% similar to bcpA gene product in S.hygroscopicus. Function in aminophosphate, aminosugar and glycerolipid metabolism.
PA1116	3A2	---	hypothetical protein, unclassified, unknown	flanked by hypothetical proteins	48% similarity to YitL of *B.subtilis*
PA1253	8 A 2	----	Probable semialdehyde dehydrogenase, aldehyde dehydrogenase glutamic acid active site	possibly in an operon with PA 1254	65% similarity to ketoglutarate semialdehyde dehydrogenase of *P.putida*, 42% similarity to toluenesulfonate aldehyde dehydrogenase of *C.testosteroni*
PA1430	32A10	*lasR*	transcriptional regulator, function in adaption, protection; belongs to class of bacterial regulator proteins with LuxR family signature	lasR regulon, next to *lasI*	luxR-type protein, essential for quorum sensing and virulence in *P.aeruginosa*

PA2006	1 A 1	---	Probable MFS transporter, membrane protein, aromatic compound catabolism, 12 predicted transmembrane domains, verified by hydrophobicity plot.	Fourth of four ORF cluster, upstream: transcriptional regulator, conserved hypothetical and 3-hydroxy-butyrate dehydrogenase with short-chain dehydrogenase/ reductase family signature	58% similarity to pcaK gene product of *P.putida* KT2440, attributed function: carbon compound catabolism. Similarity to probable MFS transporters PA 2472, and PP 3165 (*P.putida* KT2440), function: transport of carbohydrates, organic alcohols and acids.
PA2127	7 A 1	---	Conserved hypothetical	Next to cupA1, orphan, flanked by hypotheticals upstream	no close relatives or homologues with attributed functions, best database hit : 53% similarity to hypothetical ybdN gene product, no ascribed function
PA2360	28 B 11	---	Hypothetical protein, no structural features could be detected by database searches	part of operon, No terminator or promoter structure was detected	no information was obtained by database searches
PA2402	9 B 10	*pvdI*	Probable non-ribosomal peptide synthetase, phosphopantetheine attachment site.	On antisense strand, *pvdD* is two genes away	very large gene, probable fusion product (TIGR). Putative AMP binding enzyme, close to *fpvA* and *pvd* genes.
PA3059	2A11	*pelF*	Glycosyl transferase group 1 (weak) structural domain.	Part of *pelABCDEFG* cluster involved in biofilm matrix formation in *P. aeruginosa* PA14 and ZK2870.	
PA3268	8 B 10	---	Probable *tonB*-dependent receptor, membrane protein, transport of small molecules	One of four antisense strand genes, possible operon	61% similarity to *fecA* gene from *E.coli*
PA3704	2A8,5A10,6A1, 10A1,10A3, 11A12,11B12, 13A12,22A7	*wspE*	Probable chemotaxis sensor/effector fusion protein, motility and attachment, chemotaxis, signal carboxy-terminal domain, response regulator receiver domain	Part of the *wspRFEDCBA* operon, involved in autoaggregation in a WspF mutant	48% similarity to gliding motility regulatory protein FrzE of *M.xanthus* two-component regulatory systems;

PA4070	4 A 2	----	Probable transcription regulator	Among a group of 6 genes on the antisense strand, some intergenic space to both sides, in a region of hypotheticals	45% similarity to the regulator FeaR of *E.coli*
PA4265	8 A 12	*tufA*	Translation, post-translational modification, degradation, GTP-binding elongation factor signature	Next to *fusA* and *rpsJ.* In a region of translation / transcription genes.	Similarities and identities with numerous elongation factors. Integral enzyme in protein synthesis.
PA4277	8 A 12	*tufB*	same mutant, 2 hits in database, identically annotated genes	located within a region of ribosomal genes, the structure of the genomic region is conserved among many species.	*tufA* and *B* only differ by a few nucleotides, the protein sequences are identical according to *pseudomonas.com*
PA4753	2 A 2	proposed gene name *yhbY*	Hypothetical, unclassified, unknown, homologies to novel class of RNA binding proteins, YhbY domain	Only gene on sense strand, 9 genes right, 16 genes left on antisense strand, orphan	75% similar to conserved hypothetical protein of *H.influenzae, E.coli*; there: probable RNA binding proteins
PA4971	23 B 6	*aspP*	Adenosine diphosphate sugar pyrophosphatase, energy metabolism, bacterial mutT protein domain	located in a region of hypotheticals, next gene with attributed function is PA 4973: thiamine biosynthesis protein ThiC; PA 4975: NAD(P)H quinone oxidoreductase	mutT proteins: dNTPases, hydrolysis of 8-oxo-dGTP. 67% identity and 84% similarity to PP 4919 (*P.putida* KT 2440) and 69% identity and 84% Similarity to PSPTO 4973 (*P.syringae* pv. tomato), both *mutT* gene family
PA5166	22 B 11	---	Probable two-component response regulator, transport of small molecules, σ^{54}-interaction domain and response regulator receiver domain predicted from structure	Clustered with *dctBPM*, upstream: cell wall/capsule synthesis genes, 5167-5169 are genes involved in C4-dicarboxylate metabolism, most likely operon structure, conserved neighbourhood prediction for PA 5152-5173 (COG).	64% similarity to C4 dicarboxylate transport system regulatory protein DctD of *R.meliloti.* Signal transduction pathways, no ascertained function.

PA5291	23 B 4, 2 A 12	betT2	Probable choline transporter, transport of small molecules	next to two hypothetical proteins,	61% similarity to *E.coli betT2* gene product
PA5375	2 B 10	*bet*T1	Choline transporter BetT, membrane protein, transport of small molecules	Adjacent to and transcribed oppositely from *bet*IAB ORFs, like in *E.coli*	63% similarity to *E.coli betT1* gene product
10A12	non-PAO gene	---	probable integrase	non-PAOI gene	homology to pKLC, similarity to Tn21, integron In2, and the mercury resistance operon of *E.coli*

Table 11: Genes with an impact on quorum sensing-controlled phenotypic traits found in the protease screen.

Elastase production (*lasB* expression) is known to be directly regulated by the two major quorum sensing systems of *P.aeruginosa*, the *lasR-lasI* and the *rhlR-rhlI* system[160]. Therefore, the elastase production of the mutants was assessed by the elastin-congo red conjugate assay[161]. Each mutant was assayed in triplicate and the mean value of all three experiments was calculated, compared with that of the wildtype and the percentage of wildtype elastase production was determined. All mutants produced less elastase than the wildtype SG17M strain and expectedly, the *lasR* mutant was most attenuated in the production of this virulence factor, but instead of a completely abrogated elastase secretion, 8% of wildtype levels were still observed. The mutants that proved to be most attenuated in the production of this virulence factor other than the *lasR* mutant had transposon insertions in the genes PA 3704 (the mutants 5A10, 10A3, 13A12 and 19A8 are identical), which codes for *wspE* (attributed a function in chemotaxis, motility and attachment), PA 3059 (*pelF*, carbohydrate metabolism and biofilm formation), PA 4971 (*aspP*, carbohydrate metabolism), PA 2006 (a probable transporter protein which might be involved in aromatic compound catabolism), PA 0796 (a putative carboxyenolpyruvate phosphomutase involved in carbon compound metabolism) and PA 5291 (probable choline transporter, two individual mutants, 2A12 and 23B4). Isogenic mutants of the original STM mutants have been constructed during this study and show the same phenotype regardless of their parental strain: PA 5291 mutants generated from strain TB proved to be as attenuated as those generated from strain SG17M in elastase and pyoverdin production as well as haemolysis, and their phenotype was comparable to that of the original STM mutant. All the genes listed above seem to have a function in carbon compound catabolism and mutations in these genes results in elastase production of only 20% of the wildtype levels. Mutations in the probable regulator genes PA 4070 and PA 5166 showed levels of 24% and 31% of wildtype elastase production, suggesting that their regulatory function could be possibly involved in either elastase production or secretion, whereas gene PA 4753, coding for a hypothetical protein that might belong to a novel class of RNA binding proteins seems to be of only marginal importance for elastase production (77% of wildtype elastase levels).
Figure 22 summarizes the elastase levels produced by the STM mutants in comparison to the SG17M wildtype.

[160] Pasador L, Cook JM, Gambello R, Rust L, Iglewski BH: Expression of Pseudomonas aeruginosa virulence genes requires cell-to-cell communication. Science 260, 1993, 1127-1130
[161] Ohman DE, Cryz SJ, Iglewski BH: Isolation and characterization of a Pseudomonas aeruginosa mutant that produces altered elastase. J. Bacteriol. 142, 1988, 836-842

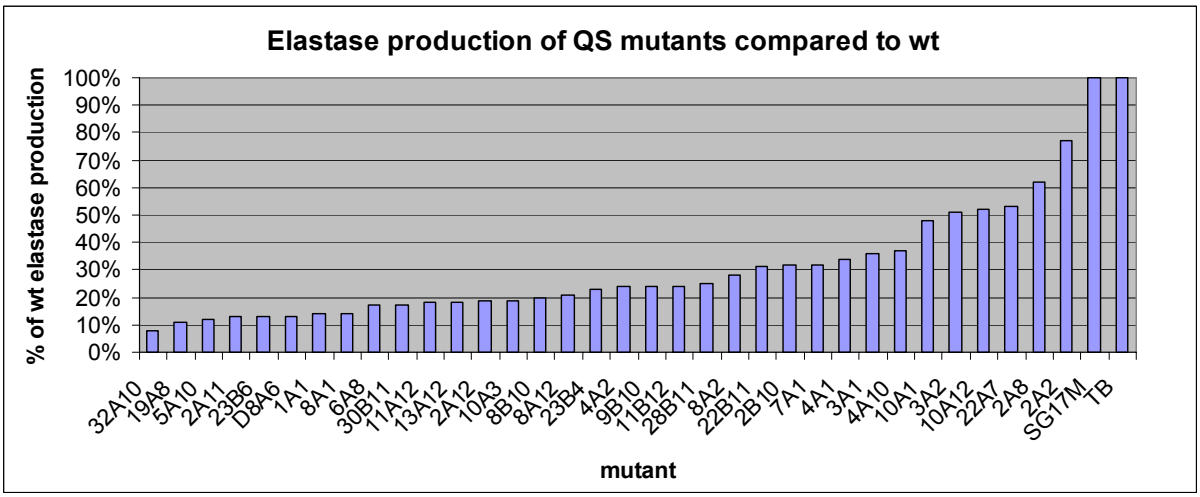

Figure 22: Percentage of wildtype elastase levels produced by transposon mutants.

Also assessed was the siderophore production of the transposon mutants according to a protocol developed by Schwyn and Neilands[162] that was slightly modified for greater sensitivity (see Materials and Methods). Bacterial siderophores represent low molecular weight Fe(III) specific ligands that have a high affinity to the metal and are excreted by bacteria to sequester iron from the environment which in the case of infection can very well be a mammalian host. Hence, siderophores have been ascribed a function as virulence factors[163] and their synthesis and regulation are a popular topic of recent research[164]. *P.aeruginosa* produces two siderophores, pyochelin and pyoverdin, which are both produced by several extra-ribosomal peptide synthethases[165] and bind to specific receptors that serve to translocate the cognate siderophore-iron complex into the bacterial cell. The method of Schwyn and Neilands does not allow for the identification of the siderophore but permits a qualitative assessment of general amount of siderophores excreted by the bacteria. The STM mutants that proved to be most attenuated in siderophore production represented by a small, sharp nearly invisible halo or almost no halo around the bacterial culture were the *wspE* mutants, mutant 22B11 (PA 5166), the *pelF* mutant (PA 3059), the mutants of PA 4971(*aspP*) and PA 5291. In the case of the PA 5291 mutants, the isogenic mutants generated by allelic replacement showed a comparable phenotype.
Not affected were mutants of PA 4753 and PA 5375, coding for a hypothetical protein and the choline transporter *bet*T1, respectively.
This assay is suitable for a quick screening for siderophore production, but mutants that show a different phenotype compared to the wildtype should be examined using more sensitive assays that also allow for the differentiation between the two siderophores produced by *P.aeruginosa*.
All genes tested in these preliminary assays showed no homoserine lactone production at all as verified by identification by luciferase assay and thin-layer liquid chromatography, done by B.Huber in Munich (see chapter 2.5.). Transcritpome data were generously provided by P. Salunkhe, Hannover.

The results of the bioassays presented here served the purpose of a quick and simple identification of genes that play a major role in quorum sensing. Database and literature research shed more light on some of the genes and yielded information for further characterization.

3.5.2. Short characterization of the genes found to be involved in quorum sensing activity of SG17M transposon mutants

PA 2127 is a conserved hypothetical gene that has not been attributed any function so far. On one side, it is flanked by another conserved hypothetical, PA 2126, and both genes show slight similarities to hypothetical gene products of *E.coli*: PA 2126 is 66% similar to the hypothetical *ybdM* gene product of *E.coli* while PA 2127 is 53% similar to the *ybdN* gene product. A similar hypothetical protein (85% similarity and 74% identity) is found in *R.solanacearum* GMI1000 on a megaplasmid as well as in the genome of *S.typhi* (56% similarity and 41% identity). Domain and function prediction in the Swissprot

[162] Schwyn B, Neilands JB: Universal chemical Assay for the Detection and Determination of Siderophores. Analytical Biochemistry 160, 1987, 47-56
[163] Ravel J, Cornelis P: Genomics of pyoverdine-mediated iron uptake in Pseudomonads. Trends in Microbiology 11 (5), 2003, 195-200
[164] Cf. 163
[165] Crosa JH, Walsh CT: Genetic and Assembly Line Enzymology of Siderophore Biosynthesis in Bacteria. Microbiol Mol Biol Rev. 66 (2), 2002, 223-249

database assign a PAPS domain to these genes, a domain characteristic for phosphoadenosine phosphosulfate (PAPS) reductase. This enzyme is a member of the adenine nucleotide α-hydrolase superfamily that uses thioredoxin as an electron donor for the reduction of PAPS to phosphoadenosine-phosphate (PAP) and is a rare example of an enzyme that is able to store two electrons. PAPS are involved in the sulphur metabolism of prototrophic organisms[166]. The only link of an enzyme like that to quorum sensing could be through S-adenosyl-methionine, which is the starter building block for homoserine lactones, but other than that, no obvious function involved in quorum sensing could be attributed to this gene. An interesting detail is that for this gene, no homologs in other *Pseudomonads* could be found, only several *Salmonella* and *E.coli* as well as *S. pyogenes*, *L.lactis* and *L.inocua* possess at least one copy of a PA 2127 homolog. What makes this gene so interesting in the context of quorum sensing is its genomic location: It is located right in front of the *cup* cluster, a group of genes that is involved in adhesion of *P.aeruginosa* to abiotic and biotic surfaces such as plastic or epithelial cells[167]. Indeed, preliminary experiments for biofilm formation revealed that this gene might have a function in *P.aeruginosa* that is related to biofilm formation, as this mutant proved to form less biofilm than the wildtype, assessed by crystal violet staining. The *cup* cluster, genes PA 2128-2132, is located on the sense strand whereas PA 2127 is located on the antisense strand flanked on the other side by PA 2126, also located on the antisense strand. The next genes upstream are found on the sense strand and code for dehydrogenases, a transcriptional regulator and another hypothetical gene. Given the role of the *cup* cluster in adherence and taking into account that the mutant 7A1 did indeed show an altered phenotype in respect to biofilm formation compared to the wildtype SG17M strain, it could be concluded that PA 2127 does influence the *cup* cluster and thus adhesion. Should the phenotype of the mutant be verified by construction of an isogenic mutant by allelic replacement which is already under way, a link between quorum sensing and adhesion in *P.aeruginosa* could have been discovered.

Another mutant that could present a link between the “novel adhesins” (*cup* genes and *pel* genes) and quorum sensing in *P.aeruginosa* is mutant 2A11. The gene inactivated by the transposon insertion is *pelF* which belongs to the *pel* operon of seven adjacent genes (PA 3058-3064), recently having been found to be involved in the synthesis of a glucose rich biofilm matrix in *P.aeruginosa* strains PA14 and ZK2870[168]. The C-terminal region of the gene PA 3059 shows sequence similarity to a family of glycosyl transferases that contains the RfaG (COG0438) glycosyl transferase motif and the pfam00534 glycosyltransferase group 1 motif[169]. Mutants in the *pel* operon have been examined for solid surface-associated biofilm formation by crystal violet (CV) staining[170] and were found not to differ significantly from the wildtype. The *pelF* mutant was also examined for biofilm formation by CV staining and was found to produce roughly the same amount of biofilm material as strain SG17M, assessed by the absorption of solubilized crystal violet. The mutant 7A1 (PA 2127), however, produced only about 50% of biofilm matrix material compared to the *pelF* mutant and the wildtype strain. Five of the seven *pel* operon gene products exhibit amino acid similarities with proteins of known functions in carbohydrate processing (*pelA, C, D, E, and F*), whereas genes *pelE* and *G* have predicted transmembrane helices and could therefore serve as transporters of carbohydrates, a feature that is also found in *pelD*. To date, the enzymatic reactions or substrates of the *pel* genes have not been identified, but it has been hypothesized that these genes either produce molecules that are essential for biofilm matrix formation, a biofilm matrix precursor, or that they produce a regulatory molecule required for the inititiation of biofilm matrix production by a series of redundant genes[171]. The regulatory molecule hypothesis does seem to be rather appealing in the case of the *pelF* mutant examined in this study, however speculative it might be, as there apparently is a link between quorum sensing and biofilm matrix production: A mutation in a gene that is known to be involved in biofilm matrix formation, *pelF*, abrogates HSL production and thus the secretion of several quorum-controlled virulence factors. Polar effects aside, this could be a very interesting path of research to follow. To ascertain the function of the *pelF* gene and to rule out polar effects exerted by the transposon insertion, an isogenic mutant should be constructed and examined. Furthermore, if the phenotype is verified, biochemical characterizations of the *pelF* gene product should unravel connections between biofilm matrix material production and quorum sensing.

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[166] Savage H, Montoya G, Svensson C, Schwenn, JD, Sinning I: Crystal structure of phosphoadenylyl sulphate (PAPS) reductase: a new family of adenine nucleotide alpha hydrolases. Structure 5(7), 1997, 859-906

[167] Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A: The chaperone/usher pathways of Pseudomonas aeruginosa: Identification of finbrial gene clusters (*cup*) and their involvement in biofilm formation. Proc. Natl. Acad. Sci. USA 98 (12), 2000, 6911-6916

[168] Friedman L, Kolter R: Genes involved in matrix formation in P.aeruginosa PA 14 biofilms. Mol Microbiol. 51(3), 2004, 675-690

[169] Mulder NJ, Apweiler R, Atwood TK, Bairoch A, Barrell D, Bateman A et al: The InterPro Database 2003 brings increased coverage and new features. Nucleic Acids Res. 31, 2003, 315-318

[170] Riedel K, personal communication, 2004

[171] Cf. 125

Not long ago, a link between quorum sensing and iron homoeostasis and metabolism was established[172]. In the presented screen, two genes that could also play a role in both quorum sensing and iron metabolism were identified: PA 2402, a probable non-ribosomal peptide synthetase and **PA 3268**, a probable *tonB*-dependent receptor membrane protein that shares 61% similarity and 46% identity with the *E.coli fecA* gene. This gene is an outer membrane receptor which binds ferric citrate and relays the signal of the bound ligand to FecR which itself transmits the signal across the cytoplasmic membrane and converts the gene FecI in the cytoplasm into an active sigma factor[173]. A homolog of PA 3268 is also found in the *P.putida* KT 2440 genome (77% identity, 88% similarity) where PP 0867 has been annotated as a *fecA*-like outer membrane receptor that is involved in binding and transporting iron and cations in general. Furthermore, PA 3901 displays 62% similarity and 46% identity with PA 3268 and has been putatively identified as Fe(III) dicitrate transport protein *fecA*. In P.luminescence, the gene plu4446 has been identified as the iron (III) dicitrate outer membrane transporter precursor protein FecA and it shows 65% similarity and 47% identity to PA 3268. The corresponding transposon mutant found in this screen, 2A11, is attenuated in siderophore production (qualitative assessment), and the elastase secretion is reduced to only 20% of wildtype level. Together with the fact that this mutant does not produce HSL, a function of this gene not only in iron metabolism but also in quorum sensing could be expected. The phenotype awaits verification by the generation of an isogenic mutant via allelic replacement to further characterize this gene.
The protein sequence reveals a rather complete *tonB*-dependent receptor domain[174] as well as a *tonB*-siderophore and a tRNA ligase domain and several putative functions have been linked to this gene: ATP binding, receptor activity, transporter activity, tRNA ligase activity and tRNA aminoacylation for protein translation. It seems that this protein could be multifunctional and therefore, an involvement in quorum sensing is not too farfetched given that although extensively researched, many features of the QS systems in *P.aeruginosa* still are not completely understood, the link to iron metabolism and homoeostasis being two of them.
The second gene that was found in this screen to possibly have a function in iron metabolism as well as quorum sensing is **PA 2402**. According to the *P.aeruginosa* genome project database, this large gene has not yet been assigned a name; as a possible functional attribution, the database lists a putative non-ribosomal peptide synthetase (NRPS). NRPS are enzymes that are essential for example in the synthesis of siderophores, making the identification of this mutant as quorum sensing-deficient as highly spectacular as well as speculative. Should the phenotype of this mutation be verified, it would imply that there is a direct regulatory link between siderophore production and quorum sensing: A gene responsible for siderophore synthesis would be essential for quorum sensing regulation or HSL synthesis as well. The closest homolog to this gene in the *P.aeruginosa* genome is PA 2400*, pvdJ*, another NRPS. In *P.putida*, the gene PP 4219 (63% similarity, 50% identity) has been annotated as *ppsD* and been assigned the function of a non-ribosomal siderophore peptide synthetase and has been linked to toxin production and resistance. In *P.syringae DC3000*, a five module gene cluster has been identified of which the five genes share 63% similarity and 49% identity with PA 2402. The genes of this cluster are responsible for the production of the *P.syringae* siderophore pyoverdine. The respective operon structure in *P.aeruginosa* is similar to that in *P.syringae*, and similar operons are also conserved in *P.putida* and *R.solanacearum*. Literature research revealed that the gene PA 2402 has been assigned the name *pvdI*[175], was identified by IVET and found to be induced in both a mouse and rat infection model. Further characterization of this gene has identified it as the NRPS that adds the amino acid sequence serine- arginine-serine-N^5-formyl-N^5-hydroxyornithine to the peptide chain of pyoverdine[176]. It is a large ORF (15450nt) coding for a 549 amino acid residue protein for which several structural domains have been identified in the Swissprot database; an AMP binding as well as an amino acid acetylation domain seem to be present in the proteine, furthermore, a phosphopantetheine attachment site and a condensation domain also were two top-scoring domains. Transcriptome data suggests that the gene is generally lowly expressed under normal growth conditions (late exponential phase in LB medium) as well as in the presence of hydrogen peroxide, PMN and paraquat in comparison to other genes. In fact, of all the quorum sensing-related genes identified in this study, PA 2402 had the lowest absolute expression level signal intensity. These

[172] Juhas M, et al: Global regulation of quorum sensing and virulence by VqsR in Pseudomonas aeruginosa. Microbiology 150, 2004, 831-841
[173] Angerer A, Enz S, Ochs M, Braun V: Transcriptional Regulation of ferric citrate transport in E.coli K-12. FecI belongs to a new subfamily of σ^{70} –type factors that respond to extracytoplasmic stimuli. Mol. Microbiol. 18, 1995,163-174
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[176] Ravel J, Cornelis P: Genomics of pyoverdine-mediated iron uptake in Pseudomonads. Trends in Microbiology 11 (5), 2003, 195-200

findings are in correspondence with the observation that the pyoverdine synthesis genes are repressed by the Fur repressor under favorable growth conditions and expressed once the iron levels are low as part of the iron starvation response. Transcriptome analysis of strain PAOI under iron starvation conditions has revealed that PA 2402 is upregulated in the same range with other genes involved in pyoverdine production. Although iron metabolism and quorum sensing have been linked on a regulatory level, a functional implication of PA 2402 has not yet been made for quorum sensing, and structural analysis has neither revealed a regulatory domain that could have anything in common with genes known to have regulator functions in quorum sensing, nor has any domain been found that could be involved in HSL production or transport. Therefore, as far as quorum sensing is concerned, further characterization of this gene could either lead to an expansion of current understanding of iron-metabolism and quorum sensing co-regulation, or further analysis of the mutant 9B10 could reveal another transposon insertion within the genome of the mutant that causes the quorum sensing-deficient phenotype. As the transposon insertion site has been determined by sequencing a PCR product that was obtained using the y-linker method, that should not be the case, however, direct genomic sequencing employing a primer specific for the transposon should be done to confirm the insertion site.

A gene identified in this screen that may not directly be responsible for the regulation of quorum sensing itself is **PA 0104**. It is located upstream of four genes on the sense strand of which three represent cytochrome oxidases (*coxAB* and *coIII*), and the fourth is a conserved hypothetical that shows some resemblance to a cytochrome assembly protein from *P.denitrificans*. PA 0104 seems to be the first gene of a 13 gene operon of which 5 have been identified as involved in cytochrome oxidation/energy metabolism. The genes PA 0105-0109 have been reported to be quorum-activated[177], so if the first gene in the operon is knocked out, transcription of the other genes could be seriously hampered so that the impact of PA 0104 on quorum sensing could be indirect. Database searches did not yield any helpful information towards the attribution of functions or structural domains, and the only relative of this gene is PP 0102 of *P.putida* KT2440 with 54% similarity and 41% identity. In this organism, it has also been annotated as hypothetical without any hint to function or structural features. Further characterization should provide more information on the role of this gene in quorum sensing; in this case, complementation *in trans* could verify whether a mutation of the gene is responsible for the abrogation of HSL production or whether it just has a polar effect of the whole operon.
Another hypothetical protein is encoded by **PA1116**, which lies on the sense strand as the only gene inmidst of a neverland of genes located on the antisense strand coding for hypothetical proteins (PA 1114-1121, although an operon structure of the whole cluster has not been verified), interrupted by a gene coding for a probable outer membrane precursor (PA 1119). A function of PA 1116 could be RNA binding, inferred from electronic annotation[178]. The region is completely unknown and has not been linked to quorum sensing yet. Genes homologous to PA 1116 have been identified in *P.syringae pv tomato* (PSPTO 1539, 74% identity and 85% similarity) and *P.putida* KT2440 (PP 4563, 70% identity and 82% similarity) as well as in *B. bacteriovorus* (Bd1395, 51% identity, 70% similarity). All these genes have been annotated as hypotheticals or conserved hypotheticals and have been attributed a function in RNA binding. Complementation studies or the construction of an isogenic mutant should be of help to understand the possible function of this gene in quorum sensing. Another gene coding for a conserved hypothetical protein is PA 2360, although an ImpA-related N-terminal domain signature could be detected. The ImpA protein is an inner membrane protein of *A. actinomycetemcomitans* involved in the export of proteins associated with colony variation in this organism. Homologs or related genes to PA 1116 could not be found, it seems that this gene is rather unique to *P.aeruginosa* PAOI. The next genes exhibiting some similarity were hypotheticals from *B.mallei* (33% identity and 47% similarity) and *R.solanacearum* (32% identity and 48% similarity). However, no other hint at a function could be found for this gene and its role in quorum sensing remains elusive.
Another interesting gene coding for a conserved hypothetical protein is PA **4753**, which shows homologies to a novel class of RNA binding proteins with a YhbY domain. *E.coli yhb*Y has 67% similarity and 43% identity to PA 4753 and belongs to a conserved family of genes represented in eubacteria, archaea and plants. Bioinformatic data suggests a role for these proteins in translation (Expasy.org database). No relation to quorum sensing has been attributed, but the gene is highly

[177] Schuster M, Lostroh CP, Ogi T, Greenberg EP: Identification, Timing and Signal Specificity of Pseudomonas aeruginosa Quorum-Controlled Genes: a Transcriptome Analysis. J.Bacteriol. 185 (7), 2003, 2066-2079
Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH: Microarray analysis of Pseudomonas aeruginosa Quorum Sensing Regulons: Effect of Growth Phase and Environment. J. Bacteriol. 185 (7), 2003, 2080-2095
[178] www.genome.jp/KEGG, www.tigr.org

conserved in *P.syringae* and *P.putida* (70 and 66% identity and 80% similarity to PA 4753, respectively). Interestingly, the knock-out mutant does not produce HSL but the elastase levels are 77% of wildtype elastase production and in terms of siderophore production, no difference has been observed for the mutant in comparison with the wildtype. To investigate its role in quorum sensing would be a start in elucidating the function of this gene.

A remarkable number of genes that have been found in the quorum sensing screen are involved in carbon compound metabolism, such as PA 0796 (a carboxyphosphoenolpyruvate phosphonomutase, CPEP), PA 1253 (a possible semialdehyde dehydrogenase), PA 4971 (attributed the function of an adenosine diphosphate sugar pyrophosphatase) and **PA 4791**, an adenosine diphosphate sugar pyrophosphatase that has also a very weak MutT signature (Pfam data). MutT is a protein first discovered in *E.coli* and homologs have been identified in eukaryotes and viruses. Its function is to remove a form of oxidatively damaged guanine (7,8-dihydro-8-oxoguanine) from DNA and the nucleotide pool[179]. The role of PA 4971 in quorum sensing is not obvious, but further experiments could clarify whether it has a function similar to MutT or is a completely different protein- after all, the MutT signature is recognizable but weak (expasy.org database).
PA 0791, *prpB*, is annotated as a CPEP and seems to play a role in carbon compound catabolism, fatty acid and phospholipids metabolism and aminophosphate-and aminosugar metabolism and shows an isocitrate lyase structural domain. A CPEP was first described in 1990 by Hidaka[180] et al and was identified to play a role in the biosynthesis of the antibiotic bialaphos by *S.hygroscopicus*. The function of the gene product of PA 0796 and its relation to quorum sensing could not be further clarified, but the gene itself is rather conserved among *Pseudomonads* as well as *Ralstoniae* and several *Salmonella* strains. PA 0796 is the second gene in an operon of 6 genes preceded by a probable transcriptional regulator with a *gntR* signature. However, there is no closer characterization of this gene available. The other members of the operon are *prpC*, a citrate synthase, PA 0794, a probable aconitate hydratase, PA0793 codes for a hypothetical protein and *prpD* is a propionate catabolic protein. Searches of databases that display metabolic pathways did not lead to the discovery of a link to quorum sensing, so that it can only be speculated that this mutation either generates a polar effect on downstream genes or the protein is important for providing building blocks for HSL biosynthesis.
PA 1253 encodes for a probable semialdehyde dehydrogenase. This enzyme belongs to a family of enzymes that oxidize aliphatic or aromatic aldehydes using NADP as a cofactor. The PA 1253 gene product could be necessary in the synthesis of the aliphatic side chain of HSL, but no obvious link to the known HSL synthesis pathway has been discovered.
Another group of genes that seem to have a relation to quorum sensing are transporters. Three mutants with transposon insertions in genes coding for betaine/choline transporters have been identified, the gene **PA 5291**, *betT2* is represented by two individual mutants and one mutant was found for the gene PA 5375 (*betT*1). At a first glance, a role for these transporters in quorum sensing is not at all obvious, but given the fact that betaine and choline play a role in osmoprotection and thus stress adaptation of the bacterium, it can be speculated that bacteria which have a defect in these genes cannot shuttle these molecules out of their cells anymore could therefore be deficient in a response towards osmotic stress. A stop in HSL production would result in the abrogation of quorum sensing and this way, the mutant could prevent other individual bacteria from “invading its space” and using up nutrients, leaving behind byproducts and waste that could represent stressors for the *betT* mutants. It could be possible that *P.aeruginosa* with mutations in the *bet* genes can simply not cope with multicellular lifestyle to which HSL production is a first step. Stopping HSL production could simply be a protection mechanism of these mutants.
A screen for quorum sensing-deficient *P.aeruginosa* mutants in strain TB has revealed that a mutation in the gene *betB*, which codes for a betaine aldehyde dehydrogenase also completely abrogates HSL production. The *bet* genes are organized in an operon (PA 5370-PA 5378) and are flanked by a Major Faciliator Superfamily (MFS) transporter downstream and an ABC transporter upstream. This operon must somehow be important for quorum sensing as a mutation in a structural gene as well as mutations in genes encoding transporter proteins lead to a complete shutting down of quorum sensing. It might be interesting to perform mutational analyses using the technique for allelic replacement adapted to both *P.aeruginosa* TB and SG17M in this study to generate mutations in every single gene of this operon and assess their phenotype in quorum sensing.
Another transporter that seems to be important for quorum sensing is the gene product of **PA 2006**. This protein shows structural features of a MFS transporter, hydrophobicity plots revealed 12

[179] Michaels ML, Miller JH: The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine. J.Bacterial 174 (20) 1992, 6321-6325
[180] Hidaka T, Imai S, Hara O, Anzai H, Murakami T, Nagaoka K, Seto H: Carboxyphosphonoenolpyruvate phosphonomutase, a novel enzyme catalyzing C-P bond formation. J.Bacteriol. 172 (6), 1990, 3066-3072

transmembrane domains and carbohydrates and aromatic compounds were predicted as substrates by the KEGG database. The gene PA 2006 lies in midst of an operon consisting of genes involved in carbon compound catabolism next to a transcriptional regulator (PA 2005) and a maleylacetoacetate isomerase. It can be suggested that this gene either has a polar effect on downstream genes which are important for the biosynthesis of HSL, or that the gene itself has a function in shuttling the HSL out of the cell. A link to the biosynthetic pathway of HSL has not been discovered by database search, and here as well as with many other genes, complementation *in trans* of the gene in question could provide more information, as could the analysis of mutants in every gene of the operon to rule out polar effects.
The last group of genes affecting quorum sensing that was identified in the screen at hand is made up of different genes involved in protein biosynthesis, transcriptional regulation and signalling.
The gene **PA 4265** is almost identical with the gene **PA 4277** (*tufA* and *tufB*, respectively). These genes are 99.7% similar with only 8 nucleotide and no amino acid exchanges and code for elongation factors. Both lie apart from each other but in operons of high similarity. They show a GTP-binding elongation factor domain and are flanked by numerous genes that are involved in transcription and translation, whether both genes code for monomers of a dimeric protein could not be determined. A direct link to quorum sensing is not obvious, and so far, a mutation in an elongation factor that abrogates quorum sensing in bacteria has not been reported in the literature. It is possible that this mutant does have a transposon insertion in the sequenced gene which is not responsible for the quorum sensing-associated phenotype but a second one is that has not been detected. For this mutant, Southern blotting and hybridization of the blotted DNA to a transposon-specific probe should reveal whether a double insertion of the transposon has occurred, and if so, the mutation can be identified. If not, it will be very interesting to uncover a link between general protein biosynthesis and quorum sensing.

The gene **PA 4070** codes for a probable transcriptional regulator with an *araC* family signature. It is located in a genomic region of genes that are either involved in carbohydrate and amino acid metabolism or encode unknown hypothetical gene proteins. Database search does not allow much insight into the function of the gene or that of its relatives. Proteins that are similar to the PA 4070 gene product are those encoded by PA 4094 which has also been annotated as a probable transcriptional regulator with an *araC* family signature (37% identity, 55% similarity), so have PSPTO 3050 of *P.syringae pv. tomato* and a set of genes from diverse *Salmonella*, these genes show the greatest extent of similarity and identity to PA 4070 (41% identity, 66% similarity). All these proteins share some degree of sequence homology in their C-terminal end (assessed by blasting the protein sequences) as described for *araC* family transcriptional regulators[181], a helix-turn-helix DNA binding motif was indicated by Pfam data for all proteins, and they seem to be involved in the regulation of carbon compound catabolism as deducted from the respective putative annotations. Probably the most prominent *araC*-family regulator is ExsA, the transcriptional regulator that positively regulates the expression of the TTSS regulon[182] in *P.aeruginosa*. PA 4070 has so far not been directly linked to quorum sensing and remains elusive; literature search has not uncovered links of *araC* family transcriptional regulators with obvious functions in quorum sensing. However, a recent study has described a regulatory protein with a function in biofilm formation in *S.aureus*[183] belonging to this family. A connection of this gene to quorum sensing in *S.aureus* has not yet been discovered in that study, but work in that direction is ongoing. As an operon structure in the genomic region of PA 4070 is likely, the transposon mutant should be examined further for polar effects of this gene by complementation and construction of an isogenic mutant, so that it can be uncovered whether the quorum sensing defect is due to regulation of the synthesis of building blocks for HSL or occurs on a different regulatory level.

The gene **PA 5166** has also been identified as encoding a probable regulator; according to the *P.aeruginosa* genome annotation database, it could be a two-component response regulator, a σ^{54} interaction domain as well as a response-regulator domain were predicted from the protein structure. Together with PA 5165, it belongs to an operon of possibly 10 genes, of which genes 1-4 are involved in cell wall/LPS and capsule synthesis (*rmlBDAC*), followed by PA 5165 and 5166, which are flanked by another 4 genes downstream that have been annotated as playing a role in C4-dicarboxylate metabolism and transport. The genes PA 5165 and 5166 could well encode a two-component

[181] Gallegos M-T, Michán C, Ramos JL: The XylS/AraC family of regulators. Nucleic Acid Research 21(4), 1993, 807-810
[182] Hovey AK, Frank DW: Analyses of the DNA-binding and transcriptional activation properties of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* exoenzyme S regulon**.** J Bacteriol. 177(15), 1995, 4427-4436
[183] Lim Y, Jana M, Luong T, Lee CY: Control of Glucose- and NaCl-Incuced Biofilm Formation by rbf in Staphylococcus aureus. J.Bacteriol. 186 (3), 2004, 722-729

regulatory system for both sets of genes as they themselves have also been attributed a function in C4-dicarboxylate transport. The bacterial σ^{54} transcriptional regulators are involved in several regulatory systems, especially in those associated with nitrogen metabolism[185] and the degradation of organic compounds[186]. In *P.aeruginosa*, it has been reported that the gene *rpoS*, a stationary phase alternative sigma factor, is regulated by quorum sensing[187]. A later study, however, contradicted this observation and concluded that RpoS itself influences the transcription of *rhlI*. RpoS has also been linked to biofilm formation[188] and to the control of the expression of the gene *exoS*, encoding the *P.aeruginosa* exoenzyme S, a recognized virulence factor. Database research has revealed that most of the bacterial proteins which contain the conserved σ^{54} interaction domain of about 230 residues belong to signal transduction two-component systems and also possess a domain that can be phosphorylated by a sensor kinase in their N-terminal section. Pfam data indicate that this is also the case with the gene product of PA 5166. A hydrophobicity plot indicated the presence of transmembrane domains and the original annotation of this gene also attributed a function as transporter of small molecules to the protein, so apart from functioning in signal transduction of quorum-sensing related pathways, it could also play a role in the transport of HSL.
There are several possibilities how this gene could be linked to quorum sensing, and after the verification of the phenotype by either complementation of the construction and examination of an isogenic mutant, further experiments should elucidate the role of this gene in quorum sensing.
Within this study, a transposon mutant library of almost 3000 mutants generated from the environmental isolate SG17M was screened for the production of homoserine lactones, proteases, elastase and siderophores. 23 individual mutants showing a phenotype different from the parental strain were identified and compared to mutants found in a similar screen performed with a STM mutant library of strain TB[189]. Surprisingly, only one mutant was found in both mutant libraries to be affected in the same gene, PA 3704, *wspE*. The SG17M transposon mutant library contains 3 individual mutants with different transposon insertions in this gene, all showing a very similar phenotype of rough colony growth and autoaggregation at low growth temperatures, no HSL production and diminished levels of siderophore and elastase production. The direct impact on quorum sensing needs to be elucidated and polar effects have to be ruled out in order to attribute a function to this gene as it is located just upstream of a GGDEF-domain containing regulator *wspR*. This regulator has proven to be responsible for altered growth behaviour in *P.aeruginosa*; similar regulatory proteins have also been found to regulate different colony morphologies in other bacterial species. The fact that only one identical gene was found to be knocked out in the construction of both mutant libraries might suggest that both libraries, which were constructed to comprise enough mutants to knock out every non-essential gene at least once, do in fact not contain enough mutants for that purpose. As a comprehensive transposon library constructed from strain PAOI has become recently available[190], this mutant library could be screened for mutants deficient in quorum sensing in the same way as the two libraries constructed from strains TB and SG17M and possibly reveal even more genes that have an impact on this regulatory network.
The phenotypic properties of the mutants that were found to differ in their quorum sensing abilities from the wildtype have to be verified in order to reliably assign a function to the genes either by complementation or the construction of isogenic mutants in the respective parental strain. A method for allelic replacement in the clinical isolates TB and SG17M has been established during the course of this study and isogenic mutants to the original STM mutants with a transposon insertion in the gene PA 5291, *betT*2, have been constructed and revealed the same phenotype as the STM mutant. Therefore, it can be concluded that the gene *betT2* is either essential for the production of HSL or has an as yet elusive regulatory function in quorum sensing or the general lifestyle of *P.aeruginosa*. Its function remains to be identified and will be part of ongoing work. Also, the genes that are involved in iron metabolism and have been found to also play a role in quorum sensing will be subjected to further

[185] Buck M, Gallegos M-T, Studholme DJ, Guo Y, Gralla JD: The Bacterial Enhancer-Dependent σ^{54} ($\sigma^{N)}$ Transcription Factor. J. Bacteriol. 182 (15), 2000, 4129-4136
[186] Ehrt S, Ornston LN, Hillen W: RpoN (σ^{54}) is required for conversion of phenol to catechol in Acinetobacter calcoaceticus. J. Bacteriol. 176, 1994, 3493-3499
-Cases I, de Lorenzo V: Genetic evidence of distinct physiological regulation mechanisms in the σ^{54} Pu promoterof Pseudomonas putida. J.Bacteriol. 182, 2000, 956-960
[187] Latifi A, Foglino M, Tanaka K, Williams P, Lazdunski A: A hierarchical quorum-sensing-cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase σ-factor RpoS. Mol. Microbiol. 21, 1996, 1137-1146
[188] Xu KD, Franklin MJ, Park CH, McFeters GE, Stewart PS: Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continuously fed Pseudomonas aeruginosa biofilms. FEMS Microbiol Lett 199, 2001, 67-71
[189] Wiehlmann L, see ref. 78
[190] Jacobs MA et al: Comprehensive transposon mutant library of Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. USA, 100(24), 2003: 14339-14344

studies which will begin with the construction of isogenic mutants and their characterization. Phenotypic comparisons should shed light on the role of the genes PA 2402 and PA 3268 in quorum sensing. Another important set of genes found in this screen represents a possible connection with HSL production and quorum sensing as well as the regulation of novel adhesins in *P.aeruginosa*. These adhesins have been recognized as virulence factors and it will be very interesting to see how the genes PA 2127 and PA 3059 are linked or co-regulated with quorum sensing. The genes representing transcriptional regulators found to be essential for HSL production will also give more insight to the regulatory mechanisms of quorum sensing. More and more interactions on a regulatory level between quorum sensing circuits and different metabolic pathways as well as virulence factor expression and transitions from one lifestyle to another are discovered, hence, it will take a great effort to elucidate the scope of this vast network and understand its intricate details. The genes discovered in this study might provide valuable information for this task.

3.6. Allelic Replacement in P.aeruginosa SG17M and TB

The major objective of functional genomics is to attribute functions to genes. Once the genome sequence of an organism is known, algorithms can be applied in order to analyze the genome for open reading frames (ORFs). When an ORF is identified, the analysis of sequence/structure relationships can shed light onto the function of the gene encoded by the ORF. In order to attribute and analyze gene function, mutational approaches are of great value as the function of the gene can either be studied directly by disrupting the gene itself or indirectly by disrupting its genomic context, i.e. neighboring genes and regulatory elements. During the last years, a reliable system for directed mutagenesis has been established for the genetic reference strain *P.aeruginosa* PAO 1 and has also been used with *P. aeruginosa* PAK[191]. In general and regardless of the bacterial species in question, all strategies for rendering a gene unfunctional rely on the introduction of an artificial sequence into the target ORF via homologous recombination and thus its disruption. The artificial sequence is commonly plasmid-borne and accompanied by a positive selection antibiotic as well as a *sacB* gene allowing for negative selection. This method is easily applied to organisms such as *H.pylori*[192] or other organisms which have a naturally high recombination frequency. It does have its limitations in *P.aeruginosa* whose natural recombination frequency is lower. Therefore, the introduction of mutations into *P.aeruginosa* that relies on homologous recombination is difficult.

3.6.1. Allelic Replacement strategies

P.aeruginosa mutants are commonly generated according to two strategies; the more common one being an introduction of an antibiotic cassette into the target gene with or without complete deletion of the gene from the chromosome (termed “allelic replacement” or “gene replacement”), the other relying on the complete deletion of the target gene (termed “gene deletion”) without introducing an antibiotic resistance into the genome. Both strategies involve PCR-generated stretches of DNA which serve as target sequences for homologous recombination, a gene cassette conferring antibiotic resistance and a *sacB* gene, all of which are combined in a suicide vector that can not be replicated in the organism of choice due to *ori* incompatibilities. The *sacB* gene needs to be under the control of a strong, constitutively expressed promoter and encodes a levansucrase which cleaves sucrose and shuttles the monomers into the cell. In order to maintain the osmotic equilibrium, the cell reacts to the intake of sucrose with increased water uptake which leads to cell swelling and finally its bursting.
The difference between the two strategies is the construction of the shuttle plasmid: The position of the antibiotic cassette in respect to the recombination target sequences is crucial as the allelic replacement aims to integrate it into the genome whereas the gene deletion strategy generates an “antibiotic-free” mutation.

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[191] -Schweizer HP, Hoang TT: An improved system for gene replacement and xylE fusion analysis in Pseudomonas aeruginosa. Gene 158, 1995, 15-22
-Priebe GP, Brining MM, Hatano K, Grout M, Coleman FT, Pier GB, Goldberg, JB: Construction and Characterization of a Live, Attenuated aroA Deletion Mutant of Pseudomonas aeruginosa as a Candidate Intranasal Vaccine. Infect Immun 70 (3), 2002, 1507-1517
-Rahim R, Burrows LL, Monteiro MA, Perry MB, Lam JS: Involvement of the rml locus in core oligosaccharide assembly in Pseudomonas aeruginosa. Microbiology, 146, 2803-2814

[192] Yuan JP et al: Deletion of Helicobacter pylori vacuolating cytotoxin gene by introduction of directed mutagenesis, World J Gastroenterol, 9 (10), 2003, 2251-2257

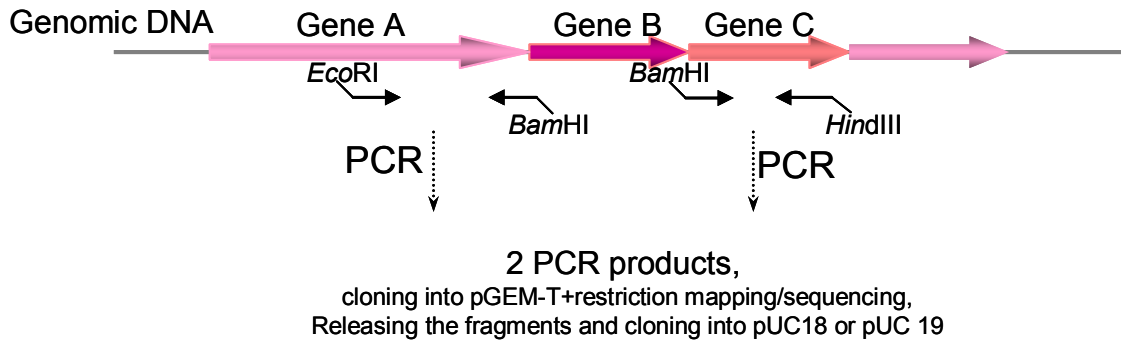
[195] Promega Corporation: Technical Manual for pGEM®-T and pGEM®-T *Easy* vector systems, 03/2003, Madison, WI, USA

3.6.1.1. Strategy I: Allelic replacement

The allelic replacement strategy aims at replacement of the functional gene by one that is disrupted by an antibiotic cassette or by replacing the whole gene with the resistance cassette, resulting in a knock-out phenotype.

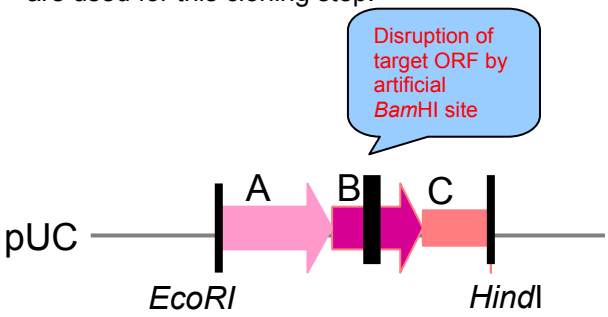
For the allelic replacement strategy, the shuttle plasmid is constructed as follows: Two PCR products of at least 500 bp are generated downstream and upstream of the gene to be analyzed using primers that introduce specific restriction sites on both ends of the PCR products. The restriction sites at the outer ends are designed to be compatible with selected sites from the shuttle vector and serve to link the DNA stretches with the vector; the inner sites are designed to be compatible with sites from a suitable resistance cassette and are used to integrate the cassette between the two PCR products.

Step I: Generation of two PCR products on genomic DNA from regions flanking the gene of interest ("target ORF", gene B).



The PCR products are cloned into a subcloning vector that has a single *T* overhang to complement a single added *A* overhang to each end of the PCR product by specific polymerases[195]. In order to control whether the products have the desired properties, sequencing or informative restriction mapping should follow the subcloning procedure.

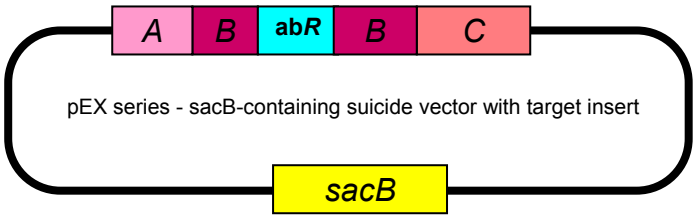
Step II: The generated PCR products are excised from their subcloning vectors, purified and ligated into a second vector so that they are combined and make up the deletion sequence. Commonly, vectors of the pUC series[196] are used for this cloning step.



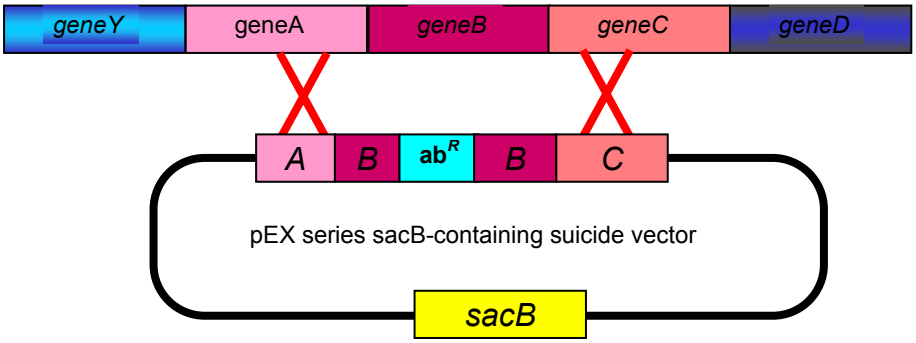
A suitable antibiotic cassette can now be introduced into the *Bam*HI site and further disrupt the target ORF.

Step III: Excision of the complete deletion sequence, in the illustrated case with *Eco*RI and *Hind*III and ligation into a non-replicative, *sacB* containing suicide vector, i.e. pEX 100T, pEX 18 series[197]. The accordingly generated allelic replacement vector is then transformed into *E.coli* and shuttled into *P.aeruginosa* via triparental mating.

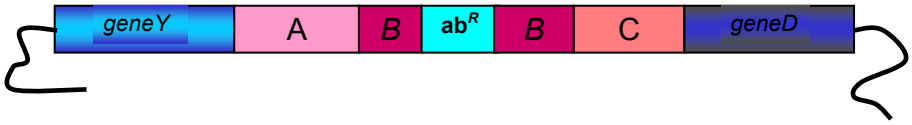
[196] New England Biolabs, Catalog 2005
[197] GeneBank accession numbers U17500 (pEX100T), AF004910 (pEX18Ap), AF047518 (pEX18Gm), AF047519 (pEX18Tc)



Homologous recombination can then take part: The first recombination step is driven by the selection pressure exerted by the antibiotic; the second step is promoted by *sacB*-expression.



The mutant chromosome, with the antibiotic cassette (ab^R) disrupting gene B:



An obvious disadvantage of this strategy is the need for numerous subcloning steps; each subcloning procedure can possibly produce rearranged plasmids[198], the procedure is time-consuming and the loss of material is high. Furthermore, it involves two PCR products and hence is more prone to mutations generated by the polymerase. The PCR products of the flanking regions of the gene of interest have to be thoroughly analyzed as mutations could lead to secondary effects which would make any phenotypic attributions to the disrupted gene difficult. However, most of the mutants constructed so far in *P. aeruginosa* PAOI as well as in a few clinical isolates[199] have been the result of applying this strategy[199]. Nevertheless, the mutagenesis in clinical isolates proves to be difficult and does not work for every target gene[201].

3.6.1.2. Strategy II: Gene Deletion

A different strategy has been developed by Cornelis et.al.[202]. The objective of this strategy is the deletion of the whole target gene from the genome to be examined. It relies on the construction of a PCR product from flanking regions of a target ORF that do not belong to the ORF itself containing an artificial stretch of DNA as deletion sequence and an antibiotic cassette outside of this sequence. For this application, the non-replicative plasmid pKNG 101 has been constructed; a *sacB* gene is encoded on this plasmid as well as a streptomycin resistance. The deletion sequence can be inserted into several unique restriction sites all over the plasmid. A drawback of this plasmid is its size of almost 7 kb, furthermore, a streptomycin resistance is not applicable to a wide range of *P.aeruginosa* isolates, and particularly clinical isolates of this species often exhibit a high intrinsic resistance to this antibiotic.

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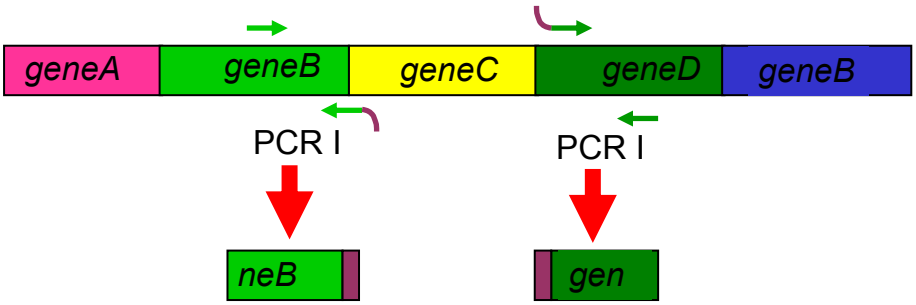
[198] Attree, I., Larbig, K., Schweizer,H.P., Wiehlmann, L.: Personal communications 2001-2004
[199] Dacheux D, Attree,I, Schneider C, Toussaint B: Cell Death of Human Polymorphonuclear Neutrophils induced by a Pseudomonas aeruginosa Cystic Fibrosis Isolate Requires a Functional Type III Secretion System. Infect. Immun. 67(11), 1999, 6164-6167
[199] Ha UH, Wang Y, Jin S: DsbA of Pseudomonas aeruginosa Is Essential for Multiple Virulence Factors. Infect. Immun. 71(3), 2003,1590-1595
[201]Schweizer HP, personal communication, 2002, Ohman D, Storey D, personal communication, 2003
[202] Kaniga K, Delor I, Cornelis GR: A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the blaA gene of Yersinia enterocolitica.
Gene. 20;109(1), 1991, 137-41

For this approach, a suitable vector needs to incorporate a marker for selection in *E.coli*, one for selection in *P.aeruginosa*-or if possible, the same antibiotic could be used for selection in both organisms-should not be too large and contain unique restriction sites that facilitate the introduction of the artificial sequence. Furthermore, a *sacB* gene is needed to select against integration of the complete plasmid into the target genome, as in strategy I. In this case, the antibiotic cassette used for selection in *P.aeruginosa* is located outside of the recombination target sites as a part of the suicide vector, so that the gene replacement can function without integrating the selection antibiotic into the chromosome.
The gene deletion according to this strategy is a multistep process as well. Compared to the allelic replacement strategy, it is a less straight-forward approach involving even more PCR constructs and cloning steps:

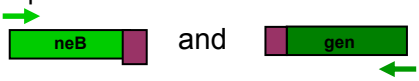
Step I: constructing two PCR products on genomic DNA of the strain to be analyzed, each with a suitable restriction site at its one end and an overhang complementary to that of the second product on the other:



Purple: compatible overhangs

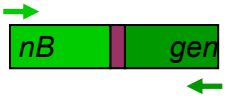
Step II: An asymmetric PCR primed by the oligonucleotide designed for the ends of the product in step I bearing the restriction sites is conducted. This step generates two single-stranded products that have the 5' restriction site and the 3' complementary overhang.

PCR II: Generation of single-strand products



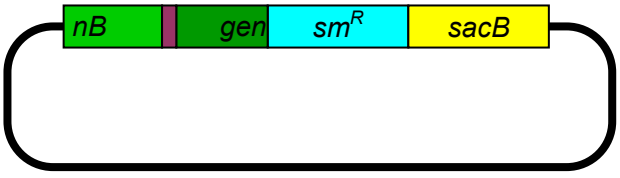
Step III: A third PCR is carried out in two steps: The first 10 cycles are self-primed; the single-stranded molecules can anneal and form double strands which then are filled in by the polymerase. After this phase is completed, the primers with the restriction sites are added and the PCR is carried out for 30 or more cycles to yield the double-stranded deletion product.

PCR III: Generation of the recombination target sequence



Step IV: The deletion product is cut with the specific endonucleases and ligated into the equally cut pKNG 101 or any other suicide vector which in turn is then transformed into *E.coli* to be analyzed.

Ligation of the target sequence into a vector for gene deletion:



After confirming the properties of the insert sequence, this construct can be transferred to *P.aeruginosa* by triparental mating. The first recombination step integrates the whole plasmid into the genome and is triggered by selection pressure (in this case, streptomycin). The second recombination step is promoted by the *sacB* expression as in strategy I.

This method involves several PCR-based steps and might produce deletion sequences with mismatched nucleotides which could lead to mutations. Furthermore, polar effects can never be completely ruled out as the gene itself is not mutated but completely deleted, and flanking regions serve as recombination target sites. Recombination events can produce secondary mutations and rearrangements[203]. Therefore, it has to be cautiously verified that the mutation that was introduced into the genome is indeed the desired and only one. However, a mutagenesis that does not introduce an antibiotic cassette into the genome of interest is a rather attractive notion as the mutant strain can be subjected to further mutagenesis. However, the drawbacks of this method have prevented it from being widely applied[204].
As mutagenesis in clinical isolates of *P.aeruginosa* is generally not a trivial task, an allelic replacement strategy that relies on as few intermediary steps as possible would be beneficial. Therefore, the allelic replacement strategy of Schweizer was modified and tested on a large gene not present in the PAOI genome.

3.6.2. Allelic Replacement employing a whole-gene approach

A technique fulfilling these criteria for allelic replacement in *P.aeruginosa* clinical isolates would have to rely on a gene-spanning PCR product and the use of naturally occurring restriction sites. These sites can be used to either delete a part of the gene, religate the remaining parts so that the recombination target sites are present but a part of the original gene is missing, or an antibiotic cassette with suitable restriction sites can be introduced into the recombination target sequence. The resulting deletion sequence is similar to that of the original allelic replacement strategy established by Schweizer, but one PCR should be sufficient to yield the product, and the downstream applications consist of only two cloning procedures.
An outline for this method has been designed by the author. It consists of several steps and is illustrated using the non-PAOI gene D8A6 as example. This gene is essential for quorum sensing in the *P.aeruginosa* clinical isolate TB and is as yet almost uncharacterized.

1. Analysis of the gene to be replaced concerning DNA structure, naturally occurring restriction sites and compatibility with a suitable antibiotic cassette so that no restriction sites in the gene chosen for cloning procedures occur within the antibiotic cassette and the delivery vector. The results serve as a basis for constructing the deletion fragment. An analysis of this sort can easily be carried out using the NEB cutter software version 2.0 free of charge, available on the NEB homepage (www.neb.com).
2. Selecting a *sacB*-containing vector with suitable restriction sites to match those that are naturally occurring in the sequence of the gene in question so that a DNA stretch of 1000 bp or more can serve as deletion sequence. Generally, the suicide vectors of the pEX18 series developed by H. Schweizer are equipped with the widest range of restriction sites and have therefore been frequently used for allelic replacement approaches.
3. PCR of the target sequence on the genomic DNA of the organism in question, if possible with gene-spanning primers to avoid polar effects. If the gene is large enough, a part of the gene will also be sufficient as recombination site.

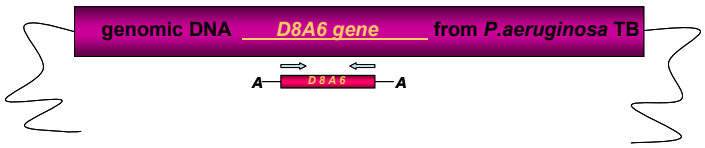


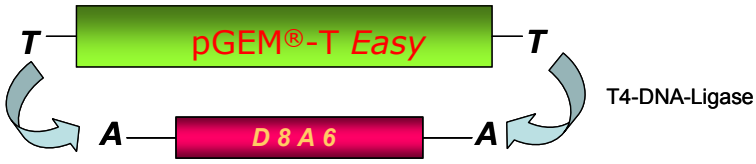
Figure 23: Generation of a recombination target sequence from genomic DNA of P.aeruginosa TB

4. In order to introduce an antibiotic resistance cassette into the recombination target sequence, a PCR product of the antibiotic cassette with primers that generate a suitable restriction site at the ends of the resistance-conferring gene can be constructed. Here, it is crucial to allow for the amplification of a fragment that is larger than the resistance-conferring gene itself in order to avoid inactivating the gene by mismatch mutations due to the primer-generated restriction site. Alternatively, if a suitable restriction site for a blunt-end cutting endonuclease or selected sticky-end cutting enzymes is present close to or in the middle of the target sequence, a gentamicin resistance cassette can be cut out of the vector pUC-*Gm*. This cassette is flanked by two identical multicloning sites with the recognition sequences of several useful restriction endonucleases; the one closest to the gene is that of the blunt-

[203] Schweizer HP, personal communication, 2003
[204] Ohman D, personal communication, 2003

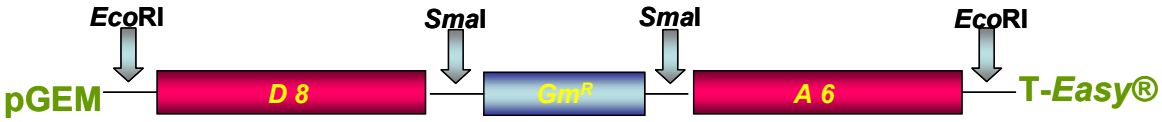
end cutting endonuclease *Sma*I. If the target sequence can be cut with a suitable restriction enzyme and the cassette ligated into the recombination target sequence, this approach would be preferable to generating a PCR product of the resistance cassette: As a polymerase statistically inserts a misplaced nucleotide every 1000 bp, these mismatched nucleotide(s) can lead to a mutation that could render the cassette unfunctional.

5. Subcloning of the deletion fragment and a PCR-generated resistance gene into linearized subcloning vectors that are commercially available. This greatly facilitates the excision of the PCR products and broadens the choice of restriction sites as most of the commercially available subcloning vectors contain convenient multi-cloning sites with rare-cutting endonucleases. However, the deletion sequence can also be cloned directly into a suicide vector of the pEX series and the subcloning step can be omitted:



6. Excision of the resistance cassette and gel-purification, restriction digest of the subcloning vector containing the target DNA sequence with restriction enzymes compatible with sites flanking the resistance gene. Thorough dephosphorylation of this vector prevents vector religation and increases ligation efficiency. If the target sequence is cloned directly into the suicide vector, this step is carried out with the purified cassette and the suicide vector-target DNA insert construct.

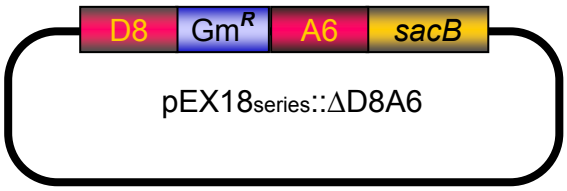
7. The gel-purified PCR product or restriction fragment of the resistance cassette is then ligated into the linearized vector containing the target DNA sequence and the ligation product transformed into a suitable *E.coli* strain, i.e. DH5α or its derivatives which is then grown on a medium containing the selection antibiotic.



8. Positive clones are selected for plasmid extraction and the plasmid structure is verified by restriction analysis.

The deletion sequence is cut out and ligated into the *sacB*-containing vector which in turn is then transformed into *E.coli*, extracted and analyzed:

Suicide vector-insert construct:



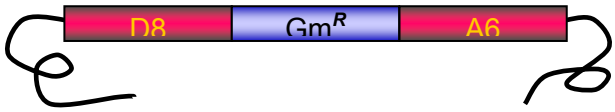
9. The vector containing the correct insert which consists of the target DNA sequence separated in two parts by the antibiotic cassette is chosen for further experiments. The two parts derived from the target gene serve as recombination sites and should consist of 400 base pairs minimum; the antibiotic cassette will serve as positive selection marker.

10. The vector is then either electroporated into the recipient *P.aeruginosa* strain or transferred via triparental mating. The bacteria are subjected to selection pressure by the antibiotic for which the resistance is encoded within the target sequence. This way, a first homologous recombination step is forced, integrating the plasmid into the genome. If the triparental mating technique is employed, selection is done on a minimal medium with a carbon source that the donor strain cannot metabolize, e.g. M9 medium with benzoate.

11. Positive transformants resistant to the selection antibiotic are picked off the antibiotic-containing medium and transferred to LB medium supplemented with 5% sucrose and the selection antibiotic to promote plasmid loss via a second recombination step.

12. Colonies that did not take up sucrose and are selection antibiotic resistant can be considered as mutants and are picked and transferred to a medium that contains an antibiotic for which a resistance-conferring gene is encoded on the plasmid. For the most common sacB-containing

vectors, this is a beta-lactamase and the antibiotic of choice for *P.aeruginosa* in this case is carbenicillin. Colonies that have acquired resistance for the antibiotic used for positive selection and are sensitive to the plasmid-encoded antibiotic have integrated the resistance gene for the selection antibiotic into the genome via homologous recombination and lost the rest of the plasmid.
The mutant *P.aeruginosa* chromosome:



13. The insertion site should be verified on the genome from both sides by PCR with one primer on the targeted gene or upstream and one in the resistance cassette and the products sequenced, or a PCR product of the whole construct can be generated and sequenced. Furthermore, Southern blotting can be used to verify the insertion and its genomic location.

This technique has been employed in the form presented here for the generation of mutants in two different *P.aeruginosa* isolates and has yielded reliable results. Nevertheless, the construction of mutants from isolates other than PAOI using this technique still remains a demanding task.

3.6.3. Results for the Gene Deletion Method

An STM mutant library of the environmental *P.aeruginosa* isolate SG17M was constructed in this study comprising about 3000 individual mutants. Furthermore, another mutant library constructed from the clinical *P.aeruginosa* isolate TB was constructed by L. Wiehlmann[205]; this mutant library consists of approximately 4600 mutants. Both mutant libraries have been subjected to several bioassays in order to reveal genes important for a distinct phenotype and a rather large number of different genes has been attributed a function as results of these screens. To confirm the phenotypical properties of the transposon mutants, isogenic mutants needed to be constructed by targeted mutagenesis and compared phenotypically to the original transposon mutants. This way, the impact of the gene found in the bioassay can be verified. Meanwhile, transposon mutant libraries generated from the isolates *P.aeruginosa* PAOI and PA14 have been constructed and are made available to the Pseudomonas community[206]. However, several genes have been found in these screens that are not present in the PAOI genome and therefore have not been sequenced. One of these genes, D8A6, was found to have a function in intracellular survival of strain TB within PMN as well as in quorum sensing. Furthermore, the D8A6 mutant was found to be attenuated in other virulence models[207]. Approaches of complementing this gene *in trans* failed due to as yet unknown reasons[208], hence the construction of an isogenic mutant was essential. The experiments with allelic replacement techniques for this study were however started with the gene PA 1288, which seemed to have the greatest impact on intracellular survival and quorum sensing on strain TB. It is an outer membrane transporter protein and was investigated when the comprehensive transposon libraries had not yet been available.
The gene deletion strategy seemed ideal for mutagenesis of strain TB as it does not introduce an antibiotic cassette into the parental strain's genome and would allow for the generation of several mutations in the same genome. Therefore, this approach was tried first to construct a PA 1288 deletion mutant.
A difficulty of this approach was to find a suitable vector for delivery of the recognition target sequence-the vector pKNG 101, which was used for an approach like this by Cornelis et al. in *Yersinia* carries a streptomycin resistance gene. Both TB and SG17M, however, are highly resistant to streptomycin, hence, this antibiotic could definitely not be used for selection of mutants from these strains. Attempts were made to change the resistance cassette, but no ligation results were obtained either with this vector and a tetracycline cassette from p*MOD-OTc*, nor with a chloramphenicol resistance cassette, both cut with suitable restriction endonucleases and gel-purified.
The vector p*MOD*-OGm had been successfully used for transposon mutagenesis in TB and SG17M, so with a few alterations, it was assumed it could also serve as a shuttle vector for a recombination target sequence: its replication origin can not be used for replication by *P. aeruginosa,* and the plasmid-encoded gentamicin cassette works well in both strains.

[205] Wiehlmann L: Dissertation
[206] Jacobs MA et al: Comprehensive transposon mutant library of Pseudomonas aeruginosa. Proc. Natl.Acad.Sci., 100(24), 2003, 14339-14344
[207] Wiehlmann L: personal communication
[208] Salunkhe P: personal communication

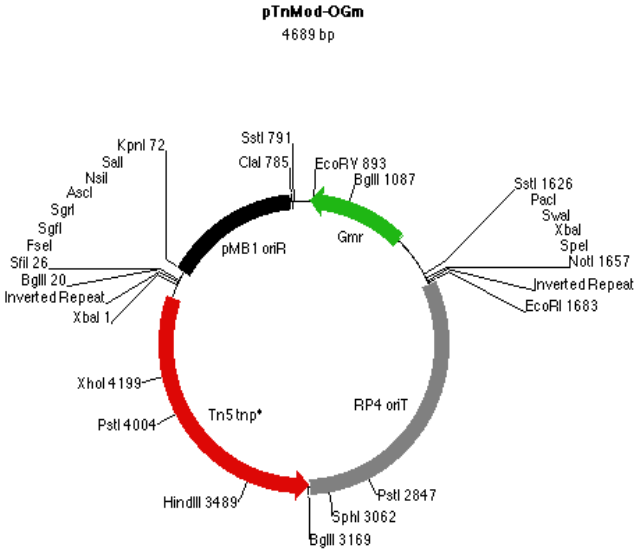


Figure 24: plasmid pMOD-OGM

Two alterations had to be made in order to use pMOD as a shuttle vector for gene deletion: The transposase encoded outside of the IS elements needed to be removed or rendered unfunctional by deleting a part of the gene so that no transposition events would thwart the homologous recombination of the plasmid-borne sequences with the genome. Furthermore, a *sacB* gene for negative selection had to be introduced, preferably in exchange for the transposase gene to keep the vector size to a minimum. Therefore, the vector was cut into two parts with *Nsi*I and *EcoR*I, yielding a 1619 bp and a 3070 bp fragment, corresponding to the upper (nt 64-1683) and lower part of the vector (nt 1683-1), respectively (see vector map). This step was necessary because the *Bgl*II restriction site at position 3169 was to be used for the introduction of a *sacB* gene and a second *Bgl*II site is present at position 1087 within the resistance gene.

The upper part of the vector contains the origin of replication and the gentamicin resistance cassette, the lower part the transposase and an RP4 *ori*T which is essential for transferring the vector by conjugation.

The 3070 bp fragment needed to be digested with *Bgl*II (sites at position 20 and 3169) in order to introduce a sacB gene flanked by a *Bam*HI and a *Pst*I site. *Bam*HI produces a compatible overhang to BglII and the overhang generated by *Pst*I is compatible to *Nsi*I, [209] and fragments cut with these enzymes could therefore be ligated. The restriction sites were subsequently lost, facilitating handling of any DNA to be inserted into the vector. A *Bgl*II digest of the 3070 bp fragment yielded a 1489 bp fragment (nt 1680-3169) and a 1529 bp fragment (transposase gene + 64 nt distance to *Nsi*I site). Fragments with such a little size difference are almost impossible to separate by gel electrophoresis, and as a ligation reaction is rather sensitive to contamination, the fragments to be ligated needed to be as pure as possible. Therefore, the 3070 bp band was digested with *Bgl*II and *Xho*I to generate three fragments: nt 1680-nt 3169 (1489 bp), 3169-4199 (1030bp) and a 490 bp fragment (4199-20). The significant size difference made it possible to separate the two larger bands by gel electrophoresis.

The *sacB* gene was released from the vector pSB5[210] with *Pst*I and *BamH*I The two fragments from pMOD-Gm and the sacB gene were ligated using the NEB Quick Ligation Kit; the fragments were ligated in a 1:1:1 ratio employing 100 µg of DNA each in a 20 µl reaction. The ligation mix was transformed into *E.coli* Top10 cells (Invitrogen) and selected on gentamicin. Positive transformants were picked and the plasmid was extracted for analysis.

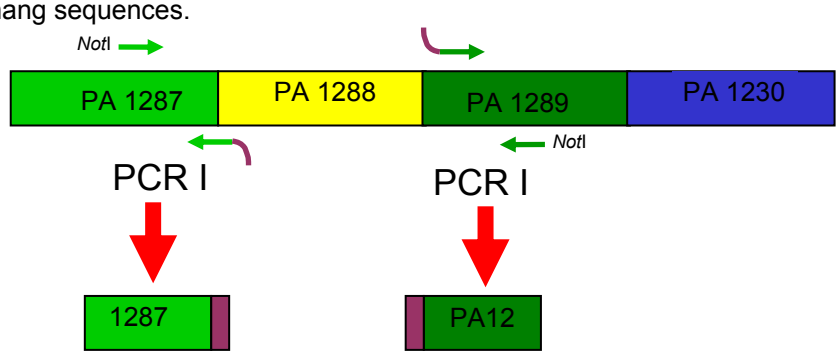
Plasmids with the following restriction patterns were considered correctly ligated and selected for further use:

Restriction enzyme	Band I size	Band II size	Band III size	Position between λ-*BstE*II-standard bands
*Kpn*I	4562	1046	---	5&6 (I) and 11&12 (II)
*Dra*I	2573	2356	612	7&8 (I), band 8 (II), below band 12 (III),
*Cla*I	3202	2126	277	7&8 (I), 8&9 (II), band 13 (III)

Table 12: Diagnostic restriction fragments for pMOD-SacB-constructs.

[209] NEB catalog 2003
[210] Generous gift of G.F.Gerlach, Hannover, Germany

With the shuttle vector pMOD-*sacB* for gene replacement available, a recombination target sequence needed to be constructed.
Two PCR products flanking the gene 1288 were generated: a 305 bp product was amplified from gene PA 1287 and a 440 bp product was amplified from gene 1289. The primers for both products were constructed as follows: the forward primers carried a *Not*I recognition site to allow for the ligation into the shuttle vector, and the reverse primers were equipped with a 50 bp complementary overhang sequence which would allow for the annealing of two single stranded products generated from the respective double-stranded DNA stretches to serve as recombination target sequence.
Step I: Generation of two PCR products to serve as templates for single stranded products. Purple: compatible overhang sequences.



The picture of the agarose gel (figure 23) shows the products in lanes 2 and 3 (1287, 647 bp) and 5 and 6 (1289, 781bp). Standard in lanes 1, 4, and 7 : λ-*BstE*II, corresponding band size: 702 bp (1.5% agarose gel).

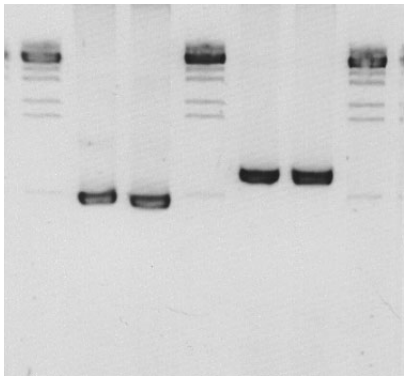


Figure 23

Step II: Generation of single-stranded PCR products:

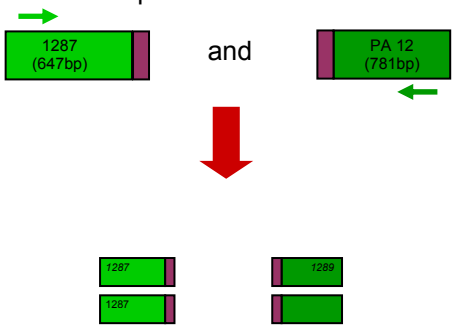


Figure 24 shows the single-stranded products, the by-bands represent the double-stranded products. A separation of the two DNA strands at this point is not possible.
The PCR reactions were purified by centricon filter devices and the eluate was used for the hybridization PCR.

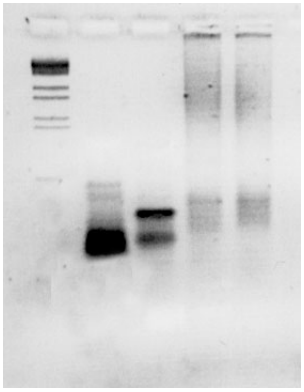
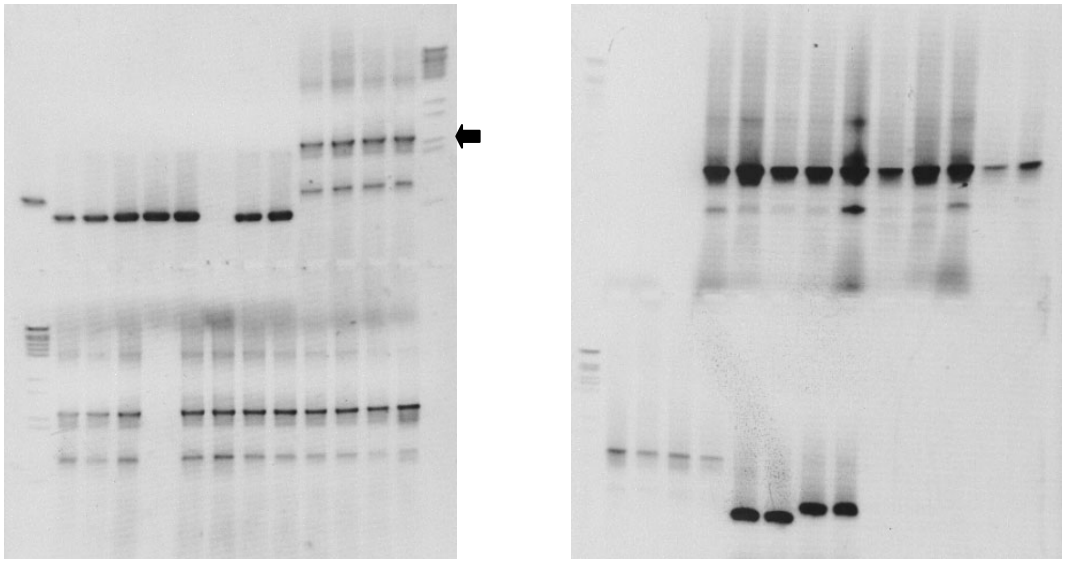


Figure 24

Step III: Generation of a recombination target sequence of the following form:

The first cycles of the PCR needed to be self-primed so that the compatible overhangs of the single-stranded products could anneal and the polymerase was able to fill in the rest of the strands. Afterwards, the *Not*I-site primers were added to the reaction mix and overall 40 cycles were run. The resulting double-stranded recombination target sequences of 1428 bp are depicted in picture25.a), red arrow. The λ-*BstE*II band in lane I corresponds to a size of 1371 bp and is slightly smaller than the larger PCR product (1.2% agarose gel).



picture a picture b

Figure 25: double stranded “hybridization” products generated from single stranded products by a combination of self-primed and conventionally primed PCR cycles.
Lanes from left to right, figure a: λ-*Bst*EII standard on lane 14 (upper half) and 1 (lower half), figure b: lane 1 on upper and lower half.
Picture a shows gel-purified single stranded products on lanes 1 (PA1289) and 2-6 (PA1287), negative control on lane 7, and two 1287 products generated with a different polymerase on lanes 8 and 9. The upper bands on lanes 10-13 represent the double stranded product of the recombination target sequence, lanes 10 and 11 after 10 self-primed cycles, lanes 12 and 13 after 15 self-primed cycles. The annealing temperature used for these PCR reactions was 64°C. Lower half of the gel: PCR products obtained at an annealing temperature of 62°C. Lanes 1, 2: 5 self-primed cycles. Lane 3: 8 self-primed cycles, lane 4: negative control; lanes 5,6: 10 self-primed cycles, lanes 7,8: 15 self-primed cycles, lanes 9,10: 20 self-primed cycles, lanes 11,12: 30 self-primed cycles.
Picture b, upper half of the gel: lanes 2-4: negative controls, lanes 5-14: double-stranded products generated from the single stranded products at an annealing temperature of 62°C with 15 self-primed cycles and 40 cycles run in total. For PCR programs, see material and methods. Lower half: lanes 2-5:

double-stranded products obtained at an annealing temperature of 64°C, lanes 6-9: double stranded templates: PA1287 (lanes 6 and 7), PA1289 (lanes 8 and 9).

The PCR products were excised from the gel and purified using the Qiagen gel elution kit, digested with *Not*I, the nuclease was extracted with phenol-chloroform and the purified DNA used in a ligation reaction with *Not*I (nt 1657)-digested and purified pMOD-*sacB*. The ligation was transformed into *E.coli* Top10 cells but no positive clones were obtained. Several ligation kits were tried, but none yielded either ligation product or transformants. Restriction mapping of the recombination target sequence did not provide a reason for the ligation failure, but several other *Not*I-digested vectors were tried for ligation with the recombination target sequence as well, and none of them yielded any ligation products or transformants. Therefore, the cause of ligation failure must be related to the *Not*I sites of the insert. At a later time point, the pMOD-*sacB* vector was tested with a recombination target sequence obtained from gene PA5291; a ligation product was obtained as were transformants in *E.coli*, but the vector/insert construct failed to yield sucrose-resistant/gentamicin resistant *P.aeruginosa* transformants that showed a phenotype different from the parental strain. This result suggests that the *sacB* gene was not properly expressed and the gentamicin-resistant transformants were merodiploids that had integrated the whole plasmid with one recombination event. The pressure forcing the transformants to lose the plasmid would have been exerted by a functional *sacB* gene, suggesting that the gene either was not expressed at all or mutated in some form that rendered it unfunctional. Therefore, the strategy was changed from gene deletion to allelic replacement employing the approach presented in chapter 3.6.2.

3.6.4. Results for Allelic Replacement

The D8A6 gene, which is not present in the PAOI genome, was selected as a target for generating a knock-out mutant isogenic to an original STM mutant from strain TB carrying a transposon insertion in at least one homolog of this gene. The isolate TB has homologs of the D8A6 gene in 3 copies, and SG17M has homologous genes, one of which is SG 57, which are present in the strain's genome in four copies[211].
The sequence of the D8A6 gene and its vicinity was analyzed for suitable restriction sites using the free NEB cutter 2.0 software available on the NEB homepage. The D8A6 region obtained by sequencing of a plasmid-rescue product from the D8A6 transposon mutant encompasses 5550 bp, the gene itself ranges from nt 1357 to 4307 (2950bp). This large gene could not be used as a whole for the generation of a recombination target sequence as it would generate an unnecessarily large shuttle vector, especially as the vector/insert construct was to be electroporated into the *P.aeruginosa* isolates. The electroporation procedure is most efficient with vectors the size of 3000-6000 bp[212], and as the pEX18 series vectors are already rather large (pEX18Ap: 5842 bp), a balance between an insert as small as possible with a recombination target sequence as large as possible had to be found. Therefore, a PCR product from nt 1600 to nt 3252 of the 5550 bp sequence was generated (figure 26) by conventional PCR with a *Sma*I restriction site located conveniently at position 2493, thus separating the PCR product into two parts of almost equal size:

[211] Klockgether J: personal communication, 2004.
[212] V.Götz, F ; v.Pall-de Tolna, S. : Personal communication, 2003

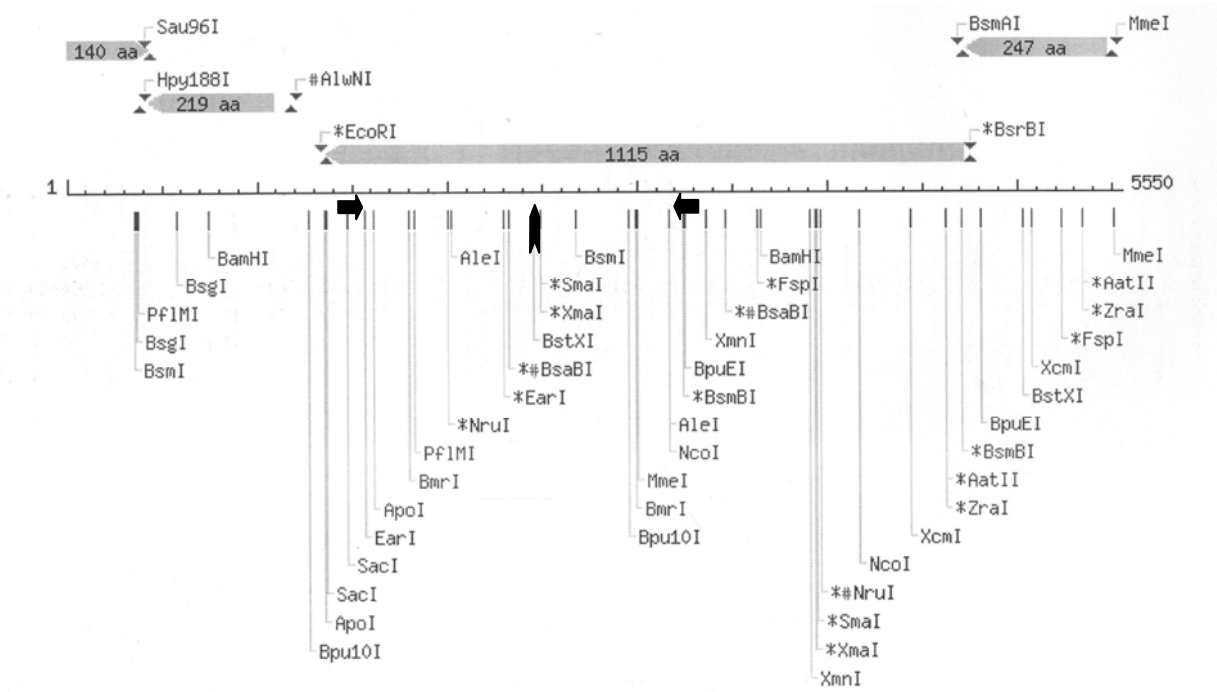


Figure 26: Restriction map for double-cutting enzymes obtained from NEB cutter 2.0 (www.neb.com). Primers for the generation of the 1652 bp PCR product are represented by horizontal arrows, the *Sma*I recognition site is marked by a vertical arrow.

The PCR product was subcloned into pGEM-T*Easy* and this vector/insert construct transformed into *E.coli* Top10 cells.

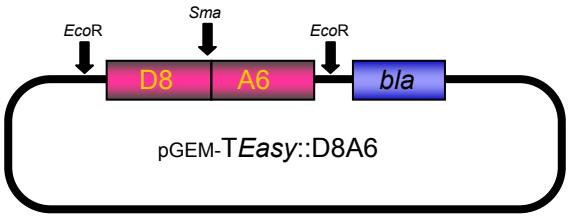


figure 27: vector pGEM-T*Easy* containing the PCR-generated D8A6 recombination target sequence, *bla* = beta lactamase, Amp^R selectable marker encoded on the vector.

Positive transformants were selected for plasmid extraction; the insert was released from the vector by digestion with *Eco*RI and examined for its size; moreover, the construct was linearized with *Sma*I to verify the presence of the restriction site.
Figure 28 shows the *Eco*RI digest: fragment I corresponds to a size of approximately 3000 bp (vector band, pGEM-T*Easy*, 3015 bp), the smaller band represents the recognition target site a size of approximately 1700 bp (D8A6 recognition target sequence: 1652 bp, 1% agarose gel).

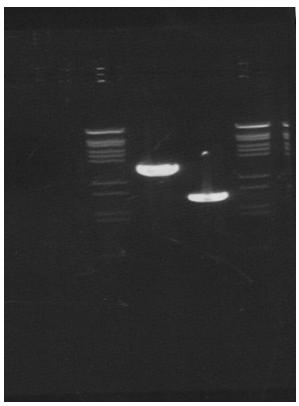


Figure 28: Lanes 1 and 4: λ-*Bst*EII standard, lane 2: 3015 bp fragment of pGEM-T*Easy*, obtained by releasing the insert with *Eco*RI. Lane 3: 1652 bp fragment of the D8A6 recombination target sequence released from pGEM-T*Easy*
The vector/insert construct was then digested with *Sma*I, dephosphorylated twice and phenol-chloroform purified. A fragment of 855 bp containing the gentamicin resistance gene was released with *Sma*I from vector pUC-*Gm* and gel-purified.

Figure 29 shows both fragments, the SmaI-linearized vector pGEM-T*Easy* containing the D8A6 recombination target sequence on lane 2 of the gel, and the gentamicin cassette fragment on lane 3 (1% agarose gel). The gentamicin cassette was then ligated into the linearized vector and the ligation reaction was transformed into *E.coli* Top10 cells. Gentamicin-resistant transformants were selected and plasmid extraction yielded pGEM-T*Easy*::ΔD8A6. An *Eco*RI control digest confirmed the size of the D8A6 recombination target sequence with the inserted gentamicin cassette (ΔD8A6) to 2507 bp as seen in figure 29 (second fragment in lane 2, the first fragment corresponds to the vector band; a plasmid with an insert of the wrong size can be seen on lane 3, lanes 4 and 5 obviously show partial digests of a correct plasmid).

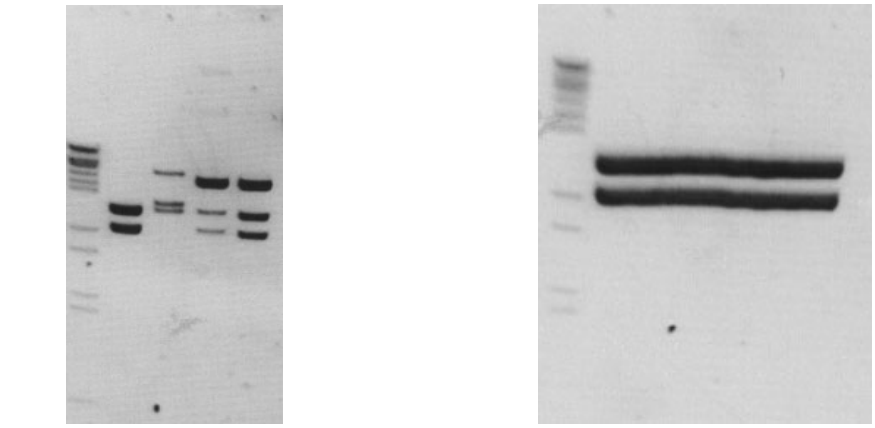


Figure 29: Control and preparative digests of pGEM-T*Easy*::ΔD8A6
picture a: *Eco*RI control digests, correct plasmids on lanes 2,4 and 5 , lanes from left to right.
picture b: preparative *Eco*RI digest of pGEM-T*Easy*::ΔD8A6

The plasmid carrying the insert with the correct size was chosen for further reactions and digested with *Nsi*I (vector-borne recognition site) and *Sph*I (insert-borne recognition site). Vector and insert fragments were separated on a 0.9% agarose gel, the insert was eluted from the gel and purified and ligated into the accordingly digested and dephosphorylated vector pEX100T. The ligation mix was transformed into *E.coli* Top10 cells and positive transformants were selected on LB medium containing 25µg/ml gentamicin. Plasmid extraction and digestion with *Bgl*II revealed one correct plasmid from 30 selected transformants which was then also digested with *Nsi*I and *Sph*I to assess the insert size. Figure 30 shows a picture of the plasmid with the correct insert size (*Sph*I/*Nsi*I digest on lane 3), and the *Bgl*II digest depicted on lane 1 shows that the gentamicin cassette is incorporated into the D8A6 recombination target sequence (lane 1 and 5).

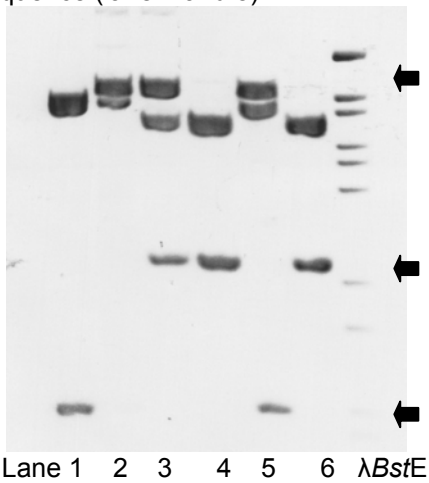


Figure 30: pEX100T carrying the ΔD8A6 recombination target sequence.
Arrows from top top bottom: appr. 5800 bp, 2400 bp, 1400 bp
Lane 1: *Bgl*II digest of pEX100TΔD8A6.1, lower fragment size appr. 1400-1500 bp, larger fragment size appr. 5800bp, (vector band: 5842), this plasmid seems to be correct. Lane 2: *Bgl*II digest of pEX100TΔD8A6.2, a plasmid with a wrong insert (*Bgl*II site missing), lane 3: digest of this plasmid with *Nsi*I and *Sph*I: ΔD8A6 recombination target sequence seems to be present. Lane 4: Digestion of plasmid pEX100TΔD8A6.3 with *Nsi*I and *Sph*I, vector band corresponds to approx. 5600 bp, seems to be too small. Lane 5: BglII digest of pEX100TΔD8A6. The insert band size seems to be correct. Lane 6: *Nsi*I and *Sph*I digest of pEX100TΔD8A6.1, ΔD8A6 insert size is correct at appr. 2400 bp.

The vector pEX100TΔD8A6.1 was electroporated into *P.aeruginosa* TB and SG17M, but no gentamicin-resistant transformants were obtained. A second attempt to introduce this construct into the host strains was made applying triparental conjugation, but to no avail. Either the gentamicin cassette was not properly functional in *P.aeruginosa* in this case, or some problem with the vector did not allow for plasmid integration in the *P.aeruginosa* strains so that no recombination took place at all.
To either rule out or confirm a vector-derived problem, the D8A6 insert was released from pGEM-T*Easy* with *Eco*RI. Subsequently, the recombination target sequence was gel-purified and ligated into an *Eco*RI-digested and dephosphorylated pEX18Ap[213] vector. This plasmid is a *sacB*-containing suicide vector of the pEX18 series, kindly provided by H. Schweizer, the physical properties of this vector had been verified beforehand in the laboratory of Prof. Schweizer. The ligation reaction was transformed into *E.coli* Top10 cells and gentamicin-resistant transformants were selected for plasmid extraction. The plasmids were digested with *Eco*RI to verify the insert size and with *Bgl*II, the digest with this enzyme does not only verify the size of the resistance cassette but its orientation as well: Due to the position of the *Bgl*II sites in the D8A6 insert and the gentamicin cassette, two different restriction fragments can be expected, as illustrated by figure 31:

Figure 31:
Picture a: sense-orientation of the gentamicin cassette, picture b: antisense orientation of the cassette within the recombination target sequence.

As can be seen in figure 31, a *Bgl*II digest of the construct with the gentamicin cassette inserted in sense orientation would yield a fragment of 925 bp, an antisense orientation would generate a fragment of 1469 bp; however, orientation of the cassette is not an issue as it contains a strong promoter[214].
This construct was electroporated into *P.aeruginosa* TB and SG17M and transformants selected on LB agar containing 50µg/ml gentamicin. Electroporation of the construct yielded transformants which were resistant to gentamicin and therefore had at least undergone one recombination step. 54 transformants were obtained from strain TB, strain SG17m yielded 38 transformants. The colonies were transferred to LB agar containing 50µg/ml gentamicin and 5% sucrose to promote a second recombination event and plasmid loss. 21 gentamicin-and sucrose resistant colonies were obtained from strain TB, 24 from strain SG17M.

3.6.5. Analysis of the phenotypic and genotypic characteristics of the mutants

3.6.5.1. Phenotypic characterization

An easy and fast phenotypic assay for a D8A6 knockout mutant is to test whether the mutant is capable of degrading casein. The D8A6 gene is known to be essential for quorum sensing in strain TB, and if the gene is knocked out, all quorum sensing related capabilities are shut down, including protease secretion. Therefore, a D8A6 knockout mutant should not be able to degrade casein and should not show a halo on M9 agar containing 0.5% casein as sole carbon source. As can be seen in figure 32, 21 out of 45 mutants do show a halo indicating casein degradation, however, in the case of TB, 13 mutants do not, in the case of SG17M, 11 mutants show no casein degradation halo. These mutants should have at least one or even all D8A6 homologs knocked out and possess no quorum sensing-related capabilities. All colonies on the plates were numbered consecutively and the numbers are referred to in the following paragraphs.

[213] GeneBank accession number AF047518
[214] Zylstra GJ: personal communication, 2004

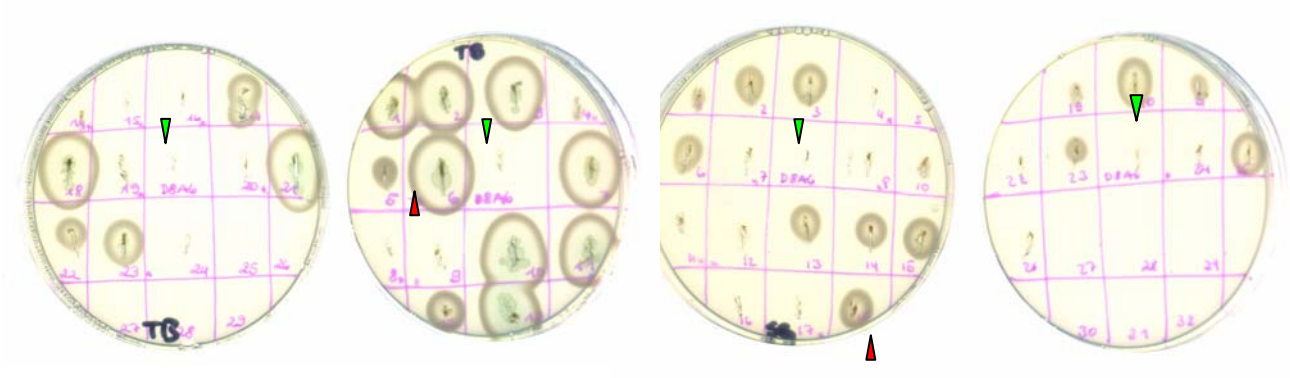


Figure 32: D8A6 mutants on casein agar. 2 plates on the left: parental strain TB, right: parental strain SG17M. Green arrows: original transposon mutant, red arrows: the respective wildtype strain.

3.6.5.2. Gene-spanning PCR

Genomic DNA was prepared from the mutants and the insertion of the 855 bp gentamicin cassette was verified by PCR. Gene-spanning primers of D8A6 were employed to generate a 3441 bp product in the case of no insertion into the gene and 4296 bp if the cassette is inserted into the D8A6 gene. In figure 32, two products of approximately this size can be detected in lanes 4 and 8. The PCR product on lane 2 was generated from the TB wild type strain and shows a band significantly smaller than the λ-*Bst*EII band no. 7 (3675 bp). The products on lanes 4 and 8 are about the same size as the λ-*Bst*EII band no.6 (4324 bp), most likely representing the mutant D8A6 gene. The PCR product depicted on lane 8 (Mutant S4, parental strain SG17M) was cloned into pGEM-T*Easy*, the ligation mix transformed into *E.coli* Top10 cells, the resulting plasmid extracted, examined by restriction digests and dispatched for sequencing.

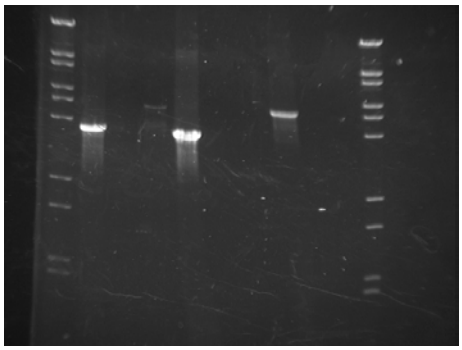


Figure 32 : PCR products obtained on genomic DNA of TBwt and knock out mutants: On lane 2, the PCR product of TBwt with gene-spanning primers can be seen, its size corresponds to 3441 bp. The standard band on lane 1 running slightly above the PCR product has a size of 3675 bp. PCR products corresponding to the size of the mutant allele can be detected on lanes 4 and 8: they have a size of 4296 bp. Corresponding standard band: 4324 bp.

3.6.5.3. Southern Hybridization

To determine whether these mutants verified by gene-spanning PCR were the only ones with the cassette inserted into the genome correctly, Southern hybridization was performed on the same genomic DNA that was used as PCR template with a probe specific for the gentamicin cassette. The blots shown in figure 32 display hybridization signals for most of the mutants, suggesting that the PCR on the genomic DNA of these mutants did not function properly, presumably due to the large size of the product to be generated.

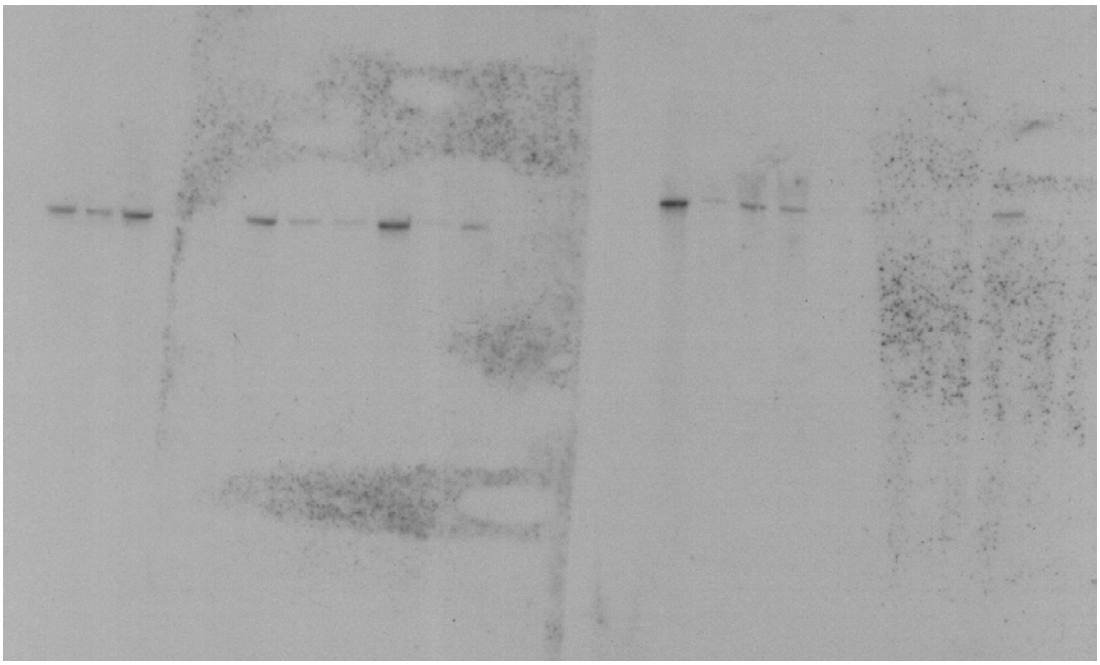


Figure 33: Southern blots of knock-out mutants
Blot 1: *Eco*RI digested genomic DNA of TB D8A6ko mutants, lane 1: λ-*Bst*EII standard, TB mutants 4, 5, 8, 9, 14, 15, 16, 19, 20, 22, 23, 24 on lanes 2-13, lane 14: TBwt.
Blot 2: lane 1: λ-*Bst*EII standard, lane 2: TBwt, lane 3: D8A6 original STM mutant, lane 4: SG17M mutant S4, lanes 5-13: S6, S7, S8, S9, S10, S11, S12, S16, S17

Table 12 summarizes the properties of the analyzed mutants:

Mutant No.	Casein degradation	Pyoverdin production	Southern signal	PCR product	Mutant No.	Casein degradation	Pyoverdin production	Southern signal	PCR product
T1	-	-	n.e.	n.e.	**S1**	-	-	n.e.	n.e.
T2	+	+	n.e.	wt	**S2**	+	+	n.e.	n.e.
T3	+	+	n.e.	wt	**S3**	+	+	n.e.	n.e.
T4	-	-	+	-	**S4**	-	-	+	mut
T5	-	-	+	-	**S6**	+	+	+	-
T6	+	+	n.e.	-	**S7**	-	+	+	-
T7	+	+	n.e.	wt	**S8**	-	+	-	-
T8	-	+	+	wt/mut	**S9**	-	+	-	mut
T9	-	(+)light green	-	mut	**S10**	-	+	(+) faint	n.e.
T10	+	+	n.e.	n.e.	**S11**	-	+	-	n.e.
T11	+	+	n.e.	n.e.	**S12**	-	-	-	n.e.
T12	+	+	n.e.	n.e.	**S13**	+	+	n.e.	n.e.
T13	+	+	n.e.	n.e.	**S14**	+	+	n.e.	n.e.
T14	-	-	-	mut	**S15**	+	+	n.e.	n.e.
T15	-	-	-	wt/mut	**S16**	-	-	-	n.e.
T16	-	-	+	mut	**S17**	-	-	-	n.e.
T17	+	+	n.e.	n.e.	**S18**	+	+	n.e.	n.e.
T18	+	+	n.e.	n.e.	**S19**	-	-	n.e.	n.e.
T19	-	+	+	n.e.	**S20**	+	+	n.e.	n.e.
T20	-	-	+	n.e.	**S21**	+ (retarded)	(+)light green	n.e.	n.e.
T21	+	+	n.e.	n.e.	**S22**	-	-	n.e.	n.e.
T22	+ (retarded)	(+) light green	+	n.e.	**S23**	-	-	n.e.	n.e.
T23	+	+	(+) faint	n.e.	**S24**	-	-	n.e.	n.e.
T24	-	-	-	n.e.	**S25**	+	+	n.e.	n.e.
D8A6	-	-	+	mut	**S26**	-	(+)light green	n.e.	n.e.

Table 12: Properties of D8A6 mutants generated in strains TB and SG17M. n .e. = not examined, strain D8A6: original STM mutant. Obtained mutants were numbered consecutively, regardless of phenotype.

Not all the mutants which were not capable of casein degradation yield a PCR product and/or a hybridization signal with the gentamicin probe. A few mutants which did not degrade casein do not show the genomic hybridization signal, i.e. mutants TB 9, 14, 15 and 24, illustrating the need for genotypic experiments to corroborate phenotypic analyses. However, it has to be taken into account that both Southern blotting as well as PCR, although often reliable, can fail, and a thorough assessment of the constructed mutants is necessary before it is subjected to any experiments, or before an apparent "false positive" is discarded.

3.6.5.3. Diagnostic Restriction Digest in Combination with Southern Blotting

A more exact approach was undertaken to determine the integration site of the gentamicin cassette in the genome of the mutants. In a recent screen, several homologues of the gene D8A6 have been found to be present in the genomes of a large panel of *P. aeruginosa* strains. In strain TB, at least 3 copies yield a hybridization signal specific for a part of the D8A6 gene, and in strain SG17M, genes homologous to D8A6 are present in 4 copies[215]. The sequence of the D8A6 gene from TB and those of the known homologs, ORF SG57 from SG17M as well as the ORF CP81 which is encoded on a plasmid present in strains belonging to clone C were analyzed employing NEB cutter 2.0. In order to obtain detailed information into which homologue of the D8A6 gene the gentamicin cassette was inserted in strains TB and SG17M by homologous recombination, the genomic DNA of the mutants was digested with *Nru*I as well as *Eco*RV. The expected diagnostic restriction fragment sizes according to the sequence analysis performed with NEB cutter were:

	*Nru*I	EcoRV
D8A6 gene	2820 bp	10360 and > 3600 bp
SG 57	9250 bp	4970 and > 2220 bp
CP 81	8100 bp	1920 and > 4420 bp

The genomic DNA of the mutants was digested with the respective enzyme, the fragments separated by gel electrophoresis and Southern blotting was performed and a DIG-labeled probe specific for the gentamicin cassette was hybridized to the blotted DNA.
The Southern blot of the *Nru*I digest depicted in figure 34 shows the results of the restriction digest of genomic DNA of the allelic replacement mutants generated in strains TB and SG17M:

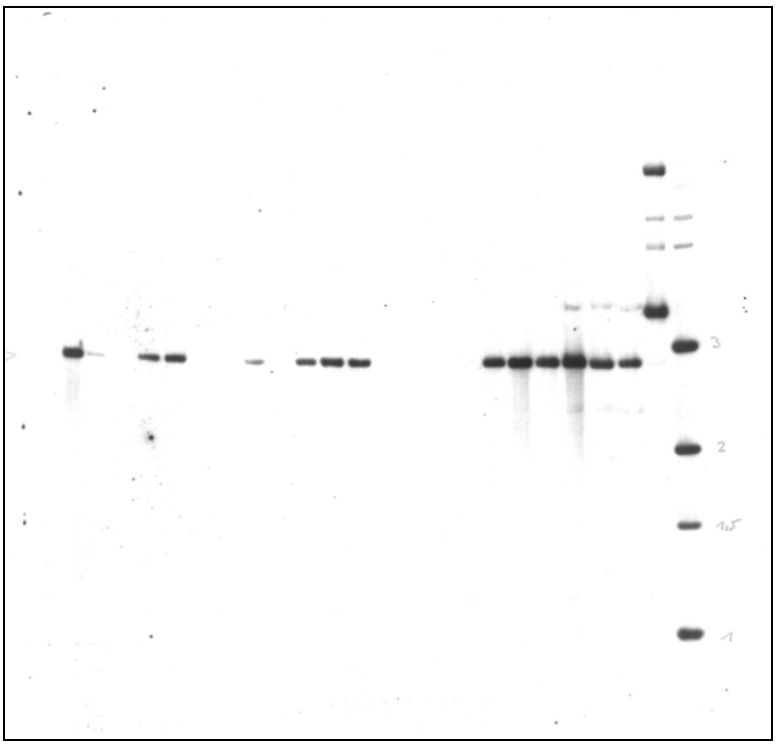


Figure 34: diagnostic *Nru*I digest of genomic DNA isolated from mutants generated employing allelic replacement, table 13 summarizes the results. EcoRV blot not shown.

[215] Klockgether J : Personal communication, 2004.

Mutant NR	*Nru*I Southern	*Eco*RV Southern
T1	+	+
T4	+	-
T5	-	-
T8	+	+
T9	+	+
T19	-	-
T20	(+)	-
T22	+	+
T24	-	n.e.
T14	+	+
T15	+	+
T16	+	+
S4	not enough DNA	not enough DNA
S7	-	-
S8	-	-
S9	-	-
S11	+	+
S16	+	+
S17	+	+
S22	+	+
S23	+	+
S24	+	+
D8A6	+, different size,different Gm-Cassette	+, different size

Table 13: Results of the diagnostic Southern blots.

The mutants that are highlighted show a hybridization signal representing the correct fragment size on both diagnostic Southern blots (*Eco*RV blot not shown).
It can be seen on the diagnostic *NruI* Southern blot that a signal of all mutants that did hybridize with the gentamicin probe show a band in the same location, which suggests that the fragments that yielded the hybridization signals are of equal length. Judging from the standard size, this band has a size of about 3000 bp and therefore is highly specific for an insertion in the TB D8A6 gene and not in any of the other homologous genes.

3.6.4. Sequencing of a PCR product representing the mutant allele

The mutant S4 has been selected for further experiments as it shows a gentamicin signal on the non-diagnostic Southern blot, and a PCR product of the size corresponding to the D8A6 gene with the inserted gentamicin cassette was obtained. The PCR product representing the mutant allele was easily subcloned into a plasmid which could then be sent for sequencing. Sequence data for a large part of its insertion has been obtained, verifying a genomic insertion of the gentamicin cassette at exactly the predicted location. Furthermore, no genomic rearrangements were observed by analyzing the sequenced parts of the insertion and its flanking regions (figure 35).

Sequencing results:
The D8A6 knock-out mutant S4 was sequenced with two primers (red, bold letters), one assessed the presence of the gentamicin cassette within the genome, the other was designed to span the recombination point downstream of the *antisense*-oriented gentamicin cassette. Both primers are forward primers, oriented towards the end of the gene (TCA, highlighted in red). Figure 35 illustrates the sequencing results:

TCATTCGTCCTGGCACTCCACTGCCGGTTGGTCTGGGAAATTAATTGGGAAGGGCGGCAGGCCG
CGCGCCTTGTCGAGATCCGCTGCGACCTGCAAGGCCGCCTCGAGCTCGTCGCAGCCGGTGGCT
TGCATGATGTTGTAGCGCTGGTTCTTTTCTTCGTTTTCGGTCATGGCCAGGGCCAGGTAGAGACT
CGGGGGAACCACACGGAAGAGGTACTCCTTGCCCTTGGCCAGG**AGCACGCCCTCGGTGAATTT**
GCCGCTTTCCTTGCGGGCCGAGAGCATCATCGACTTCTGCGCCGGCGACAGCTCGCGGAACCT
GGAGATCTTCTCTACCTCGTCTGGGGGCATGTTCAGGCACAACCACCACTCGATCATGTTCAGCA
TCGGCGCCCCGGAGGCTGGGATGTCGTCGATGTTCTGGGTGGCGAGCCAGAACCAGGCGCCCA
GTTTCCGCCACATCTTGGTGATCTTCATGGCGTAGGGCAGCAGCAGCGGGTGCTTGGTGATGAT
GTGTCCCTCATCGGTGATCTTGACGATGGGCCGGCCCTTGAATTGGTCGCGTTCGGCGATGTTG
TTTACGGTGTTCAGCAGCGAGATGTAGGCGATCCCGAGCTGGGCGGCGTAGCCTTCGCGCGCG
TAAGTCGCGAAATCCACCACGGTGAGGTCGGCTTCAGGCCAGGGCGTGCCTTTGCGATTGAACA
TCTCGCCGTCGGCGCCCATGCAGAACATCTGCATGGCTTCCGCCATTTCGGCCAGGCGCGCGC
GCCGTTCTGGCGCGGAGCCATCGCTCCTGGAGGCCTCGTAGAGCGCATCGCGCACGTCCTGGG
TGAGCACCGTGCGGTTCGCGGCGGCGCAGGTCCTGGCCGCCGCCAGAATCGCCTGGCGGATG
GCGCTGCGATCGGCACGGGTCAGGCGCGCGTCTTCCTTCTCTTCGCCGCCGGTGATCATCAGG
CGCGCGACGATCTCCATCTCGCCGAGGATGTCTCGTTGATCGTCCTCGAGGTCGGCCTTGCTGC
CCTGGACCGAGTCCGAGGCCTCGATGTCTTCGGCATCCAGGACCTTCACCTGGTCGGGACTCTC
GACCAGCTTGATGGCGTCCGCGAACGGCGCCAGGCTGACGCCGGAG**CCC**gggtaccgagctcgaattga
cataagcctgttcggttcgtaaactgtaatgcaagtagcgtatgcgctcacgcaactggtccagaaccttgaccgaacgcagcggtggtaacg
gcgcagtggcggttttcatggcttgttatgactgttttttgtacagtctatgcctcgggcatccaagcagcaagcgcgttacgccgtgggtcgatgttt
gatgttatggagcagcaacgatgttacgcagcagcaacgatgttacgcagcagggcagtcgccctaaaacaaagttaggtggctcaagtatg
ggcatcattcgcacatgtaggctcggccctgaccaagtcaaatccatgcgggctgctcttgatcttttcggtcgtgagttcggagacgtagccacc
tactcccaacatcagccggactccgattacctcgggaacttgctccgtagtaagacattcatcgcgcttgctgccttcgaccaagaagcggttgtt
ggcgctctcgcggcttacgttctgcccaggtttgagcagccgcgtagtgagatct**a**tatctatgatctcgcagtctccggcgagcaccggaggca
gggcattgccaccgcgctcatcaatctcctcaagcatgaggccaacgcgcttggtgcttatgtgatctacgtgcaagcagattacggtgacga**tc**
ccgcagtggctctctatacaaagttgggcatacgggaagaagtgatgcactttgatatcgacccaagtaccgccacctaacaattcgttcaa
gccgagatcggcttcccggccgcggagttgttcggtaaattgtcacaacgccgcggccaattcgagctcggtaccc**GGG**GCGAGGCG
GACACGGTGGACCGAGAGGCCAAACCGCTTGGCGAAGTCGGCCAGCAGGCCGAAGCTGTTGCC
CGCTTCCGCGACGAACATCCGTGGCAGGTACATGGCGAGCATCTGGCTGATGAGGTTGGTCAG
GGACGCTGACTTGCCGGACCCGGTTGGGCCGAAGATGAAGCCGTGGGCATTCATCTGCCGGTC
CAGCTTGTTGAACGGGTCGAAGGTCAACGGCGCGCCGCCACGGTTGAACAGCGTGAAGCCGGG
GTGTCCGGTACCGGTGGTGCGCCCCCAGATGGGCGACAGGTTGGCGATGTGCTGCGCGAACAT
CATCTGGGTGTACCACTCCAGGGCTCGCTTTTCGTTCGGATCGAAATTGCAGGGCAGCCAGCGC
AGGTAGCTGTTCAGCGGTCCCACTTCGTTCTGCGGTTCGACCGGCACCAGGCCGGCGCCGAGC
AGCACGTTGCTCAGGGTGATGCAGCGCTCCTCCAACTGGGTATGGTCGCGGCCGCGCACGAAC
AGAGCGATCGAACCGCGATAGAGCTTGTGCTCCCGGCCGATCAGGCGGCGAACGGTGGCCACG
TCCTCGCGGGTGTGGATCGAGGCCTGGTTGTCACCAACGGCCTTTTTCGAGAGCTGCTGCAGAT
GCCCTTCCAGCATGTCCTGCGGCGTCACCACCATGGTGATACACAGCAGCGTATCCTCGGGCAT
G**C**GATCGAACAGGGCGTTCAGGCCAT**CACCCTTGAGCGTCTCGCCGG**TGAAATGCCCTGTCAG
CGGCGCCTTGTTCAACTGGTCGACCACGAT**CACTCGGTGTGGCATGGCATCG**

Figure 35:
Sequence of the complete D8A6 gene (capital letters, gene end highlighted in red, TCA) with the 855 bp gentamicin cassette (small letters) added in antisense direction into the *Sma*I site: sequencing results for the PCR product obtained from mutant S4. The *Sma*I sites, into which the cassette was ligated, are highlighted in green. Sequencing primers are printed in bold, red letters, each facing forward towards the gene end (TCA, highlighted in red).
The primers used to construct the recombination target sequence are printed in bold letters.
The underlined part highlighted in yellow represents genomic sequence from the sequenced mutant that was not included in the artificial recombination target sequence. Dark grey: Sequence gained with the sequencing primers

Figure 35 shows that S4 is a true knock-out mutant, as the gentamicin cassette has been integrated into the genome. The flanking sequences are exactly those of the PCR product and furthermore, as can be seen, no genomic rearrangements have occurred within the gene sequence so that secondary effects can be ruled out. The *Eco*RI sites introduced by the subcloning vector can not be found in the sequenced stretch of DNA anymore, indicating that homologous recombination has indeed taken place.
A phenotypic and genotypic characterization of the mutants has revealed that more than 50 % of the constructed mutants carried the gentamicin gene which was integrated into the genome by homologous recombination. Furthermore, this is proof that the protocol for targeted mutagenesis employing allelic replacement as established in this study does indeed work in *P.aeruginosa* strains other than PAOI.

3.7. Construction of a PA 5291 deletion mutant

Now that in a pivotal experiment it has been proven that the refined method for allelic replacement works in the mucoid *P.aeruginosa* isolates TB and SG17M, the universal applicability can be proven by the construction of a second knock-out mutant. The gene PA 5291, *betT2,* was found to be essential for quorum sensing, the original STM knock-out mutant was neither capable of secreting homoserine lactones (HSLs), had greatly reduced elastase secretion levels and produced no pyoverdine. The gene has been annotated as a probable choline transporter and might also have a function in the transport of small molecules. Choline is a small tertiary amine; $CH_2CH_2N(CH_3)_3^+$, which is believed to play a role in osmoprotection of the bacterial cell. Although choline has a significant structural difference compared to homoserine lactones, it nevertheless consists of a non-polar hydrocarbon side chain and a slightly more polar amine group at the end, similar to short-chain HSLs such as the C4-HSLs synthesized by *P.aeruginosa*. It could therefore be possible that the PA 5291 gene product is at least partly responsible for shuttling HSLs out of the cell, or that HSL production is abrogated when PA 5291 is not functional. To verify the phenotype, a knock-out mutant was constructed in a similar manner to that of the D8A6 gene.

The whole gene sequence was obtained from the *pseudomonas.com* database and primers were designed 200 bp upstream and downstream of the start and end of the gene. A 2601 bp PCR product was generated that encompassed the promoter region as well as 235 extra nucleotides at the end of the gene and could therefore also be used not only for the construction of knock out mutants but also for complementation experiments. As with the D8A6 gene, the sequence was analyzed for suitable restriction sites using the NEB cutter software (figure 36).

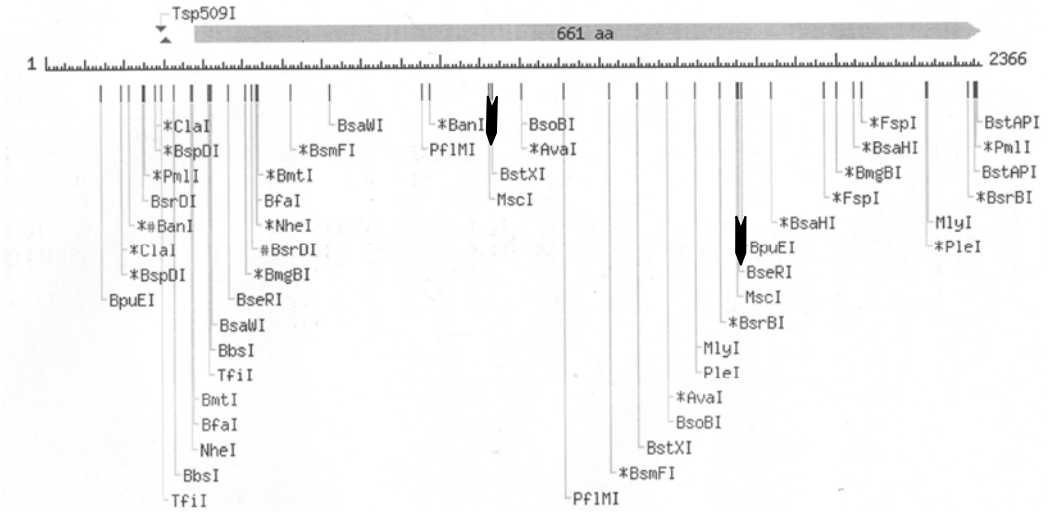


Figure 36: NEB cutter map of the PA5291 region for double-cutting enzymes. *Msc*I sites are indicated by red arrows.

At positions 1122 and 1749, the software located two *Msc*I sites. This enzyme cuts blunt ended and would therefore cut out a 627 bp fragment of the gene which could in turn be replaced with the 855 bp gentamicin cassette released from pUC-Gm with *Sma*I.

The PCR product was generated using the FailSafe PCR kit, as generating a product with a size of about 2601 bp has proven to be a challenge employing conventional PCR reactions[216]. The product was easily amplified and subcloned into pGEM-T*Easy* which added two *Eco*RI sites to the product. The size of the construct was verified by linearization with *Nru*I and confirmed to approximately 5600 bp as well as by releasing the insert with *Eco*RI (data not shown). The construct was then digested with *Msc*I and the two fragments were separated by gel electrophoresis. The larger band corresponding to the right size was cut out, eluted from the gel and dephosphorylated twice before a ligation reaction with the equally purified *Sma*I fragment from pUC-*Gm* was started. The ligation mix was transformed into *E.coli* Top10 and the bacteria were selected on LB agar supplemented with 25µg/ml gentamicin. Positive transformants were selected for plasmid extraction and the construct was verified by *Sma*I digest to release the cassette and *Eco*RI digest to gauge the size of the recombination target fragment (figure 37 shows the *Eco*RI digested plasmids on lanes 1-7).

[216] Charizopoulou N, Salunkhe P, personal communications, 2003

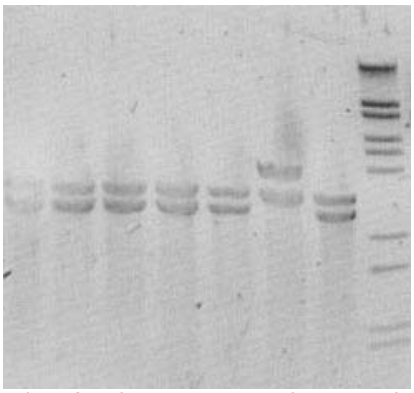


Figure 37: *Eco*RI digest of the ΔPA5291-recombination target sequence. The plasmid on lane 6 has the wrong size, the others are correct and seem to be identical.

The fragment was then released from pGEM-T*Easy* with *Eco*RI and ligated into pEX18Ap which had been digested with *Eco*RI, dephosphorylated and phenol-chloroform purified. The ligation reaction was transformed into *E.coli* Top10 cells and transformants were selected on LB medium containing 25 µg/ml gentamicin. Positive transformants were selected for plasmid extraction, and the plasmid was digested with *Eco*RI to verify the presence of the insert and its size (data not shown). The correct plasmid was then selected for electroporation into *P.aeruginosa* SG17M and TB. Transformants were selected first on gentamicin, then on sucrose as described for D8A6. Several positive transformants were tested for casein degradation:

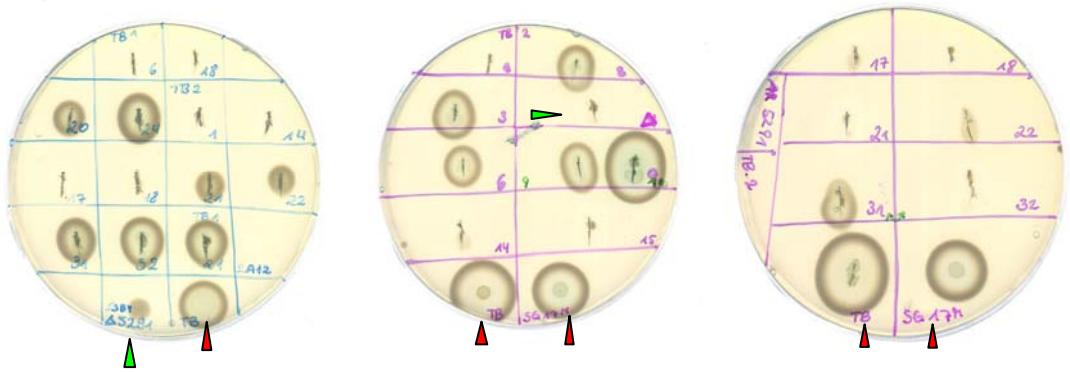


Figure 38: PA5291 knock-out mutants on casein agar, left, middle: mutants generated from TB, right: mutants generated from SG17M. Red arrows: parental wildtype strains, green arrows: original STM knock-out mutant generated from SG17M.

The mutants TB 1.6, 1.18, 2.1, 2.14, 2.15, 2.17, 2. 18, S17, S18, S21, S22 and S32 (mutants T2.1. and T2.14 are represented twice in figure 38) were selected for further phenotypic assays and for Southern blot analysis. All of the examined mutants showed a phenotype comparable to the original knock-out mutant: none of the mutants produced pyoverdine, the elastase production was reduced in comparison to both wildtype strains and comparable to that of the original STM mutant and the hemolytic activity was diminished. Figure 39 shows a Southern blot analysis of genomic DNA of 16 PA5291 knock-out mutants digested with *Eco*RI.

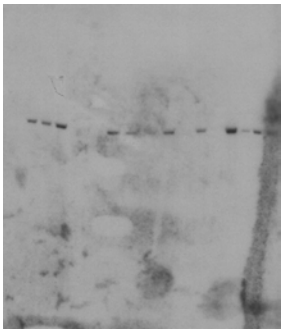


Figure 39: Southern blot of 16 PA 5291 k.o. mutants; genomic DNA was digested with *Eco*RI and hybridized with a probe specific for the gentamicin cassette from pUC-*Gm*.
Lane 1: SG17M wildtype, lanes 3-18: TB and SG k.o.mutants: T1.6, T1.18, T2.1, T2.31, T2.32, T1.21, T2.18, S17, S18, S21, TB wt, S22, S31, S32, T.1.1, T1.2, original STM mutants 2A12 and 23B4.

The properties of the mutants are summarized in table 14:

Mutant No.	Casein degradation	Pyoverdine production	Southern signal	Mutant No.	Casein degradation	Pyoverdine production	Southern signal
T1.6	-	-	+	**T2.17**	-	-	n.e.
T1.18	-	-	+	**T2.18**	-	-	+
T1.20	+	+	n.e.	**T2.21**	(+) faint halo	light green	n.e.
T1.21	+	+	+	**T2.22**	(+) faint halo	light green	n.e.
T1.24	+	+	n.e.	**T2.31**	+	+	n.e.
T2.1	-	-	+	**T2.32**	+	+	n.e.
T2.2	+	+	n.e.	**S17**	-	-	+
T2.6	+	+	n.e.	**S18**	-	light green	+
T2.7	(+) faint halo	-	n.e.	**S21**	-	-	+
T2.8	+	+	n.e.	**S22**	-	-	+
T2.14	-	-	n.e.	**S31**	+	light green	-
T2.15	-	-	n.e.	**S32**	-	-	+

Table 14: Properties of TB and SG 17M PA5291 knock-out mutants, n.e. = not examined

Discussion

The first system for allelic replacement in *P.aeruginosa* that allowed for positive selection of mutants during all steps of the process was designed by H. Schweizer in 1992[217]. This system consisted of a vector that for the first time included the *sacB* gene from *B.subtilis*, and only few restriction sites so cloning of foreign DNA fragments into the vector and working with these inserts was greatly facilitated. A drawback of this system was the necessity for multiple cloning steps in order to generate the "ready to use" vector/recombination target sequence construct which could then be transferred into *P.aeruginosa*. Another disadvantage was that there were still several commonly used restriction sites present within the vector which made access to cloned DNA fragments difficult, and also, this vector contained no *oriT*, therefore, mobilization of the vector was only possible with the use of a specialized helper vector. To address these shortcomings, Schweizer and Hoang[74] developed an improved system based on established pUC vectors which contained the *sacB* gene, an *oriT* for conjugation-mediated plasmid transfer, an ampicillin resistance-conferring beta lactamase gene, a *lacZα* allele for blue-white screening and unique cloning sites for *Sma*I and *I-Sce*I. Furthermore, several common restriction sites present in the vector were excised so that handling of the DNA cloned into the vector would be easier. This vector, pEX100T is still utilized by researchers for allelic replacement as it has proven to be stable, easy to handle and convenient for the cloning of even large fragments[218]. A further improvement of this system resulted in a "new generation" of vectors for gene replacement, the pEX18 series[219]. These 3 vectors are pEX100T-based but in addition contain a convenient multicloning site derived from pUC18, and each of them contains a different gene conferring antibiotic resistance: On pEX18Ap, the selectable marker is a beta lactamase conferring ampicillin resistance, pEX18Tc carries a tetracycline resistance and pEX18Gm a gentamicin resistance gene. Therefore, the original sacB-containing vector for allelic replacement, pEX100T, had become decidedly more user-friendly and applicable to more bacterial species. This vector series has gained great popularity and is the most widely used system for the generation of mutants in *P.aeruginosa* PAOI; a few mutants from host strains other than PAOI have also been generated. Mutagenesis of clinical isolates has generally proven to be much more demanding than that of the genetic reference strain, especially if the isolates to be manipulated are mucoid[220]. Both *P.aeruginosa* strains employed in this study are mucoid, even though strain SG17M was not obtained form a patient and is an environmental isolate. Transposon mutagenesis of the isolate SG17M has also proven to be far more difficult than with PAOI[221], although some simple washing steps could partly remedy the problem.
The first attempt at constructing knock out mutants in strains TB and SG17M for this study was made employing the gene deletion method based on a plasposon-*sacB* construct, pMOD-Gm-*sacB*. This

[217] Schweizer HP: Allelic exchange in P.aeruginosa using novel ColE1-type vectors and a family of cassettes containing a portable oriT and the counterselectable Bacillus subtilis sacB marker. Mol. Microbiol. 6, 1992,1195-1204
[218] Attree I, Schweizer HP: personal communication, 2003
[219] see ref. 84 and 85
[220] De Lorenzo V, Timmis KN: Analysis and Construction of Stable Phenotypes in Gram-Negative Bacteria With Tn5 and Tn10-Derived Minitransposons. Methods in Enzymololgy, 235, 1994, 386-405
[221] Limpert AS, unpublished results, 2001

approach seemed to be promising for the generation of unmarked mutants, as it incorporated a gentamicin cassette, contained convenient restriction sites and a *sacB* gene for promoting a second recombination event to resolve merodiploids generated via whole plasmid integration into the genome. This vector/insert system did most likely not perform properly due to two reasons: As the construct failed to yield transformants that were phenotypically different from the parental strain, expression of the *sacB* gene was perhaps not strong enough to ensure the loss of the plasmid. As the *sacB* gene used for the integration into the pMOD vector was not under the control of a strong and constitutively expressed promoter[222], the low or no expression explanation for its failure seems plausible: the gene was most likely not expressed in the *P.aeruginosa* strains in large enough quantities, if at all, to force the bacterium towards plasmid loss. To further use the pMOD-*sacB* construct, a strong, *P.aeruginosa*-compatible promoter should be cloned into the pMOD-*sacB* construct to ensure sufficient *sacB* gene expression in *P.aeruginosa*. This could for example be achieved by generating a PCR product of a constitutively expressed promoter from the *P.aeruginosa* genome and clone this product upstream of the *sacB* gene. The orientation of the *sacB* gene needs to be verified for this purpose and the multicloning site from nt 1-72 of the vector should allow for inserting a promoter in the right direction. These changes to the vector construct would only solve the problem if the failure of the *sacB* gene is really due to low or no gene expression. It has been reported for several bacterial species that mutations in the *sacB* gene occur easily, which is why many researchers stay away from *sacB* containing vectors as long as possible within a gene replacement procedure[223,224] and frequently check *E.coli* strains used to store or mobilize *sacB*-containing vectors for sucrose susceptibility as spontaneous *sacB* mutations occur and are easily selected for[225]. A mutation in the *sacB* gene that prevented it from to functioning properly is another possibility for its failure in the presented experiments. This problem could be overcome by only storing vector DNA and transforming it every time right before use which is a practice that has been carried out throughout this study as earlier experience especially with the pMOD vector showed that rearrangements of the vector occur frequently and can significantly alter its properties.

Another problem seemed to be the non-functional *Not*I sites introduced to the ends of the recombination target sequence. *Not*I is generally a reliable and easy to use restriction enzyme, furthermore, its recognition site is positioned right in front of the gentamicin gene which would be a perfect position for introduction of a recombination target sequence: The close proximity to the gene conferring the antibiotic resistance would facilitate the uptake of the whole plasmid by the first recombination event, which would take place in the target sequence part right next to the resistance gene. A subsequent second homologous recombination would occur promoted by *sacB* expression in the other part of the target sequence and result in the loss of the plasmid. Unfortunately, the restriction sites on the insert did not work properly and no ligation products were obtained. It would therefore be an improvement of this system to rely on restriction sites existing within the DNA to be introduced into the vector as a recombination target sequence, as it was done in the allelic replacement approach.

All in all, it can be said that the presented pMOD-based vector system could work for gene deletion and the generation of unmarked mutants, but several significant alterations would have to be made. It should be considered whether these changes would be worth making, or if the gene replacement system of Schweizer et al. could be used for the gene deletion strategy as well. As it seemed easier to generate inserts to be used in an allelic replacement technique, the gene deletion method was not pursued further; instead, the possibilities for gene replacement utilizing the vectors constructed by Schweizer were explored.

The construction of a recombination target sequence as already described has several advantages over the one used for gene deletion: First of all, it relies on restriction sites occurring within the target sequence, so that one PCR reaction is sufficient to generate a product that could be used for ligations. The subcloning step can be omitted in this case, it is not necessary to clone the PCR product into pGEM-T*Easy* if it is obtained in a generous enough amount that permits subsequent purification steps. Nevertheless, subcloning the insert into either pGEM-T*Easy* or similar vectors designed for inserting PCR products adds useful restriction sites to the insert which can be utilized for further applications. The pGEM-T*Easy* vector proved to be an excellent choice for both the D8A6 gene replacement strategy as well as for knocking out the gene PA 2591. The primers for generating the PCR product from the gene D8A6 were designed not to incorporate the EcoRI restriction site present in the gene at position 1352 of the whole sequence (see figure 26, gene end at nt 1360) so that this site could be

[222] Gerlach GF, personal communication, 2004
[223] Attree I, Hassett D, Ohman D, Schweizer HP, personal communications, 2003
[224] Sherman DR, Voskuil M, Schnappinger D, Liao R, Harrell MI, Schoolnik GK: Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding α-crystallin. Proc. Natl. Acad. Sci. 98(13), 2001, 7534-7539
[225] Gene replacement © Herbert Schweizer 2001

used to release the PCR product from pGEM-T*Easy* and clone it into the *Eco*RI site of pEX18Ap. The gene PA 5291 did not contain any *Eco*RI site, so that the same strategy could be applied in its case.
The first attempt at allelic replacement of D8A6 was made with the insert generated as described above (3.6.), which was cloned into pGEM-T*Easy* and released with *Sph*I and *Nsi*I. *Sph*I is a site that is also present at the very end of the amplified sequence (nt position 3202 of the whole sequence, gene start at nt 4307, start of PCR product at nt 3254), so a small part of the sequence is lost after an *Sph*I digest.
The purified insert was ligated into pEX100T, the construct was verified and subsequently electroporated into *P.aeruginosa* TB and SG17M. No mutants were obtained by this method, so triparental conjugation as described by Schweizer and earlier in this study for the generation of STM mutants was employed, however to no avail. As the insert proved to function properly when integrated into pEX18Ap, the pEX100T vector might not be ideal for the delivery of recombination target DNA into the selected strains due to as yet elusive reasons. This vector has been frequently used in PAOI and was also applied to generate an exsA$^-$ mutant of the clinical isolates CHA and TB[226], so it should be functional in *P.aeruginosa* in general. Restriction mapping of the vector did not show any irregularities, so that it can most likely be concluded that pEX100T does not work in TB and SG17M in conjunction with the recombination target sequence derived from the D8A6 gene.
A change of vector was all it needed to accomplish allelic replacement for the gene D8A6 in the selected *P.aeruginosa* strains.
The pEX18 series vectors are an improvement of pEX100T and contain a multicloning site derived from pUC18, numerous other restriction sites have been destroyed to facilitate handling of cloned DNA inserts, and they are available with different selectable markers whereas no changes in promoter structures or other significant alterations have been made. The vectors are of nearly identical size as pEX100T and incorporate the *sacB* gene also present on pEX100T for selection of double recombinants. pEX18Ap was chosen as the beta lactamase encoded on this plasmid can be used with ampicillin in *E.coli* and carbenicillin in *P.aeruginosa*, therefore facilitating selection of mutants that have lost the delivery plasmid. Homologous recombination takes place at one side of the insert-encoded antibiotic cassette, in this case gentamicin; this process integrates the plasmid into the genome and results in the formation of a merodiploid. The second recombination step is forced by *sacB* expression from the plasmid, promoting the excision of the construct and subsequent plasmid loss. As stated before, spontaneous mutations in the *sacB* gene occur and are selected for by this procedure, therefore, it is helpful to have a plasmid-encoded marker to verify plasmid excision. The beta lactamase represents such a marker. If the plasmid is not lost due to a mutated *sacB* gene and the transformant is nevertheless sucrose resistant, a selection on carbenicillin will show whether the plasmid is still present or not. All recombinants obtained in this study were checked for carbenicillin sensitivity, and only those that proved to be carbenicillin sensitive were retained. Another advantage of the pEX18 series vectors is that they are easily electroporated into *P.aeruginosa*, which makes the transferring of the vector a lot less “messy” as no *E.coli* contaminations occur, furthermore, the mating procedure takes three to four days whereas electroporation only takes 4 hours and overnight selection in the case of strains TB and SG17M.
The properties of the knock-out mutants were assessed and compared to the respective wildtype and original STM mutants. However, it needs to be stated that as with most mutagenesis methods, mutants obtained with allelic replacement have to be examined thoroughly by PCR and Southern blotting, if possible in combination, as well as for their phenotypic properties. In the case of D8A6, several mutants showed hybridization signals with a probe specific for the gentamicin resistance cassette but no PCR product. For example mutants T4 and T5 of D8A6 failed to yield a PCR product, and if this had been the only method of verification in addition of the phenotypic proof, they would have been discarded as false positives. On the Southern blot, a strong signal can be detected for both mutants; therefore, with this very specific evidence, they can be regarded as true knock-out mutants. In contrast, the mutant T24 does not yield a hybridization signal, whereas the phenotype seems to be that of a knock-out mutant. To assess whether a mutant is a false positive or the Southern blot did not function properly, a PCR and another Southern blot experiment should clear up any doubts before the mutant either undergoes further experiments or is discarded. Sequencing of the insertion site is the ultimate genotypic proof, but the method has several drawbacks, especially associated with *P.aeruginosa*: This species has a very large and G+C-rich genome, and both isolates used for this study have a genome that is even larger than that of the genetic reference strain, therefore, genomic sequencing is rendered decidedly difficult. Applying the procedure for direct genomic sequencing developed in this study, it is possible to obtain stretches of sequence with up to 300 bp by sequencing with one primer directly on genomic DNA. While this is an excellent and fast method to identifiy short stretches of DNA, say, a transposon insertion into the genome, it is not suitable for the verification of

[226] Attree I, unpublished data, 2003

insertions spanning several hundred base pairs. One possibility would be to sequence directly on the genome by primer walking from a part both upstream and downstream of the expected recombination point, but this procedure would be costly and rather time consuming. Sequencing of a PCR product as done in the D8A6 case seems to be the optimal method to gather stretches of DNA long enough to verify the exact genomic location of the inserted cassette and exclude genomic rearrangements that could interfere with attributing a specific mutation to a distinct phenotype. As in the case presented in this study, this technique still relies on the generation of a PCR product from the whole recombination target sequence plus several hundred base pairs upstream and downstream which is difficult for large genes. If the product is obtained, it has to be present in such quantity that it can be purified and dried for sequencing; this is also difficult with large products. Given all these drawbacks of sequencing, Southern blotting with an insert-specific probe seems to be the method of choice for verifying knock-out mutants; the experiment should nevertheless be performed at least in duplicate to exclude negative results due to technical failure.
A good combination for the genotypic verification is a diagnostic restriction digest in combination with a Southern blot and hybridization with a gentamicin-cassette specific probe as applied to the D8A6 mutants. Hybridization signals of fragments with distinct length and therefore height of the Southern blot verify the insertion of the cassette into the desired genomic context.
Another method for the verification of an insertion into the genome of a bacterium is to generate a PCR product from a primer pair of which one is specific for the inserted part and the other for a sequence stretch on the genome. This approach should yield highly reliable results and its application to the knock-out mutants constructed during the course of this study is currently under way.
The only real drawback of the method for allelic replacement presented here is the fact that mutants resistant to one of the few selection markers applicable to *P.aeruginosa* are constructed. Therefore, the construction of double mutants is made nearly impossible. Apart from the gene deletion technique, a solution to that problem has been suggested by Schweizer et al[227]. The Flp-*FRT* system of *S.cerevisiae* has been successfully applied to generate mutants from PAOI and consists of two main components: The Flp recombinase target sites (*FRT* sites) and the Flp enzyme itself. The FRT sites are symmetrical genetic elements that can either be in inverted orientation or in the same orientation respective to each other. In both cases, the *FRT* sites are separated by a naturally occurring 8 bp insert, and their orientation has direct impact on their function: If the *FRT* sites are oriented invertedly to each other, the DNA stretch separating them is inverted, if the *FRT* sites are in the same orientation, the separating DNA is excised by the Flp recombinase and lost. This feature can be used for allelic replacement if a selectable marker is placed in between of two *FRT* sites that are in the same orientation in respect to each other. A recombination target sequence is generated and the *FRT*-selectable marker-*FRT* construct is inserted into a convenient restriction site. The whole construct is cloned into a *sacB*-containing suicide vector and introduced into the genome by homologous recombination promoted by the selectable marker; the plasmid is then cured by sucrose selection. The Flp enzyme is introduced on a plasmid to the mutants resistant to sucrose as well as to the selectable marker; it functions if supplied *in trans*, conveniently excising the selection antibiotic and leaving a small “scar” in the genome. The scar consists of 13 base pairs which is a naturally occurring sequence following religation of the DNA between two *FRT* sites in the same orientation respective to each other. Vectors for this method have been developed and applied to *P.aeruginosa* PAOI in the Schweizer lab, but this technique is still not widely applied, most likely due to general difficulties with allelic replacement in *P.aeruginosa*- the labs that have successfully constructed mutants with the established technique are reluctant to change to a new method[228]. This technique could nevertheless be of great use for allelic replacement in *P.aeruginosa* strains TB and SG17M, as the generation of unmarked mutants is a very promising concept. This way, several genes can be mutated and step-by-step mutations in operons can help elucidating the function of every single gene in one strain. The established method requires either one individual mutant per gene or the introduction of several selection markers into one genome, and in *P.aeruginosa*, the number of mutants to be introduced into a single genome can very well be limited by the small quantity of available selection markers. Hence, the Flp-*FRT* technique will be applied to construct several mutants in genes of the *exsA* operon in strain TB to determine the function of these genes for interactions of strain TB with eukaryotic cells.
Another different technique has recently been applied to conduct systematic mutagenesis of the *E.coli* genome. This technique relies on a combination of *in vitro Tn5* insertion and λ-Red-mediated recombination: PCR products spanning every identified ORF are generated into which a modified *Tn5*

[227] Schweizer HP: Applications of the Saccharomyces cerevisiae Flp-FRT System in Bacterial Genetics. J.Mol. Microbiol.Biotechnol. 5, 2003, 67-77
- Hoang TT, Karkhoff-Schweizer RA, Kutchma AJ, Schweizer HP: A broad host-range Flp-FRT recombination system for site-specific excision of chromosomally located DNA sequences: application for isolation of unmarked Pseudomonas aeruginosa mutants. Gene 212, 1998, 77-86
[228] Schweizer HP, personal communication, 2003

transposon is randomly inserted *in vitro*, the reaction mixture is electroporated into *E.coli* strains expressing the λ-Red proteins which allow for homologous recombination of the artificial DNA into the chromosome. A second PCR product is generated with a 3' and a 5' homology to the regions flanking the gene of interest. This construct is then co-electroporated with a high-copy plasmid encoding the yeast meganuclease *I-Sce*I into the transposon mutant. *I-Sce*I mediates cleavage of the genome within the transposon sequence and would introduce a lethal double strand break unless repaired or homologous recombination with the incoming fragment took place.
The principles underlying this technique are slightly similar to the Flp-*FRT* system, but the method relies on numerous cloning and transformation steps which is a major drawback. It has been used to generate a large number of mutants in *E.coli*, and could possibly be adapted to *P.aeruginosa* with some alterations, but it might be difficult to adapt the original plasmids to the requirements of *P.aeruginosa*. Consequently, the method of choice for the construction of unmarked *P.aeruginosa* mutants would be the Flp-*FRT* system and its application to strains TB and SG17M is under way.
In this study, a straight-forward and easy to use method for the generation of knock-out mutants in *P.aeruginosa* isolates other than PAOI has been established, minimizing PCR-based and cloning steps thus leaving fewer possibilities for unwanted mutations and rearrangements. Furthermore, the subcloning step of the PCR product still included in the approach presented here serves the sole purpose of introducing convenient restriction sites to the ends of the recombination target sequence. In the two described mutagenesis approaches, the additional *Eco*RI sites of the subcloning vector were highly advantageous for the strategy as a single restriction enzyme could be used to release the insert from the vector, furthermore, the vector pEX18Ap contains a recognition site for this enzyme, which was otherwise not present in the insert, thus greatly facilitating the ligation of the recombination target sequence into the suicide vector. Nevertheless, a PA5291 PCR product with an *Eco*RI and a *Hind*III site introduced by PCR primers has been cloned directly into pEX18T employing these restriction sites, proving that the subcloning step is facultative.
The mutants obtained employing this method showed a phenotype different from the parental strain and similar to the respective knock-out mutants obtained by STM mutagenesis. Sequencing of one mutant yielded a stretch of DNA including the almost complete sequence of the gentamicin cassette inserted into the genome along with a large part of the recombination target sequence and reaching into the genome region that was not contained in the artificial recombination target sequence.
Furthermore, diagnostic restriction analysis verified the insertion site into the targeted gene and proved that homologous genes were not affected, thus providing a very specific and accurate method for the generation of knock-out mutants and verified the importance for the D8A6 gene for quorum sensing regulation in strain TB.

4. Perspectives

The results obtained in this study lay the groundwork for ongoing research on the topic of quorum sensing and host-pathogen interactions. Several genes have been identified that have not been linked to quorum sensing in *P.aeruginosa* yet, so ongoing research should be first of all the verification of the mutation and its impact on the phenotype, preferably by allelic replacement. Furthermore, the mutants should be characterized and their exact role either within the intricate network of quorum sensing or in the biosynthesis and transport of the homoserine lactones analyzed.

The further analysis of these genes could broaden the scope of current understanding of quorum sensing as a step towards coordinated multicellular behavior.

The STM mutant library that was constructed for this study is a valuable tool for further screening for virulence of *P.aeruginosa* SG17M in specific habitats of interest, especially in animal models. Furthermore, as a mutant library of the clinical isolate TB has been constructed in a previous study, both libraries together should contain knock-out mutants of almost every non-essential gene of *P.aeruginosa* PAOI as well as a few genes that are not present in the genetic reference strain. Therefore, both libraries together represent a powerful tool for further functional genome analysis.

The identification of mutants attenuated in various habitats is made possible by screening the STM libraries, the tool for the verification of the mutation and its impact on the phenotype has been established within this study. Allelic Replacement employing a whole-gene approach should be easily applicable to other *P.aeruginosa* strains and present a means to generate mutants in virtually every gene of every strain.

5. Summary

The study presented here had several objectives that all served to further functional genome analysis in *P.aeruginosa*. One of these objectives was the construction of a STM mutant library that is screenable in various bioassays. The method of Signature Tagged Mutagenesis was applied to the environmental isolate SG17M and 3000 mutants were constructed. Random mutants were examined for their statistical transposon insertion and after a few modifications to the original protocol, minimal redundancy of the mutant library was ensured.

The mutant library was then screened in an assay assessing the quorum sensing capabilities of the mutant in order to yield more insight in the workings of this intricate intercellular signaling network. For this purpose, the mutants were screened for protease secretion which is known to be regulated by quorum sensing, and almost 200 mutants were found to be deficient for this phenotype. These mutants were then tested for the production of quorum sensing autoinducers by a luciferase test, furthermore, their culture supernatant was examined for homoserine lactone production by thin-layer chromatography in combination with detection by a HSL- specific dye. In total, 23 mutants did not show any HSL production, suggesting they had a defect in either the regulation or the production of HSL. These mutants were further characterized for their virulence factor production and it was found that all mutants produced less elastase and siderophores than the SG17M wildtype; furthermore, most of the mutants were attenuated in haemolysis as well. The mutant in PA 2127, 7A1, was also found to be deficient in biofilm formation, and as the gene PA 2127 has not yet been attributed a function and is located just next to a cluster of recently identified novel adhesions. Further characterization of this mutant should be very promising and possibly reveal an insight into the regulation of adhesion by quorum sensing. Two mutants have been identified that have a transposon insertion in a gene linked to iron metabolism (PA 3268) and to siderophore production (PA 2402). The latter finding is very interesting as it could provide a direct link between siderophore production and iron metabolism and quorum sensing. However, the impact of all mutations on the respective phenotypes needs to be verified by constructing isogenic mutants and their genotype and phenotype has to be compared to that of the original STM mutant.

Other interesting quorum-sensing deficient mutants that were found carried transposon insertions in regulators, of which all except for *lasR* have not yet been linked to quorum sensing. Furthermore, several mutants in whose genome the transposon had inserted into genes putatively annotated as transporters as well as genes involved in C4 carboxylate metabolism were identified as quorum-sensing deficient, which provides more ground for ongoing research in this field. Another mutant that was identified as quorum-sensing deficient carried a mutation in a gene belonging to the second recently identified cluster of genes encoding for novel adhesins in *P.aeruginosa*, further hinting at a regulatory link between quorum sensing and adhesion. A detailed characterization of these genes however was beyond the scope of this dissertation, and these genes will be the subject of ongoing research.

A cooperative study with a team of researchers from Hannover and Grenoble, France, aimed at the phenotypic and genotypic comparison of two of the most virulent *P.aeruginosa* clinical isolates known to date. The author coordinated tasks to be carried out for this research project between the teams and also supplied experimental data, the results of this project are presented as a chapter within this thesis. The clinical isolates CHA and TB, although phenotypically similar in many traits, proved to be genotypically unrelated and also, several fundamental phenotypical differences were discovered, the most important finding being that strain CHA is not able to survive intracellularly in PMN while strain TB does. It has been hypothesized that Type III Secretion System activity could play a role in intracellular survival; therefore, a mutant of strain TB with completely abrogated expression of TTSS secreted effectors was also examined for this striking phenotype. Intracellular survival proved to be independent of TTSS activity. However, the damage towards the PMN exerted by the wildtype strain was much greater in comparison to the TTSS-deficient mutant, proving that strain TB indeed uses TTSS secreted effectors to mediate its cytotoxicity towards the PMN.

Identification of mutants generated by random insertional mutagenesis in screens directed towards the elucidation of a specific phenotype has lead to the elucidation of numerous gene functions in the past years. However, any randomly generated mutation needs to be verified for its importance and impact on the respective phenotype, which is most commonly done by complementation of the gene *in trans*.

This method often does not yield stable phenotypes so that the phenotypical assessment of the complemented mutant is not always possible. The construction of a mutant isogenic to the original transposon mutant circumvents this problem.

In this study, an easy, hands-on protocol for constructing knock-out mutants in *P.aeruginosa* isolates other than the genetic reference strain PAOI has been established and successfully applied. Nearly 50 mutants from two strains were obtained for a gene that is not present in the PAOI genome and was

found to regulate quorum sensing in *P.aeruginosa* TB. The phenotype and genotype of the mutants were examined. Several mutants that did respond to both selections applied in the process of mutant construction were false positives, nevertheless, screening for a quorum sensing deficient phenotype in combination with restriction analysis and Southern blotting revealed that more than 50% of the selected mutants were true knock-outs.
This is a work in process and some refining will be added to the method, nevertheless, the pivotal experiment served to prove both the applicability of the method to different *P.aeruginosa* strains as well as its value for the verification of the phenotype of a transposon mutant: The knock-out mutant in this gene generated from strain TB was found in a screen for quorum sensing capabilities and proved to be of special interest as the transposon insertion had occurred within a gene that was not present in the PAOI genome and still led to a complete shutdown of quorum sensing. Complementation experiments had failed so that the construction of mutants isogenic to the original transposon mutant was an essential step for the further characterization of this fascinating mutant.
Furthermore, a protocol for the direct genomic sequencing of *P.aeruginosa* transposon mutants was designed and established, which allowed for the sequencing of several transposon mutants without the help of PCR or plasmid-based techniques for the isolation of the affected gene. It was complemented by the application of a method for the identification of transposon insertions independent of the insert sequence, and the gene affected by the transposon insertion was identified this way in all transposon mutants that exhibited phenotypical differences from the parent strain in the quorum sensing screen as well as a screen for cytotoxicity.

This study presents an approach to functional genome analysis by integrating bioassays to screen for virulence determinants as well as novel techniques for the identification of transposon insertion sites and the construction of knock-out mutants in different *P.aeruginosa* isolates. It should be a step further on the way of understanding of bacterial lifestyle as well as host-pathogen interactions.

Appendix

Abbreviations

ADP	Adenosine diphosphate
Amp	Ampicillin
AMS	Antimycotic/Antibiotic Solution
ATP	Adenosine triphosphate
Bp	base pair
CF	Cystic Fibrosis
CIP	Calf Intestine Phosphatase
dATP	Desoxyadenine triphosphate
dCTP	Desoxycytidine triphosphate
dGTP	Desoxyguanidine triphosphate
dUTP	Desoxyuridine triphosphate
dNTP	Desoxynucleotide triphosphate(s)
dTTP	Desoxythymidine teiphosphate
DIG	Digoxygenin
DMSO	Dimethyl Sufoxide
EDTA	Ethylenediamine tetraacetate
Et al.	and others
FCS	Fetal Calf Serum
g	gram
GST	Glutathione-S-transferase
h	hour
HSL	Homoserine Lactone(s)
IVET	*In vitro* expression technique
MCS	Multicloning Site
Mg	milligram
MOI	Multiplicity of Infection
MTT	Methylthiazolyltriazolium bromide
ng	nanogram
nm	nanometer
NRPS	Non-Ribosomal Peptide Synthetase(s)
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PMA	Phorbol Myristyl Acetate
PMN	Polymorphonuclear Neutrophils
PQS	Pseudomonas Quinolone Signal
QS	Quorum Sensing
sec	second
SNP	Single Nucleotide Polymorphism
STM	Signature Tagged Mutagenesis
TTSS	Type III Secretion System
V	Volt
vol	Volume
w/vol	Weight/Volume
µg	Microgram
µm	Micrometer

Sequences of genes for allelic replacement

47D7 Sequence

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NID g3135550
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ORGANISM Plasposon pTnMod-OGm
artificial sequence; cloning vectors.
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AUTHORS Dennis,J.J. and Zylstra,G.J.
TITLE Plasposons: modular self-cloning mini-transposon derivatives for
the rapid genetic analysis of Gram-negative bacterial genomes
JOURNAL Appl. Environ. Microbiol. (1998) In press
REFERENCE 2 (bases 1 to 4689)
AUTHORS Dennis,J.J. and Zylstra,G.J.
TITLE Direct Submission
JOURNAL Submitted (29-APR-1998) Biotechnology Center for Agriculture and
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Dudley Rd., New Brunswick, NJ 08901-8520, USA
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1861 tcggccgccg tggctccggc gtcttgggtg ccggcgcggg ttccgcagcc ttggcctgcg
1921 gtgcgggcac atcggcgggc ttggccttga tgtgccgcct ggcgtgcgag cggaacgtct
1981 cgtaggagaa cttgaccttc cccgtttccc gcatgtgctc ccaaatggtg acgagcgcat
2041 agccggacgc taacgccgcc tcgacatccg ccctcaccgc caggaacgca accgcagcct
2101 catcacgccg gcgcttcttg gccgcgcggg attcaaccca ctcggccagc tcgtcggtgt
2161 agctctttgg catcgtctct cgcctgtccc ctcagttcag taatttcctg catttgcctg
2221 tttccagtcg gtagatattc cacaaaacag cagggaagca gcgcttttcc gctgcataac
2281 cctgcttcgg ggtcattata gcgatttttt cggtatatcc atcctttttc gcacgatata
2341 caggattttg ccaaagggtt cgtgtagact ttccttggtg tatccaacgg cgtcagccgg
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2581 ctatcaaggt gtactgcctt ccagacgaac gaagagcgat tgaggaaaag gcggcggcgg
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2701 tcgtggacta tgagcacgtc cgcgagctgg cccgcatcaa tggcgacctg ggccgcctgg
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3481 gcagcgtgaa gctttctctg agctgtaaca gcctgaccgc aacaaacgag aggatcgaga
3541 ccatccgctc cagattatcc ggctcctcca tgcgttgcct ctcggctcct gctccggttt
3601 tccatgcctt atggaactcc tcgatccgcc agcgatgggt ataaatgtcg atgacgcgca
3661 aggcttgggc tagcgactcg accggttcgc tggtcagcaa caaccatttc aacggggtct
3721 caccttgggg cgggttaatc tcctcggcca gcaccgcgtt gagcgtgata ttcccctgtt
3781 ttagcgtgat gcgcccactg cgcaggctca agctcgcctt gcgggctggt cgatttttac
3841 gtttaccgcg tttatccacc acgccctttt gcggaatgct gatctgatag ccacccaact
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4141 catccgggcg catccaccac tcctgatgca gtaatcctac ggtgcggaat gtggtggcct
4201 cgagcaagag aacggagtga acccaccatc cgcgggattt atcctgaata gagcccagct
4261 tgccaagctc ttcggcgacc tggtggcgat aactcaaaga ggtggtgtcc tcaatggcca
4321 gcagttcggg aaactcctga gccaacttga ctgtttgcat ggcgccagcc tttctgatcg
4381 cctcggcaga aacgttggga ttgcggataa atcggtaagc gccttcctgc atggcttcac
4441 taccctctga tgagatggtt attgatttac cagaatattt tgccaattgg gcggcgacgt
4501 taaccaagcg ggcagtacgg cgaggatcac ccagcgccgc cgaagagaac acagatttag
4561 cccagtcggc cgcacgatga agagcagaag ttatcatgaa cgttaccatg ttaggcaggt
4621 cacatggaag atcagatcct ggaaaacggg aaaggttccg ttcaggacgc tacttgtgta
4681 cgggatcgg

Primers employed for experiments of this dissertation

Y-Primer new: CTGCTCGAGCTCAAGCTTCG

D8A6 P1(400)fwd: CTT CGT CAA GAC GCT CCT AG

D8A6 P1 (693)fwd: CTG AGC CAA GAT CGC ATC G

D8A6 P1(1514)rev: CCA GCG CTA CAA CAT CAT GC

D8A6 P1 (1375)rev :CAG ACC AAC CGG CAG TGG AGT C

D8A6 P2 (3566) fwd: GGA TAC GCT CGC AGA TGG AC

D8A6 P2 (4307) rev: CGC ACG GAG GAC TAC TGA TG

D8A6 P2 (4364)fwd: GAA CAC GGT GGT ATA GCC CG

D8A6 P2 (4853)rev: GTC TAC GTG CAA CCT GGC GCA C

D8A6 P2 (4901)rev: GAC ATC CGG CAG TGG ATG AAC

D8A6 QC1fwd: GCA CGC CCT CGG TGA ATT TG

D8A6 QC1rev: GAG AGT CCC GAC CAG GTG AAG

D8A6 QC2fwd: CGA ACA TCC GTG GCA GGT AC

D8A6 QC2rev: CAT TTC ACC GGC GAG ACG CTC

gene spanning primers for Sequencing PCR product:

C47-L1: CGC TAA GCT TCT CAG TTG AAT CTG CTG CAG
C47-RP: ATA TCG TAC GAC CAG CAA GGA GGA GAT GCT

Sqeuencing primers D8A6 as found in sequence:
Gm1 (fwd) TCC CGC AGT GGC TCT CTA TAC
D8A6 inside fwd: CAC TCG GTG TGG CAT GGC ATC G

47D7 P1(76)fwd: GTG CTA GCC GAA TCC TCC TG

47D7 P1(500)rev: CAA CGG TCA GTT CGG TTC CG

47D7 P1(76)rev: CAG GAG GAT TGG GCT AGC AC

47D7 P1(500)fwd: CGG AAC CGA ACT GAC CGT TG

47D7 P2(1422)fwd: GTC CGG AGC ATG CAA ACG AC

47D7 P2(1030)rev: GGG AGT TCT TCT TGG CCG AC

Sequencing primers for genomic sequencing (Qiagen)

Left primer:
CGCGGCCGGCCTAGGCGGCCAGATCTGATCAAGAGACAG

Right primer: TAATTAATTTAAATCTAGACTAGTGCGGCCGCACTTGTGTATAAGAGTCAG

Transposon and y-linker primer

Tn2: TGC GTT CGG TCA AGG TTC TGG
Tn3: AGC CAT GAA AAC CGC CAC TGC

y-linker primer new: CTG CTC GAG CTC AAG CTT CG

Comparison of sequenced genes of strains CHA and PAOI, sequence data

CHA/PAO comparison gene PA 3841. *LasB*

3 sequencing errors, 4 nt exchange, gap of 318 nt
There is a significant exchange in amino acids in the peptide sequence that aligns with that predicted from the forward PCR product. It's relevance for the structure of the protein remains to be determined.

Forward primer:
>iv55_3724_3724F.fasta
TAGACCGGGTTCGCGGAGGCCAACGGCCCAAGCGACATAAAGCAGCCGCCCTCCTGGCGG
AAGACGGCTTGAGCGACCCCGACCGGCATTCCTTCCTGGAGTGCCAGCCGGGACCACCGA
GCTTACAACGCGCTCGGGCAGGTCACGCCGACGGTGCTGAACGCCCGGGTGACGTCAGCC
GCCGAGTAGTTGCGGTTCTGCGCCGAGCGAATCACCCCGCAGGCGCCGCTGTTGTAGTTG
CTGGTGGCGGTCCAGTAGTAGCGGTTGGCGTCGACGAACACCTCGAAGGCCTTGCGGGTA
TCCCAGCCCGGCGAATTGGCCAACAGGTAGAACGCACGGTTGTACACGCCGCTGGAGTGG
TGCACGTCGATGCCGTTGTAGTACTGCGACGCGTTGTCGATGGATCGCCCGTCGCGGCTG
GGCTGGTCCATGTAGCGCAGCGCACCGCTGCCCTTCTTGATGTCGTAGCCGATCAGGAAG
TCGTTCTTGCCGCGCATGTAGAACTCGGCTGCCTCGCCGGCCATGTCGGAGAACGCTTCG
TTCATTCCGCCTGATTGCCCGCGGTAGATCAGCCCGGAGTTCTGCTCGGTAAAGCCGTGG
CTGACCTCGTGGGCCGCCACGTCCAGCGACACCAGCGGATAGAACACGGTGGCGCCGTCG
CCGAAGAGCATCGCCGTGCCGTCCCAGTAGGCGTTCTCCACGCTGCGCCCGTAGTGCACC
TTCATGTACAGCTTGTGGGTCAGCGGGCTGGTGCCGAACCAGTCCCGGTACAGTTTGAAC
ACCACGCCGCCGAAGAAATGCGCGTCGTTCAGCGGCGAAAGGCGCCGTTGACCTGCTTGT
AGGTGTTGGTCGGGCAGCGAAGCGAACGGCGTGGTCTTGCTGTCGTCGGTGCTGCTGTTC

```
ATGTCGACGGTGATGACGT
```

Reverse primer:
>iv55_3724_3724R.fasta

```
TGATCGTCCACATGGCCCCTCGCTGAGCGCGTCCCGGAGCTGGGGGCAACCTAGCTGCCA
CCTGCTTTTCTGCTAGCTATTCCAGCGAAAACATACAGATTTCCGGCGAAATCAAGGCTA
CCTGCCAGTTCTGGCAGGTTTGGCCGCGGGTTCTTTTTGGTACACGAAAGCACCGTCGAA
AACGGGACCGAGCCAGGGGAGTGCAGTTCCTTCTACCCGAAGGACTGATACGGCTGTTCC
GATCAGCCCACAAGGCGGCGGTAAGCGTCGGCCGAGTACTTCGGCCTGAAAAAACCAGGA
GAACTGAACAAGATGAAGAAGGTTTCTACGCTTGACCTGTTGTTCGTTGCGATCATGGGT
GTTTCGCCGGCCGCTTTTGCCGCCGACCTGATCGACGTGTCCAAACTCCCCAGCAAGGCT
GCCCAGGGCGCGCCCGGCCCGGTCACCTTGCAAGCCGCGGTCGGCGCTGGCGGTGCCGAC
GAACTGAAAGCGATCCGCAGCACGACCCTGCCCAACGGCAAGCAGGTCACCCGCTACGAG
CAATTCCACAACGGCGTACGGGTGGTCGGCGAAGCCATCACCGAAGTCAAGGGTCCCGGC
AAGAGCGTGGCGGCGCAGCGCAGCGGCCATTTCGTCGCCAACATCGCCGCCGACCTGCCG
GGCAGCACCACCGCGGCGGTATCCGCCGAGCAGGTGCTGGCCCAGGCCAAGAG
```

```
BLAST result for forward primer:
Score = 1741 bits (878), Expect = 0.0
                  Identities = 914/922 (99%), Gaps = 3/922 (0%)
                  Strand = Plus / Plus
Query: 1, Sbjct: 4168865
tagaccgggttcgcggaggccaacggcccaagcgacataaagcagccgccctcctggcgg
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tagaccgggttcgcggaggccaacggcccaagcgacataaagcagccgccctcctggcgg 4168924
Query: 61
aagacggcttgagcgaccccgaccggcattccttcctggagtgccagccgggaccaccga 120
|||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
aagacggcttgagcgaccccgaccggcattccttcctggagtgccggccgggaccaccga 4168984
Query: 121
gcttacaacgcgctcgggcaggtcacgccgacggtgctgaacgcccgggtgacgtcagcc 180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gcttacaacgcgctcgggcaggtcacgccgacggtgctgaacgcccgggtgacgtcagcc 4169044
Query: 181
gccgagtagttgcggttctgcgccgagcgaatcaccccgcaggcgccgctgttgtagttg 240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gccgagtagttgcggttctgcgccgagcgaatcaccccgcaggcgccgctgttgtagttg 4169104
Query: 241
ctggtggcggtccagtagtagcggttggcgtcgacgaacacctcgaaggccttgcgggta 300
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tcccagcccggcgaattggccaacaggtagaacgcacggttgtacacgccgctggagtgg 360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tcccagcccggcgaattggccaacaggtagaacgcacggttgtacacgccgctggagtgg 4169224
Query: 361
tgcacgtcgatgccgttgtagtactgcgacgcgttgtcgatggatcgcccgtcgcggctg 420
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tgcacgtcgatgccgttgtagtactgcgacgcgttgtcgatggatcgcccgtcgcggctg 4169284
Query: 421
ggctggtccatgtagcgcagcgcaccgctgcccttcttgatgtcgtagccgatcaggaag 480
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ggctggtccatgtagcgcagcgcaccgctgcccttcttgatgtcgtagccgatcaggaag 4169344
Query: 481
tcgttcttgccgcgcatgtagaactcggctgcctcgccggccatgtcggagaacgcttcg 540
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tcgttcttgccgcgcatatagaactcggcagcctcgccggccatgtcggagaacgcttcg 4169404
Query: 541
ttcattccgcctgattgcccgcggtagatcagcccggagttctgctcggtaaagccgtgg 600
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ttcattccgcctgattgcccgcggtagatcagcccggagttctgctcggtgaagccgtgg 4169464
Query: 601
ctgacctcgtgggccgccacgtccagcgacaccagcggatagaacacggtggcgccgtcg 660
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ctgacctcgtgggccgccacgtccagcgacaccagcggatagaacatggtggcgccgtcg 4169524
Query: 661
ccgaagagcatcgccgtgccgtcccagtaggcgttctccacgctgcgcccgtagtgcacc 720
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ccgaagagcatcgccgtgccgtcccagtaggcgttctccacgctgcgcccgtagtgcacc 4169584
Query: 721
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ttcatgtacagcttgtgggtcagcgggctggtgccgaaccagtcccggtacagtttgaac 4169644
Query: 781
accacgccgccgaagaaatgcgcgtcgttcagcggcgaa-aggcgccgttgacctgcttg 839
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accacgccgccgaagaaatgcgcgtcgttcagcggcgaataggcgccgttgacctgcttg 4169704
```

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Query: 840
taggtgttggtcgggca-gcgaagc-gaacggcgtggtcttgctgtcgtcggtgctgctg 897
|||||||||||||||| ||||||| ||||||||||||||||||||||||||||||||||
taggtgttggtcgggcaggcgaagcggaacggcgtggtcttgctgtcgtcggtgctgctg 4169764
Query: 898 Sbjct: 4169765
ttcatgtcgacggtgatgacgt 919
|||||||||||||||||||||
ttcatgtcgacggtgatgacgt 4169786

Score = 1405 bits (709), Expect = 0.0
                 Identities = 712/713 (99%)
                 Strand = Plus / Minus
Query: 1       Sbjct: 4170795
tgatcgtccacatggccccctcgctgagcgcgtcccggagctgggggcaacctagctgcca 60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tgatcgtccacatggccccctcgctgagcgcgtcccggagctgggggcaacctagctgcca 4170736

cctgcttttctgctagctattccagcgaaaacatacagatttccggcgaaatcaaggcta 120
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cctgcttttctgctagctattccagcgaaaacatacagatttccggcgaaatcaaggcta 4170676
cctgccagttctggcaggtttggccgcgggttctttttggtacacgaaagcaccgtcgaa 180
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cctgccagttctggcaggtttggccgcgggttctttttggtacacgaaagcaccgtcgaa 4170616
aacgggaccgagccaggggagtgcagttccttctacccgaaggactgatacggctgttcc 240
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aacgggaccgagccaggggagtgcagttccttctacccgaaggactgatacggctgttcc 4170556
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gaactgaaagcgatccgcagcacgaccctgcccaacggcaagcaggtcacccgctacgag 4170256
caattccacaacggcgtacgggtggtcggcgaagccatcaccgaagtcaagggtcccggc 600
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caattccacaacggcgtacgggtggtcggcgaagccatcaccgaagtcaagggtcccggc 4170196
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aagagcgtggcggcgcagcgcagcggccatttcgtcgccaacatcgctgccgacctgccg 4170136
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ggcagcaccaccgcggcggtatccgccgagcaggtgctggcccaggccaagag 4170083

 Amino acid BLAST result for forward PCR product:
PA3724 >PA3724 [gene=lasB] [prot=elastase LasB] [comment=PA3724]
                           Length = 498

                  Score =  510 bits (1313), Expect = e-145
                  Identities = 249/265 (93%), Positives = 252/265 (94%), Gaps = 1/265 (0%)
                  Frame = -3

Query: 917 Sbjct: 234
VITVDMNSSTDDSKTTPFA-SLPDQHLQAGQRRLSPLNDAHFFGGVVFKLYRDWFGTSPL 741
VITVDMNSSTDDSKTTPF++++P++++++++++SPLNDAHFFGGVVFKLYRDWFGTSPL
VITVDMNSSTDDSKTTPFRFACPTNTYKQVNGAYSPLNDAHFFGGVVFKLYRDWFGTSPL 293

Query: 740 Sbjct: 294
THKLYMKVHYGRSVENAYWDGTAMLFGDGATVFYPLVSLDVAAHEVSHGFTEQNSGLIYR 561
THKLYMKVHYGRSVENAYWDGTAMLFGDGAT+FYPLVSLDVAAHEVSHGFTEQNSGLIYR
THKLYMKVHYGRSVENAYWDGTAMLFGDGATMFYPLVSLDVAAHEVSHGFTEQNSGLIYR 353
Query: 560 Sbjct: 354
GQSGGMNEAFSDMAGEAAEFYMRGKNDFLIGYDIKKGSGALRYMDQPSRDGRSIDNASQY 381
GQSGGMNEAFSDMAGEAAEFYMRGKNDFLIGYDIKKGSGALRYMDQPSRDGRSIDNASQY
GQSGGMNEAFSDMAGEAAEFYMRGKNDFLIGYDIKKGSGALRYMDQPSRDGRSIDNASQY 413
Query: 380 Sbjct: 414
YNGIDVHHSSGVYNRAFYLLANSPGWDTRKAFEVFVDANRYYWTATSNYNSGACGVIRSA 201
YNGIDVHHSSGVYNRAFYLLANSPGWDTRKAFEVFVDANRYYWTATSNYNSGACGVIRSA
YNGIDVHHSSGVYNRAFYLLANSPGWDTRKAFEVFVDANRYYWTATSNYNSGACGVIRSA 473

                  Query: 200  QNRNYSAADVTRAFSTVGVTCPSAL 126
```

```
                              QNRNYSAADVTRAFSTVGVTCPSAL
                    Sbjct: 474  QNRNYSAADVTRAFSTVGVTCPSAL 498

Amino acid BLAST for reverse PCR product:
>PA3724 >PA3724 [gene=lasB] [prot=elastase LasB] [comment=PA3724]
Length = 498

Score =  254 bits (649), Expect = 8e-069
Identities = 133/133 (100%), Positives = 133/133 (100%)
Frame = +1

Query: 313 Sbjct: 1
MKKVSTLDLLFVAIMGVSPAAFAADLIDVSKLPSKAAQGAPGPVTLQAAVGAGGADELKA 492
MKKVSTLDLLFVAIMGVSPAAFAADLIDVSKLPSKAAQGAPGPVTLQAAVGAGGADELKA
MKKVSTLDLLFVAIMGVSPAAFAADLIDVSKLPSKAAQGAPGPVTLQAAVGAGGADELKA 60

Query: 493 Sbjct: 61
IRSTTLPNGKQVTRYEQFHNGVRVVGEAITEVKGPGKSVAAQRSGHFVANIAADLPGSTT 672
IRSTTLPNGKQVTRYEQFHNGVRVVGEAITEVKGPGKSVAAQRSGHFVANIAADLPGSTT
IRSTTLPNGKQVTRYEQFHNGVRVVGEAITEVKGPGKSVAAQRSGHFVANIAADLPGSTT 120

Query: 673 Sbjct: 121
AAVSAEQVLAQAK 711
AAVSAEQVLAQAK
AAVSAEQVLAQAK 133
```

Chitin Binding protein precursor, *CbpD*, PA 0852

No difference found by comparing the nucleotide sequences of gene PA 0852 from PAO1 and CHA. However, there is a sequence gap forward read/reverse read of 124 bp!!!

Sequence data: from 930585-932047; gene range from : 931822-930653

>iv55_852_852F.fasta range from 930585-931161

```
CGCCGTCTCGCGGCGGGTGCCTGGCGGCGAAAACGACTGCCGCCCGGAGCGGGCGGCAGT
CGAGGGCGTTACAGCAGGGTCCAGGCGTCCTGCCAGGCCATGCCCTTGCCCGGGGCGTAG
TAGAGGTCCCAGCCCTTGCACCAGCCGGAGTTCGGGTAGGGCTTGCACTGGTAGCGCTTG
CCATCGGCACCGCGCACTACGGTCCCGGCGTCGTATTGCTGCAGGCCTTGCGGATAGTCG
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TCGACCTGGAAGCGGTAGCCGGCCTGGCGCACGTAGACCTGGTTGTCCTGCGAGCTGGCC
ACCGGGCTGACCGCCCCGTAGGCATCCAGCACGCCGATGTTGACCAGGGTGGAGTCCTGG
TTGACCTTCTGCGCCAGCGCCAGCGGCCATTGCTTGGCACCGTTGGCGCCCTGGGCCAGG
GTCAGGCTGTGACGCTGGGCGTCGCGGCCCTGGGCATCGAACAGACGCAGGGTGACGGTA
GCACCGGCTGGCAGGTCCTGCTGCGCGCGCAGGTTGC
Reversed:
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ATGCCCAGGGCCGCGACGCCCAGCGTCACAGCCTGACCCTGGCCCAGGGCGCCAACGGTG
CCAAGCAATGGCCGCTGGCGCTGGCGCAGAAGGTCAACCAGGACTCCACCCTGGTCAACA
TCGGCGTGCTGGATGCCTACGGGGCGGTCAGCCCGGTGGCCAGCTCGCAGGACAACCAGG
TCTACGTGCGCCAGGCCGGCTACCGCTTCCAGGTCGACATCGAACTGCCGGTCGAGGGCG
GCGGCGAGCAACCGGGCGGCGACGGCAAGGTCGACTTCGACTATCCGCAAGGCCTGCAGC
AATACGACGCCGGGACCGTAGTGCGCGGTGCCGATGGCAAGCGCTACCAGTGCAAGCCCT
ACCCGAACTCCGGCTGGTGCAAGGGCTGGGACCTCTACTACGCCCCGGGCAAGGGCATGG
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GTCGTTTTCGCCGCCAGGCACCCGCCGCGAGACGGCG
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>iv55_852_852R.fasta range from : 931285-932047

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CGAATCTGACCGGCCCGTCACATTTGGTAGGGACCTCAAACCGGCACCGAGGGCTAGGAT
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CACGGCTCGATGGAAACGCCGCCCAGTCGGGTCTACGGCTGCTTCCTCGAAGGTCCGGAG
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AAGCTGGAGAACGGCACCTACCGGATGAACTGCCCGCTGCCCC
Reversed:
GGGGCAGCGGGCAGTTCATCCGGTAGGTGCCGTTCTCCAGCTTGACGCTGGTGATCGAGC
AGAACGGCGCGGGTTCCAGGTCGCTCCAGGCCAGCGGCTTCTCGGGGTTGTAGCCGTCCT
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CGCCGGCGGCGACGGCGGCCTTGCAGGCGGCCGACTTGGGATTCTCCGGACCTTCGAGGA
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AGCAGCCGTAGACCCGACTGGGCGGCGTTTCCATCGAGCCGTGGGCATGGGCTGCCTGGG
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CAAGTCCTTTTTTCCAGAACCGGATTCATTTCGAAGCGGGCTGATCCTAGCCCTCGGTGC
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ATGTCGCGGAATGCAAAGCGGTCGGCACCCGCACTGTGAGCCGGTAGAGGCAGGCAAAGG
GCCGCCATGGGGGCGGGGAAGGGAAGCCGGCGGGAAGGTGGCG
Score = 1144 bits (577), Expect = 0.0
                 Identities = 577/577 (100%)
                 Strand = Plus / Plus
Query:1; Sbjct: 930585
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 541
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|||||||||||||||||||||||||||||||||||||
gcaccggctggcaggtcctgctgcgcgcgcaggttgc 931161

Blast-Ergebnisse für Chitin-Binding Protein (CHA gegen PAO1), reverse primer
Query: 1      ggggcagcgggcagttcatccggtaggtgccgttctccagcttgacgctggtgatcgagc
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 931285 ggggcagcgggcagttcatccggtaggtgccgttctccagcttgacgctggtgatcgagc 931344
Query: 61     agaacggcgcgggttccaggtcgctccaggccagcggcttctcggggttgtagccgtcct
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CHA-Chitinase vs.PAO1 PA2300

Sequence data from 2530301-2531092 and 2531154-2531083, sequencing gap of 62 bp

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Query: 241     gatccaccgccccgccgctggtgctgacctccaggccttcgctgggcagcgactggttgc
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CHA Chitinase gegen PAO 1 P.aeruginosa 2300 ab Base 793 (2.Teil des Gens)

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Sbjct: 2531154 ccaggcgcccttgccgccgttcgcctcctggacccacaggccgtcgccgccctggttgta 2531213
Query: 851     gtactgcggcgcgatgaagtcgtagacgccttccagggcctgcagataaggcacgtactt
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 Score = 1582 bits (798), Expect = 0.0
 Identities = 801/802 (99%)
 Strand = Plus / Plus

Query: 1       taccgaaaacgcatatggatatggcgccacccgtgcgtggctctcgccggccaaaggacc
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Sbjct: 2530901 ggccatcgtcccagttcaccgaccaggtcatcaggcccttgatcgcgtgaccggcggctt 2530960
Query: 661     ccaggcggcgaaacgcgttgctcaccgcggcgggatcgatcacgtagccggtagccgccg
               ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2530961 ccaggcgacgaaacgcgttgctcaccgcggcgggatcgatcacgtagccggtagccgccg
Query: 721
```

```
catcgacgttgctcggcaggccgatggccaggcgctgcgccgggatccgcacgaagtcgc
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
catcgacgttgctcggcaggccgatggccaggcgctgcgccgggatccgcacgaagtcgc 2531080
Query: 781     ggctgccggtggccaggctctc 802
                |||||||||||||||||||||
Sbjct: 2531081 ggctgccggtggccaggctctc 2531102
```

>iv55_2300_2300F.fasta
TACCGAAAACGCATATGGATATGGCGCCACCCGTGCGTGGCTCTCGCCGGCCAAAGGACCGGCCCGCAGCGCGGACCGG
CCCGCCCCCTCAGCGCAGCGGCCGCCAGAGGGTGAAGGCTACCGGCGGCGTCCAGCCGCTGTTGGAGGTGTGCGCCTG
CAGGCAGAGATAGCGCAGCCCCTCGTAGGTCACCCCATCGTCGACCCGATAGGCCTGGTTCTCCCGCCATTGCGGGAATT
GCGGATCCACCGCCCCGCCGCTGGTGCTGACCTCCAGGCCTTCGCTGGGCAGCGACTGGTTGCCCTGGGTATCGGTGGC
GGTGACGAAATAGCTGTAGCGGGTGTCCGCCGTCAGGCCGCTGTCGGTGGAACCCAGCGCGGCGCTCTGGCCAACCATG
GCGCCGTCGCGATAGAGGCTGTAGTAATCGATCGGACGCTGCCCGCTGGAGGCGTTCCAGGCCAGCACCAGGCTGGTCT
CGCCGCGCTCCAGCAGACGCAGGCCCTGCGGCGCCGCCGGGCGCTGGTCGCCGCCCTCGCCGTCATGGATGAGGCTGG
CGTAGCGCTTGCGGAACTCCCAGTTGTAGCGCTCGCCGCGCTTGTTCAGGCCATCGTCCCAGTTCACCGACCAGGTCATC
AGGCCCTTGATCGCGTGACCGGCGGCTTCCAGGCGGCGAAACGCGTTGCTCACCGCGGCGGGATCGATCACGTAGCCGG
TAGCCGCCGCATCGACGTTGCTCGGCAGGCCGATGGCCAGGCGCTGCGCCGGGATCCGCACGAAGTCGCGGCTGCCGG
TGGCCAGGCTCT

Sequence data reverse primer:
>iv55_2300_2300R.fasta
CGCCGAACTCAGAGAAGCCGGTTGAACCCGGCGCGGAGCATCGCCGCCGCGGCTCTGCCAATTGTGGTAGGTGAAGCCT
TCCGCCGTCTCTGCAACCATGCCCCCGCCCTGATCGCGACAAACGCCGGAATGCCCCGTGGTAGACGCTCGCGCCTGTTT
TTTCTAAGCGTTCGCGGCGGCCACGGCGCGGTTCGTCGGACAGCCCCTCCGGATGCGTTTCCTACCTTCATCCGAGGAGC
GGTAAATGATCAGGATCGACTTTTCCCAGTTGCACCAGGCCCGCGAAGATGCCGCGGCGGCCATGCCGAGCATCGCCGG
CAAGAAGATTCTCATGGGCTTCTGGCACAACTGGCCGGCCGGCGCCGCCAACGGCTACCAGCAGGGCTCGTTCGCCAAC
ATCGCGCTGGAAGACGTGCCGAGCGAGTACAACGTGGTCGCCGTGGCCTTCATGAAAGGGCGCGGCATCCCGACCTTCC
AGCCATACAACCTGTCCGACGCGGAGTTTCGCCGCCAGGTCGGCGTGCTCAACGCCCAGGGCCGCGCGGTGCTGATTTC
GCTGGGGGGCGCCGACGCGCACATCGAGTTGCACGCCGGGCAGGA
GCAGGCGCTGGCCGCCGAGATCGTCCGTCTGGTGGAAACCTACGGTTTCGACGGCCTGGACATCGACCTCGAGCAGAGC
GCCATCGACCTGGCCGACAACCAGCGGGTGCTGCCGGCGGCCCTCAAGCTGGTGCGCGAGCACTACGCCGGGCAGGGC
AAGCACTTCATCGTCAGCATGGCCCCGGAGTTTCCCTATCTGCACAAGAACGGCAAGTACGTGCCTTATCTGCAGGCCCTG
GAAGGCGTCTACGACTTCATCGCGCCGCAGTACTACAACAGGGGCGGCGACGGCCTGTGGGTCAGGAGCGAACGGCGG
CAAG

AS-BLAST:
forward primer:

```
Score =  485 bits (1248), Expect = e-138
 Identities = 236/236 (100%), Positives = 236/236 (100%)
 Frame = -3

Query: 799 SLATGSRDFVRIPAQRLAIGLPSNVDAAATGYVIDPAAVSNAFRRLEAAGHAIKGLMTWS 620
           SLATGSRDFVRIPAQRLAIGLPSNVDAAATGYVIDPAAVSNAFRRLEAAGHAIKGLMTWS
Sbjct: 248 SLATGSRDFVRIPAQRLAIGLPSNVDAAATGYVIDPAAVSNAFRRLEAAGHAIKGLMTWS 307

Query: 619 VNWDDGLNKRGERYNWEFRKRYASLIHDGEGGDQRPAAPQGLRLLERGETSLVLAWNASS 440
           VNWDDGLNKRGERYNWEFRKRYASLIHDGEGGDQRPAAPQGLRLLERGETSLVLAWNASS
Sbjct: 308 VNWDDGLNKRGERYNWEFRKRYASLIHDGEGGDQRPAAPQGLRLLERGETSLVLAWNASS 367

Query: 439 GQRPIDYYSLYRDGAMVGQSAALGSTDSGLTADTRYSYFVTATDTQGNQSLPSEGLEVST 260
           GQRPIDYYSLYRDGAMVGQSAALGSTDSGLTADTRYSYFVTATDTQGNQSLPSEGLEVST
Sbjct: 368 GQRPIDYYSLYRDGAMVGQSAALGSTDSGLTADTRYSYFVTATDTQGNQSLPSEGLEVST 427

Query: 259 SGGAVDPQFPQWRENQAYRVDDGVTYEGLRYLCLQAHTSNSGWTPPVAFTLWRPLR 92
           SGGAVDPQFPQWRENQAYRVDDGVTYEGLRYLCLQAHTSNSGWTPPVAFTLWRPLR
Sbjct: 428 SGGAVDPQFPQWRENQAYRVDDGVTYEGLRYLCLQAHTSNSGWTPPVAFTLWRPLR 483
```

reverse primer:

```
Score =  441 bits (1134), Expect = e-125
 Identities = 217/220 (98%), Positives = 220/220 (99%)
 Frame = +2

Query: 245 MIRIDFSQLHQAREDAAAAMPSIAGKKILMGFWHNWPAGAANGYQQGSFANIALEDVPSE 424
           MIRIDFSQLHQAREDAAAAMPSIAGKKILMGFWHNWPAGAA+GYQQGSFANIALEDVPSE
Sbjct: 1   MIRIDFSQLHQAREDAAAAMPSIAGKKILMGFWHNWPAGAADGYQQGSFANIALEDVPSE 60

Query: 425 YNVVAVAFMKGRGIPTFQPYNLSDAEFRRQVGVLNAQGRAVLISLGGADAHIELHAGQEQ 604
           YNVVAVAFMKGRGIPTFQPYNLSDAEFRRQVGVLNAQGRAVLISLGGADAHIELHAGQEQ
Sbjct: 61  YNVVAVAFMKGRGIPTFQPYNLSDAEFRRQVGVLNAQGRAVLISLGGADAHIELHAGQEQ 120

Query: 605 ALAAEIVRLVETYGFDGLDIDLEQSAIDLADNQRVLPAALKLVREHYAGQGKHFIVSMAP 784
           ALAAEIVRLVETYGFDGLDIDLEQSAIDLADNQRVLPAALKLVREHYAGQGKHFIVSMAP
Sbjct: 121 ALAAEIVRLVETYGFDGLDIDLEQSAIDLADNQRVLPAALKLVREHYAGQGKHFIVSMAP 180

Query: 785 EFPYLHKNGKYVPYLQALEGVYDFIAPQYYNRGGDGLWVR 904
           EFPYLHKNGKYVPYLQALEGVYDFIAPQYYN+GGDGLWV+
```

```
Sbjct: 181 EFPYLHKNGKYVPYLQALEGVYDFIAPQYYNQGGDGLWVQ 220
```

two sequencing errors in this part!

Sequence comparison CHA/PAO I ExoS locus

Sequencing of this locus has proven to be difficult as the primers generated from sequences flanking the gene did not yield results. The primers used here are 219 nt into the gene (forward primer), the reverse primer is located in the next gene, leaving a sequence gap of 105 bp.
To fully cover the sequence of the exoS locus, a smaller PCR product could be generated that spans the gap, another one could be located just before the start of the gene and extend 300 nt into the gene.

Except for one nt exchange at position 4303904 and one sequencing error, the sequenced parts are identical on nt level. On AS level, the nt exchange results in an AS exchange.

```
>v219_CHAExoS_for_wh.fasta
CTCTTTTTCTTCTTGTAGTTCGATATCCCGCTGACATCGATTCCGGACCTGCCGAACACG
GTGGATATCGTGCCCTGCCCGAAGCTCCTCGCGACACCGGGGTTCAGGGAGGTGGAGAGA
TAGCCGTCGTCGTGGCCAACCTTGCCCTCTTCCACCGCGTTGAAGGCATCCCCGCCACGG
GTGCCACGGAAAGTCTTCACTACCTGTTCAGCCTGTCCGCTCTTCTCGAAGGCCGCGGAC
ATACCTTGGTCGATCAGCTTTTGTCCCGCATCCAGCTCCTGCCCCTGACGCAGAGCGCGA
TTCAGGTCCGCGTAGTGAATGCCGGTGTAGAGACCAAGCGCCATCACTTCGGCGTCACTG
TGGATGCCACCAGGCTGTCTGCCCAGGTACTTTTCCGCATCCGCCCCGAACCGCTTCACC
AGGCCATCCGCCAGCGCCTTGTCGGCCGATACTCTGCTGACCTCGCTCTCTACCGCTTGG
CGCAACAGTGCGACCTCGCTCATTACCTGATGCAACTGGTCGGTGATTTCGCGCCGCAGT
TCCAGGCTTGCGTCGAGCACC

>v219_CHAExoS_rev.fasta
CTGCAGGGCTGGCGGTTCCAGAGCAGCCGTTCGCCGCTTTCGGGGTCGCGGCCCAGGATC
GGCTTGCAAGGGTCCTGGCTGAACAGGTTCTGCTCGCTGGCCGTAGCGTCTCCTTGTCCT
TCCAGGCGGGTGAACATCAACAGGTGATCGGTCGGATGTTCTGCCAGATGGCAGAGATGC
GGGCCCACCTGCAGGCTGAGTACGCTCTCCTCGTCATTGGGCGTCGGGAGATCGAGAGCG
AGAAAAAGCTGGTGGATGGCGGCGCGGTAGAGTGGATTCATGGCGTGTTCCGAGTCACTG
GAGGCGGCCATTAGAGCAGTGCCAGCCCGGAGAGACTGTTAATCGTGGTTCTCTTTTTTT
AGGTTTTGCCGCTGCCGATTCCAGTGAAAAAACGGCGGCCAATCCTGATAGGCGATGGGG
TTTCCCGTTCCTAGACTGGCGGAGAAACATCAGGAGAAGGCAACCATCATGCATATTCAA
TCGCTTCAGCAGAGTCCGTCTTTCGCCGTCGAATTGCACCAGGCCGCCAGTGGGCGTTTG
GGACAGATTGAGGCCCGCCAGGTCGC

BLAST results for forward primer:

Identities = 559/562 (99%), Gaps = 1/562 (0%)
                  Strand = Plus / Plus


Query: 1 Sbjct: 4303360
ctctttttc-ttcttgtagttcgatatcccgctgacatcgattccggacctgccgaacac 59
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ctcttttttcattcttgtagttcgatatcccgctgacatcgattccggacctgccgaacac 4303419
ggtggatatcgtgccctgcccgaagctcctcgcgacaccggggttcagggaggtggagag 119
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ggtggatatcgtgccctgcccgaagctcctcgcgacaccggggttcagggaggtggagag 4303479
atagccgtcgtcgtggccaaccttgccctcttccaccgcgttgaaggcatccccgccacg 179
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
atagccgtcgtcgtggccaaccttgccctcttccacggcgttgaaggcatccccgccacg 4303539
ggtgccacggaaagtcttcactacctgttcagcctgtccgctcttctcgaaggccgcgga 239
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ggtgccacggaaagtcttcactacctgttcagcctgtccgctcttctcgaaggccgcgga 4303599
cataccttggtcgatcagcttttgtcccgcatccagctcctgcccctgacgcagagcgcg 299
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cataccttggtcgatcagcttttgtcccgcatccagctcctgcccctgacgcagagcgcg 4303659
attcaggtccgcgtagtgaatgccggtgtagagaccaagcgccatcacttcggcgtcact 359
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
attcaggtccgcgtagtgaatgccggtgtagagaccaagcgccatcacttcggcgtcact 4303719
gtggatgccaccaggctgtctgcccaggtacttttccgcatccgccccgaaccgcttcac 419
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gtggatgccaccaggctgtctgcccaggtacttttccgcatccgccccgaaccgcttcac 4303779
caggccatccgccagcgccttgtcggccgatactctgctgacctcgctctctaccgcttg 479
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
caggccatccgccagcgccttgtcggccgatactctgctgacctcgctctctaccgcttg 4303839
gcgcaacagtgcgacctcgctcattacctgatgcaactggtcggtgatttcgcgccgcag 539
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gcgcaacagtgcgacctcgctcattacctgatgcaactggtcggtgatttcgcgccgcag 4303899
Query: 540 Sbjct: 4303900
ttccaggcttgcgtcgagcacc 561
|||| ||||||||||||||||
ttccgggcttgcgtcgagcacc 4303921 nt exchange at pos. 4303904
```

```
BLAST result for reverse primer:
Query: 5 ; Sbjct: 4304966
agggctggcggttccagagcagccgttcgccgctttcggggtcgcggcccaggatcggct 64
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
agggctggcggttccagagcagccgttcgccgctttcggggtcacggcccaggatcggct 4304907
Query: 65
tgcaagggtcctggctgaacaggttctgctcgctggccgtagcgtctccttgtccttcca 124
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tgcaagggtcctggctgaacaggttctgctcgttggccgtagcgtctccttgtccttcca 4304847
Query: 125
ggcgggtgaacatcaacaggtgatcggtcggatgttctgccagatggcagagatgcgggc 184
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ggcgggtgaacatcaacaggtgatcggtcggatgttctgccagatggcagagatgcgggc 4304787
Query: 185
ccacctgcaggctgagtacgctctcctcgtcattgggcgtcgggagatcgagagcgagaa 244
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ccacctgcaggctgagtacgctctcctcgtcgttgggcgtcgggagatcgagagcgagaa 4304727
Query: 245
aaagctggtggatggcggcgcggtagagtggattcatggcgtgttccgagtcactggagg 304
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
aaagctggtggatggcggcgcggtagagtggattcatggcgtgttccgagtcactggagg 4304667
cggccattagagcagtgccagcccggagagactgttaatcgtggttctctttttttaggt 364
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cagccattagagcagtgccagcccggagagactgttaatcgtggttctctttttttaggt 4304607
Query: 365
tttgccgctgccgattccagtgaaaaaacggcggccaatcctgataggcgatggggtttc 424
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tttgccgctgccgattccagtgaaaaaacggcggccaatcctgataggcgatggggtttc 4304547
ccgttcctagactggcggagaaacatcaggagaaggcaaccatcatgcatattcaatcgc 484
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ccgttcctagactggcggagaaacatcaggagaaggcaaccatcatgcatattcaatcgc 4304487
Query: 485
ttcagcagagtccgtctttcgccgtcgaattgcaccaggccgccagtgggcgtttgggac 544
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ttcagcagagtccgtctttcgccgtcgaattgcaccaggccgccagtgggcgtttgggac 4304427
Query: 545 Sbjct: 4304426
agattgaggcccgccaggtcgc 566
|||||||||||||||||||||
agattgaggcccgccaggtcgc 4304405

AS-BLAST for product sequenced with forward primer, match = exoS.
Query: 560 VLDASLELRREITDQLHQVMSEVALLRQAVESEVSRVSADKALADGLVKRFGADAEKYLG 381
           VLDAS+ELRREITDQLHQVMSEVALLRQAVESEVSRVSADKALADGLVKRFGADAEKYLG
           VLDASPELRREITDQLHQVMSEVALLRQAVESEVSRVSADKALADGLVKRFGADAEKYLG 254

                  Query: 380 RQPGGIHSDAEVMALGLYTGIHYADLNRALRQGQELDAGQKLIDQGMSAAFEKSGQAEQV 201
RQPGGIHSDAEVMALGLYTGIHYADLNRALRQGQELDAGQKLIDQGMSAAFEKSGQAEQV
RQPGGIHSDAEVMALGLYTGIHYADLNRALRQGQELDAGQKLIDQGMSAAFEKSGQAEQV 314

                  Query: 200 VKTFRGTRGGDAFNAVEEGKVGHDDGYLSTSLNPGVARSFGQGTISTVFGRSGIDVSGIS 21
VKTFRGTRGGDAFNAVEEGKVGHDDGYLSTSLNPGVARSFGQGTISTVFGRSGIDVSGIS
VKTFRGTRGGDAFNAVEEGKVGHDDGYLSTSLNPGVARSFGQGTISTVFGRSGIDVSGIS 374

                  Query: 20  NYKKKK 3
                              NYK++K
                  Sbjct: 375 NYKNEK 380

AS-BLAST result for product sequenced with reverse primer, primer is located in next gene (PA
4382)
>PA3842 >PA3842 [gene=PA3842] [prot=probable chaperone] [comment=PA3842]
                           Length = 116
Score =  194 bits (493), Expect = 7e-051
                   Identities = 91/92 (98%), Positives = 92/92 (99%)
                   Frame = -1

                  Query: 281 MNPLYRAAIHQLFLALDLPTPNDEESVLSLQVGPHLCHLAEHPTDHLLMFTRLEGQGDAT 102
MNPLYRAAIHQLFLALDLPTPNDEESVLSLQVGPHLCHLAEHPTDHLLMFTRLEGQGDAT
MNPLYRAAIHQLFLALDLPTPNDEESVLSLQVGPHLCHLAEHPTDHLLMFTRLEGQGDAT 60

                  Query: 101 ASEQNLFSQDPCKPILGRDPESGERLLWNRQP 6
                              A+EQNLFSQDPCKPILGRDPESGERLLWNRQP
                  Sbjct: 61  ANEQNLFSQDPCKPILGRDPESGERLLWNRQP 92
```

CHA/PAO PA 2591

6 sequencing errors, one in the overlapping sequence of both PCR products, other than that, the gene is identical in CHA, one AA exchange at position 1 of the protein which is most likely due to the sequencing error present in the nt sequence.

```
Gene range from : 2934388-2933528, sequenced stretch of DNA from: 2934636-2933192
```

```
Score = 1191 bits (601), Expect = 0.0
 Identities = 607/609 (99%)
 Strand = Plus / Plus
Query: 1       cgtcctcgacactgagccggccgcggatatccggtccgcgcaaccccgccaccaggcgct
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2933192 cgtcctcgacactgagccggccgcggatatccggtccgcgcaaccccgccaccaggcgct 2933251
Query: 61      ggtcgaaggcgatcggcacgtgtccctggcgcacgatgttcaacaacacctcggccagtt
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2933252 ggtcgaaggcgatcggcacgtgtccctggcgcacgatgttcaacaacacctcggccagtt 2933311
Query: 121     cgccgggcgctatgtcgaatacccggggcgccgtggccacgtcgcgcggcccaccgtcgg
                |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct: 2933312 cgccgggcgctatgtcgaatacccggggtgccgtggccacgtcgcgcggcccaccgtcgg 2933371
Query: 181     cggcctgcacctcgccctgcagcgcgcaggcgagccccaccgcacagagcacgggggtca
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2933372 cggcctgcacctcgccctgcagcgcgcaggcgagccccaccgcacagagcacgggggtca 2933431
Query: 241     acgaacgagaggaaagacgtacatcgggcatggtcgaatcgtcctggatggaattcgccc
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2933432 acgaacgagaggaaagacgtacatcgggcatggtcgaatcgtcctggatggaattcgccc 2933491
Query: 301     atgagtgcaatccgacgccggctttttcagcgtcgggcaatatgaatttcgcataaccttt
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2933492 atgagtgcaatccgacgccggctttttcagcgtcgggcaatatgaatttcgcataaccttt 2933551
Query: 361     atgcgcccgggttcctccgcaggcggcgcactagcgtgcgtcggcgagccgcggcgcggg
                |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct: 2933552 atgcgcccgggttcctccacaggcggcgcactagcgtgcgtcggcgagccgcggcgcggg 2933611
Query: 421     cgggttgatcaacagcgcgaatatctcggagtgggacttcacgttcagcttcgcgtagat
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2933612 cgggttgatcaacagcgcgaatatctcggagtgggacttcacgttcagcttcgcgtagat 2933671
Query: 481     gtgccggcgatgcaccttgatagtggcgatcgacagcgtcagccgatccgctatttcctt
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2933672 gtgccggcgatgcaccttgatagtggcgatcgacagcgtcagccgatccgctatttcctt 2933731
Query: 541     gttcgagtatccatccagcatcagccggatgacatccgactcccgcgcggtcagcggcga
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2933732 gttcgagtatccatccagcatcagccggatgacatccgactcccgcgcggtcagcggcga 2933791
Query: 601     tatcaaccc 609
                |||||||||
Sbjct: 2933792 tatcaaccc 2933800

Score = 1875 bits (946), Expect = 0.0 Identities = 974/978 (99%), Gaps = 4/978 (0%) Strand =
Plus / Minus Query: 1 aagatggacgctggtttgcgcagcccatgcactgtccgccgctgtcgcaccgat-gtcgt 59
|||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||| Sbjct: 2934636
aagatggacgctggtttgcgcagcccatgcactgtccgccgctgtcgcaccgatcgtcgt 2934577 Query:
caggatcgataacgccagtgcacaaacttttttcattggatttcctttccgaa-cgaaac 118
|||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||| Sbjct: 2934576
caggatcgataacgccagtgcacaaacttttttcattggatttcctttccgaaacgaaac 2934517 Query:
aagttggattttgcacctaccagaactggtagtttctgacctgtggct-tcttcgaaggca 177
|||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||| Sbjct: 2934516
aagttggattttgcacctaccagaactggtagttctgacctgtggctatcttcgaaggca 2934457 Query:
tcgatattatgcacattggaactcttcatgacataacgccgagtgactaatcccagagga 237
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct: 2934456
tcgatattatgcacattggaactcttcatgacataacgccgagtgactaatcccagagga 2934397 Query:
gtagttgtgtggatatcgcattgcacggcggcgcctggcacgagtcactgggaaagttgc 297
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct: 2934396
gtagttgtgtggatatcgcattgcacggcggcgcctggcacgagtcactgggaaagttgc 2934337 Query:
tcgaagcgctggaccggccgttcttctggcggatcctggcgcagaccctcggccagttcg 357
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct: 2934336
tcgaagcgctggaccggccgttcttctggcggatcctggcgcagaccctcggccagttcg 2934277 Query:
cccccgtcgacaactgggcggccctgatattcagcgattccagtcccttgattctttcct 417
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct: 2934276
cccccgtcgacaactgggcggccctgatattcagcgattccagtcccttgattctttcct 2934217 Query:
tcatggaggaggagagagaagaagtcgagccggacccgctgatcagtcgatatataaccg 477
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct: 2934216
tcatggaggaggagagagaagaagtcgagccggacccgctgatcagtcgatatataaccg 2934157 Query:
gactttatctgcaagatcctttctaccaggtatccaggaactgtcggcgcggcgggcttt 537
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct: 2934156
gactttatctgcaagatcctttctaccaggtatccaggaactgtcggcgcggcgggcttt 2934097 Query:
ttcatcttgcggatatcgtctccgaagatttcgaaacgaccgaatattacaatacgtact 597
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct: 2934096
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reverse primer:
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Sequence reversed:
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Forward primer:
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Overlapping sequence:
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Sequenced stretch of DNA :
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Gene sequence from *pseudomonas.com*:

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BLAST result for the sequenced stretch of DNA:

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Sequenced DNA from nt 2933192-2934636, gene range from 2933582-2934388
Score = 2833 bits (1429), Expect = 0.0
                  Identities = 1442/1445 (99%), Gaps = 1/1445 (0%)
                  Strand = Plus / Minus
Query: 1 Sbjct: 2934636
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Amino acid sequence:
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+DIALHGGAWHESLGKLLEALDRPFFWRILAQTLGQFAPVDNWAALIFSDSSPLILSFME
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PA 1441 CHA/PAO comparison
7 nt exchanges, one AS exchange at position 448

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Nt-Blast results:
Sequenced stretch of DNA:
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MAVAPGVLLPPTPDVKPKAAAPKSQQKTPEPSNDKTSSFSDMYAKETAKKPAERADGPAK 60 Query: 1184

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GSRDKPRDAGKDAAEAQPTDAVRQPAVAEDGKPLPADGQAKADGEDKVETPVDPLQLLGL Sbjct: 61
GSRDKPRDAGKDAAEAQPTDAVRQPAVAEDGKPLPADGQAKADGEDKVETPVDPLQLLGL 120 Query: 1004

GGAVPLLDENTQATLLPPAVPTASSAPASLTEASSDPTLVKLNGVPAVNMALEQGAQDAA 825
GGAVPLLDENTQATLLPPAVPTASSAPASLTEASSDPTLVKLNGVPAVNMALEQGAQDAA Sbjct: 121
GGAVPLLDENTQATLLPPAVPTASSAPASLTEASSDPTLVKLNGVPAVNMALEQGAQDAA 180 Query: 824

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QTAKGGPAKSADPRQANLGDALAGLTSDSLTKAVDGKALEAQLQQTAEPAVASAASESLL Sbjct: 181
QTAKGGPAKSADPRQANLGDALAGLTSDSLTKAVDGKALEAQLQQTAEPAVASAASESLL 240 Query: 644

ESKAEPRGEPFAAKLNGLTQAMAQQALTNRPVNGTVPGQPVAIQQNGWSEAVVDRVMWMS 465
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MNQLDVNVSDQSLARGWQGQQQGEGGSARGRGLAGEASGDEETLAGVSEIRSRPGASAAR 420 Query: 104

GLVDYYA 84
GLVDYYA Sbjct: 421
GLVDYYA 427
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PA 1572 CHA/PAO comparison
Sequence data from 1712819-1714260
Gene range:1712908-1714053, 3 AS exchanges, 11 nt exchanges

```
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C
Score = 2880 bits (1453), Expect = 0.0
 Identities = 1492/1505 (99%)
 Strand = Plus / Plus
Query: 1
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Sbjct: 1713000 ggccctggccagaggcgccgaaccacgtgcgctggagcgcacccggatagccgtggagtg
Query: 301     cctgttgccgctggtcgagaagatcgagttcctgacctttcccggggccatgggtggcga
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Query: 1501    gggtc 1505
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Sbjct: 1714260 gggtc 1714264


Score =  746 bits (1925), Expect = 0.0
 Identities = 378/381 (99%), Positives = 380/381 (99%)
 Frame = +2

Query: 149  MDMFRLGLVVNPLAGLGGPVALKGSDGVAAEALARGAEPRALERTRVALECLLPLVEKIE
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Sbjct: 1    MDMFRLGLVVNPLAGLGGPVALKGSDGVAAEALARGAEPRALERTRIAVECLLPLVEKIE 60

Query: 329  FLTFPGAMGGELLERMGFRHRLLGEWQGAVSSAADTRLAIERLQEAGVALILFAGGDGTA
            FLTFPGAMGGELLERMGFRHRLLGEWQGAVSSAADTRLAIERLQEAGVALILFAGGDGTA
Sbjct: 61   FLTFPGAMGGELLERMGFRHRLLGEWQGAVSSAADTRLAIERLQEAGVALILFAGGDGTA 120

Query: 509  RDVAGVVREQQPVLGIPAGVKIHSGVYAISPRAAGELARRLVEGGLVRLASGEVRDLDEE
            RDVAGVVREQQPVLGIPAGVKIHSGVYAISPRAAGELARRLVEGGLVRLASGEVRDLDEE
Sbjct: 121  RDVAGVVREQQPVLGIPAGVKIHSGVYAISPRAAGELARRLVEGGLVRLASGEVRDLDEE
Query: 689  ALRAGRVAARWYGEMTVPEEGHFMQHVKQAGMESEELVLVDLADWLAENWEDGVRYVLGP
            ALRAGRVAARWYGEMTVPEEGHFMQHVKQAGMESEELVLVDLADWLAENWEDGVRYVLGP
Sbjct: 181  ALRAGRVAARWYGEMTVPEEGHFMQHVKQAGMESEELVLVDLADWLAENWEDGVRYVLGP
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Query: 869  GSTLHGLARNLGLETTLLGVDVLENGAVIARDVDEARLFELVDGHPAYLLVTAIGGQGHV
            GSTLHGLARNLGLETTLLGVDVLENGAVIARDVDEARLFELVDGHPAYLLVTAIGGQGHV
Sbjct: 241  GSTLHGLARNLGLETTLLGVDVLENGAVIARDVDEARLFELVDGHPAYLLVTAIGGQGHV

Query: 1049 IGRGNQQISPRVLRAIGLERMRVVATKRKLGTLGGRPLLVDSGDPVLDDSFPDAIRVWAG
            IGRGNQQISPRVLRAIGLERMRVVATKRKLGTLGGRPLLVDSGDPVLDDSFPD+IRVWAG
Sbjct: 301  IGRGNQQISPRVLRAIGLERMRVVATKRKLGTLGGRPLLVDSGDPVLDDSFPDTIRVWAG

Query: 1229 YKEELLYPLGYAKDQDAPAGA 1291
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Sbjct: 361  YKEELLYPLGYAKDQDAPAGA 381
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PA 5349 CHA/PAO comparison:

PA 5349 and its surrounding is absolutely identical in PAO and CHA.

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probable rubredoxin reductase  range from 6018778-6017624
Sequence data from : 6018816-6017559
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Sequencing results fort he quorum sensing mutants

Mutant 2A12 = 28B11, PA 5291 (isogenic mutant available!)

PA 5291 betT2 PS01303: BCCT family of transporters signature. 12 predicted transmembrane domains.
Protein Name: probable choline transporter (Class 3) betT2
Function / Class: Membrane proteins;Transport of small molecules
Subcellular Localization:
Inner membrane protein (Class 2)
Range From 5955180 to 5957165
Homology: 64% similar to betT gene product of *Escherichia coli*

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Left primer
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aligned to transposon: 1721-1755 (Tnp end)
Query: 1 Sbjct: 1721

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tagactagtgcggccgcacttgtgtataagagtca 1755
Score =  591 bits (298), Expect = e-169
                 Identities = 304/306 (99%)
                 Strand = Plus / Minus

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Query: 304 cttgttgacgatgtcggacaggtaggccccggtgttctg 342
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Mutant 3A1

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Mutant 3A2

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Mutant 4A1, PA 0104

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Mutant 7A1 = PA 2127

conserved hypothetical protein (Class 4), 53% similar to hypothetical ybdN gene product of [Escherichia coli],
Range from 2341640 Range To 2340414

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ACCCCTCGCTGNTTCGGTAGCGGGCGCACNCAGTCGGNCTCGGCGCCGGGCTCCCANCAGCACCAGNTGGGTTCCTCCAGGCTGGAGGCGTTGC
GCAGGTTGAGTGGCANGCAAACCCACCAGGGGCGTACGTCGGGACGCCCGAGCATCTCGCTGACGTGATCGATGGTGGCCTGGNACTG

Alignment with transposon:
Query: 11 gggactagtggggtaccgtcgacatgcatggtngcgccggcgatngcggccggcctacgc 70
          |||      | ||||||||||||||||||||  |||||||||||| |||||||||||| |
Sbjct: 81 gggwswrkkgrggtaccgtcgacatgcatgg-cgcgccggcgatcgcggccggcctaggt 23
```

```
Query: 71 ggccagatctgatcaaganaca 92
          |||||||||||||||||| |||
Sbjct: 22 ggccagatctgatcaagagaca 1

Score =  488 bits (246), Expect = e-138
                 Identities = 269/279 (96%)
                 Strand = Plus / Plus
Query:92 Sbjct:2341161
aggctcttccctggccagccaggcgttgaagccggcgacgaactcctcgaactccatgcg 151
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
aggctcttccctggccagccaggcgttgaagccggcgacgaactcctcgaactccatgcg 2341220
Query: 152 Sbjct: 2341221
gtagcggtagaaggggaagaacgccgggtcgctgatcacccctcgctgnttcggtagcgg 211
|||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
gtagcggtagaaggggaagaacgccgggtcgctgatcacccctcgctgcttcggtagcgg 2341280
Query: 212 Sbjct: 2341281
gcgcacncagtcggnctcggcgccgggctcccancagcaccagntgggttcctccaggct 271
|||||| ||||||| |||||||||||||||||| ||||||||  ||||||||||||||
gcgcacccagtcggcctcggcgccgggctcccagcagcaccagtagggttcctccaggct 2341340
Query: 272 Sbjct: 2341341
ggaggcgttgcgcaggttgagtggcangcaaacccaccaggggcgtacgtcgggacgccc 331
|||||||||||||||||||||||||  || |||||||||||||||||||||||||||||
ggaggcgttgcgcaggttgagtggcagacagacccaccaggggcgtacgtcgggacgccc 2341400
Query: 332 Sbjct: 2341401
gagcatctcgctgacgtgatcgatggtggcctggnactg 370
|||||||||||||||||||||||||||||||| ||||
gagcatctcgctgacgtgatcgatggtggcctggtactg 2341439
```

Mutant 8A1 PA 0797?!? Identical with mutant 3A1???

```
GGGAAGCTTGGGACAAGTGGGGGACTAGTGGGGTACCGTTGACATGCATGGCGCGCCGGCGATCGCGGCGCGGCCTAGGCGGCGCAGATCTGAT
CAAGAGACAGCGACCACGCCNAACCGGCCGNNCCAGGCCTTTGNCGNNNACCANCGCATTCTCGACGCCATNGCCNANCGTGACGGCNAACTGG
NCGAGCTGTTGATGCNCCGNCNCATTANCGNGTTGNGCCGNAATATTTANCGCCANCTGGAACCCNANCCGGTGAAGCTNGCNTCCGCTTCNTC
CACCGTCTAAGAGGTCCGTGATGAGCCANACGTTCCTAACCNCCGGCCAGNGCTTCCNNGAAANCGAGGNCACCGANCAC

Alignment with transposon:

Query: 21  gggactagtggggtaccgttgacatgcatggcgcgccggcgatcgcggcgcggcctaggc 80
           |||      | |||||||| |||||||||||||||||||||||||| |||||||||
Sbjct: 81  gggwswrkkgrggtaccgtcgacatgcatggcgcgccggcgatcgcggc-cggcctaggt 23

Query: 81  ggcgcagatctgatcaagagaca 103
           ||| ||||||||||||||||||
Sbjct: 22  ggc-cagatctgatcaagagaca 1

Query: 2 Sbjct: 875300
cgaccacgccnaaccggccgnnccaggcctttgncgnnnaccancgcattctcgacgcca 61
|||||||||| |||||||||  ||||||||| | ||   |||| ||||||||||||||||
cgaccacgccgaaccggccgcgccaggccttcgccgaacaccaccgcattctcgacgcca 875241
Query: 62 Sbjct: 875240
tngccnancgtgacggcnaactggncgagctgttgatgcnccgncncattancgngttgn 121
| ||| | ||||||||| |||||| ||||||| |||||| ||| | ||| |   | || |
tcgccgaccgtgacggcgaactggccgagctgctgatgcgccgccacatcagtgcgtcgc 875181
Query: 122 Sbjct: 875180
gccgnaatatttancgccanctggaacccnanccggtgaagctngcntccgcttcntcca 181
|||| |||||  | ||||| ||||||||| | |||| |||||| || |||||||| ||||
gccgcaatatcgagcgccagctggaaccccagccggcgaagctcgcctccgcttcctcca 875121
Query: 182 Sbjct: 875120
ccgtctaagaggtccgtgatgagccanacgttcctaaccnccggccagngcttcc 236
|||||||||||||||||||||||||| |||| ||| ||| |||||||| ||||||
ccgtctaagaggtccgtgatgagccagacgtcccttacccccggccagcgcttcc 8750
```

Mutant 8A12 = PA 4277 and 4265 (identical proteins)

PA 4277 and 4265, protein elongation factor tufB (PA 4277) and A (PA 4265), the DNA sequences are identical except for 8 nt of 1194 bp, the protein sequences are absolutely identical!

```
No results with left primer
Right primer: 260 bp
>II116_8A12_rechts_wh.fasta
CTGCTGGAGCTGGTCGAGATGGAAGTTCGCGATCTGCTGAAC
```
ACCTACGACTTCCCGGGC
GACGACACTCCGATCA
```
TCATCGGTTCCGCGCTGATGGCGCTGGAAGGCAAGGATGACAAC
GGCATCGGCGTAAGCGCCGTGCAGAAGCTGGTAGAGACCCTGGACTCCTACATTCCGGAG
CCGGTTCGTGCCATCGACCAGCCGTTCCTGATGCCGATCGAAGACGTGTTCTCGATCTCC
GGTCGCGGTACCGTGGTAAC

>XI210_8A12_rechts_wh.fasta
ACCTACCACTTCCCGGGCGACGACACTCCGATCATCA
```

```
Same result for PA 4277 and 4265
Query: 1 Sbjct: 4768569
ctgctggagctggtcgagatggaagttcgcgatctgctgaacacctacgacttcccgggc 60
|||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
ctgctggaactggtcgagatggaagttcgcgatctgctgaacacctacgacttcccgggc 4768510
Query: 61 Sbjct: 4768509       gacgacactccgatcatcatcggttccgcgctgatggcgctggaaggcaaggatgacaac
120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gacgacactccgatcatcatcggttccgcgctgatggcgctggaaggcaaggatgacaac 4768450

Query: 121 Sbjct: 4768449
ggcatcggcgtaagcgccgtgcagaagctggtagagaccctggactcctacattccggag 180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ggcatcggcgtaagcgccgtgcagaagctggtagagaccctggactcctacattccggag 4768390
Query: 181 Sbjct: 4768389
ccggttcgtgccatcgaccagccgttcctgatgccgatcgaagacgtgttctcgatctcc 240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ccggttcgtgccatcgaccagccgttcctgatgccgatcgaagacgtgttctcgatctcc 4768330
Query: 241 Sbjct: 4768329
ggtcgcggtaccgtggtaac 260
|| ||||||||||||||||
ggccgcggtaccgtggtaac 4768310

PA 4277, protein elongation factor tufB
Score =  170 bits (431), Expect = 4e-044
Identities = 86/86 (100%), Positives = 86/86 (100%)
Frame = +1

Query: 1 Sbjct: 146
LLELVEMEVRDLLNTYDFPGDDTPIIIGSALMALEGKDDNGIGVSAVQKLVETLDSYIPE 180
LLELVEMEVRDLLNTYDFPGDDTPIIIGSALMALEGKDDNGIGVSAVQKLVETLDSYIPE
LLELVEMEVRDLLNTYDFPGDDTPIIIGSALMALEGKDDNGIGVSAVQKLVETLDSYIPE 205

                  Query: 181 PVRAIDQPFLMPIEDVFSISGRGTVV 258
                              PVRAIDQPFLMPIEDVFSISGRGTVV
                  Sbjct: 206 PVRAIDQPFLMPIEDVFSISGRGTVV 231
>PA4265 >PA4265 [gene=tufA] [prot=elongation factor Tu] [comment=PA4265]
                             Length = 397
Score =  170 bits (431), Expect = 4e-044
Identities = 86/86 (100%), Positives = 86/86 (100%)
Frame = +1

Query: 1 Sbjct: 146  LLELVEMEVRDLLNTYDFPGDDTPIIIGSALMALEGKDDNGIGVSAVQKLVETLDSYIPE 180
LLELVEMEVRDLLNTYDFPGDDTPIIIGSALMALEGKDDNGIGVSAVQKLVETLDSYIPE
LLELVEMEVRDLLNTYDFPGDDTPIIIGSALMALEGKDDNGIGVSAVQKLVETLDSYIPE 205
Query: 181 Sbjct: 206
PVRAIDQPFLMPIEDVFSISGRGTVV 258
PVRAIDQPFLMPIEDVFSISGRGTVV
PVRAIDQPFLMPIEDVFSISGRGTVV 231

BLAST results for PA 4277 vs 4265: teh genes are virtually identical!
Query: 2    tggctaaagaaaaatttgaacggaacaagccgcacgtcaacgtcggcaccatcggtcacg 61
             ||||||| |||||||| ||||| ||||| ||||||||||||||||||||||||||||||
Sbjct: 2    tggctaaggaaaaattcgaacgtaacaaaccgcacgtcaacgtcggcaccatcggtcacg 61
Query: 62 Sbjct: 62
ttgaccatggcaagaccactctgaccgctgcactgaccaaggtctgctccgacacctggg 121
|||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
ttgaccatggcaagaccactctgaccgctgcactgaccaaggtctgctccgatacctggg 121
Query: 122 Sbjct: 122
gtggttccgctcgtgctttcgatcagatcgacaacgcgccggaagaaaaggcccgcggta 181
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gtggttccgctcgtgctttcgatcagatcgacaacgcgccggaagaaaaggcccgcggta 181
Query: 182 Sbjct: 182
tcaccatcaacacctcgcacgttgaatacgattccgctgttcgtcactacgcccacgttg 241
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tcaccatcaacacctcgcacgttgaatacgattccgctgttcgtcactacgcccacgttg 241
Query: 242 Sbjct: 242
actgccccggtcacgccgactacgtgaagaacatgatcaccggtgctgcccagatggacg 301
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
actgccccggtcacgccgactacgtgaagaacatgatcaccggtgctgcccagatggacg 301
Query: 302 Sbjct: 302
gcgcgatcctggtttgctcggctgccgacggccccatgccgcagacccgcgagcacatcc 361
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gcgcgatcctggtttgctcggctgccgacggccccatgccgcagacccgcgagcacatcc 361
Query: 362 Sbjct: 362
tgctgtcccgccaggtaggcgttccctacatcgtcgtgttcctgaacaaggccgacatgg 421
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tgctgtcccgccaggtaggcgttccctacatcgtcgtgttcctgaacaaggccgacatgg 421
```

```
Query: 422 Sbjct: 422
tcgacgacgccgagctgctggaactggtcgagatggaagttcgcgatctgctgaacacct 481
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tcgacgacgccgagctgctggaactggtcgagatggaagttcgcgatctgctgaacacct 481
Query: 482 Sbjct: 482
acgacttcccgggcgacgacactccgatcatcatcggttccgcgctgatggcgctggaag 541
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
acgacttcccgggcgacgacactccgatcatcatcggttccgcgctgatggcgctggaag 541
Query: 542 Sbjct: 542
gcaaggatgacaacggcatcggcgtaagcgccgtgcagaagctggtagagaccctggact 601
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gcaaggatgacaacggcatcggcgtaagcgccgtgcagaagctggtagagaccctggact 601
Query: 602 Sbjct: 602
cctacattccggagccggttcgtgccatcgaccagccgttcctgatgccgatcgaagacg 661
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cctacattccggagccggttcgtgccatcgaccagccgttcctgatgccgatcgaagacg 661
Query: 662 Sbjct: 662
tgttctcgatctccggccgcggtaccgtggtaaccggtcgtgtagagcgcggcatcatca 721
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tgttctcgatctccggccgcggtaccgtggtaaccggtcgtgtagagcgcggcatcatca 721
Query: 722 Sbjct: 722
aggtccaggaagaagtggaaatcgtcggcatcaaggcgaccaccaagactacctgcaccg 781
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
aggtccaggaagaagtggaaatcgtcggcatcaaggcgaccaccaagactacctgcaccg 781
Query: 782 Sbjct: 782
gcgttgaaatgttccgcaagctgctcgacgaaggtcgtgctggtgagaacgttggtatcc 841
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gcgttgaaatgttccgcaagctgctcgacgaaggtcgtgctggtgagaacgttggtatcc 841
Query: 842 Sbjct: 842
tgctgcgtggcaccaagcgtgaagacgtagagcgtggccaggttctggccaagccgggca 901
|||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
tgctgcgtggcaccaagcgtgaagacgtagagcgtggccaggtactggccaagccgggca 901
Query: 902 Sbjct: 902
ccatcaagccgcacaccaagttcgagtgcgaagtgtacgtgctgtccaaggaagaaggtg 961
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ccatcaagccgcacaccaagttcgagtgcgaagtgtacgtgctgtccaaggaagaaggtg 961
Query: 962 Sbjct: 962
gtcgtcacaccccgttcttcaagggctaccgtccgcagttctacttccgtaccaccgacg 1021
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
gtcgtcacaccccgttcttcaagggctaccgtccgcagttctacttccgtaccaccgatg 1021
Query: 1022 Sbjct: 1022
tgaccggtaactgcgaactgccggaaggcgtagagatggtaatgccgggcgacaacatca 1081
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tgaccggtaactgcgaactgccggaaggcgtagagatggtaatgccgggcgacaacatca 1081
Query: 1082 Sbjct: 1082
agatggttgtcaccctgatcgctccgatcgccatggaagatggcctgcgcttcgcgatcc 1141
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
agatggttgtcaccctgatcgctccgatcgccatggaagatggcctgcgcttcgcgatcc 1141
Query: 1142 gcgaaggcggccgtaccgttggcgccggcgtggttgccaagatcatcgaataa 1194
            |||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct: 1142 gcgaaggcggccgtaccgttggcgccggcgtggttgccaagatcatcgagtaa 1194
```

PA 4277, 1194 bp

```
ATGGCTAAAGAAAAATTTGAACGGAACAAGCCGCACGTCAACGTCGGCACCATCGGTCACGTTGACCATGGCAAGACCACTCTGACCGCTGCAC
TGACCAAGGTCTGCTCCGACACCTGGGGTGGTTCCGCTCGTGCTTTCGATCAGATCGACAACGCGCCGGAAGAAAAGGCCCGCGGTATCACCAT
CAACACCTCGCACGTTGAATACGATTCCGCTGTTCGTCACTACGCCCACGTTGACTGCCCCGGTCACGCCGACTACGTGAAGAACATGATCACC
GGTGCTGCCCAGATGGACGGCGCGATCCTGGTTTGCTCGGCTGCCGACGGCCCCATGCCGCAGACCCGCGAGCACATCCTGCTGTCCCGCCAGG
TAGGCGTTCCCTACATCGTCGTGTTCCTGAACAAGGCCGACATGGTCGACGACGCCGAGCTGCTGGAACTGGTCGAGATGGAAGTTCGCGATCT
GCTGAACACCTACGACTTCCCGGGCGACGACACTCCGATCATCATCGGTTCCGCGCTGATGGCGCTGGAAGGCAAGGATGACAACGGCATCGGC
GTAAGCGCCGTGCAGAAGCTGGTAGAGACCCTGGACTCCTACATTCCGGAGCCGGTTCGTGCCATCGACCAGCCGTTCCTGATGCCGATCGAAG
ACGTGTTCTCGATCTCCGGCCGCGGTACCGTGGTAACCGGTCGTGTAGAGCGCGGCATCATCAAGGTCCAGGAAGAAGTGGAAATCGTCGGCAT
CAAGGCGACCACCAAGACTACCTGCACCGGCGTTGAAATGTTCCGCAAGCTGCTCGACGAAGGTCGTGCTGGTGAGAACGTTGGTATCCTGCTG
CGTGGCACCAAGCGTGAAGACGTAGAGCGTGGCCAGGTTCTGGCCAAGCCGGGCACCATCAAGCCGCACACCAAGTTCGAGTGCGAAGTGTACG
TGCTGTCCAAGGAAGAAGGTGGTCGTCACACCCCGTTCTTCAAGGGCTACCGTCCGCAGTTCTACTTCCGTACCACCGACGTGACCGGTAACTG
CGAACTGCCGGAAGGCGTAGAGATGGTAATGCCGGGCGACAACATCAAGATGGTTGTCACCCTGATCGCTCCGATCGCCATGGAAGATGGCCTG
CGCTTCGCGATCCGCGAAGGCGGCCGTACCGTTGGCGCCGGCGTGGTTGCCAAGATCATCGAATAA
```

PA 4265, 1194 bp

```
GTGGCTAAGGAAAAATTCGAACGTAACAAACCGCACGTCAACGTCGGCACCATCGGTCACGTTGACCATGGCAAGACCACTCTGACCGCTGCAC
TGACCAAGGTCTGCTCCGATACCTGGGGTGGTTCCGCTCGTGCTTTCGATCAGATCGACAACGCGCCGGAAGAAAAGGCCCGCGGTATCACCAT
CAACACCTCGCACGTTGAATACGATTCCGCTGTTCGTCACTACGCCCACGTTGACTGCCCCGGTCACGCCGACTACGTGAAGAACATGATCACC
GGTGCTGCCCAGATGGACGGCGCGATCCTGGTTTGCTCGGCTGCCGACGGCCCCATGCCGCAGACCCGCGAGCACATCCTGCTGTCCCGCCAGG
TAGGCGTTCCCTACATCGTCGTGTTCCTGAACAAGGCCGACATGGTCGACGACGCCGAGCTGCTGGAACTGGTCGAGATGGAAGTTCGCGATCT
GCTGAACACCTACGACTTCCCGGGCGACGACACTCCGATCATCATCGGTTCCGCGCTGATGGCGCTGGAAGGCAAGGATGACAACGGCATCGGC
GTAAGCGCCGTGCAGAAGCTGGTAGAGACCCTGGACTCCTACATTCCGGAGCCGGTTCGTGCCATCGACCAGCCGTTCCTGATGCCGATCGAAG
ACGTGTTCTCGATCTCCGGCCGCGGTACCGTGGTAACCGGTCGTGTAGAGCGCGGCATCATCAAGGTCCAGGAAGAAGTGGAAATCGTCGGCAT
CAAGGCGACCACCAAGACTACCTGCACCGGCGTTGAAATGTTCCGCAAGCTGCTCGACGAAGGTCGTGCTGGTGAGAACGTTGGTATCCTGCTG
CGTGGCACCAAGCGTGAAGACGTAGAGCGTGGCCAGGTACTGGCCAAGCCGGGCACCATCAAGCCGCACACCAAGTTCGAGTGCGAAGTGTACG
```

```
TGCTGTCCAAGGAAGAAGGTGGTCGTCACACCCCGTTCTTCAAGGGCTACCGTCCGCAGTTCTACTTCCGTACCACCGATGTGACCGGTAACTG
CGAACTGCCGGAAGGCGTAGAGATGGTAATGCCGGGCGACAACATCAAGATGGTTGTCACCCTGATCGCTCCGATCGCCATGGAAGATGGCCTG
CGCTTCGCGATCCGCGAAGGCGGCCGTACCGTTGGCGCCGGCGTGGTTGCCAAGATCATCGAGTAA

PA 4265 peptide sequence
MAKEKFERNKPHVNVGTIGHVDHGKTTLTAALTKVCSDTWGGSARAFDQIDNAPEEKARGITINTSHVEYDSAVRHYAHVDCPGHADYVKNMIT
GAAQMDGAILVCSAADGPMPQTREHILLSRQVGVPYIVVFLNKADMVDDAELLELVEMEVRDLLNTYDFPGDDTPIIIGSALMALEGKDDNGIG
VSAVQKLVETLDSYIPEPVRAIDQPFLMPIEDVFSISGRGTVVTGRVERGIIKVQEEVEIVGIKATTKTTCTGVEMFRKLLDEGRAGENVGILL
RGTKREDVERGQVLAKPGTIKPHTKFECEVYVLSKEEGGRHTPFFKGYRPQFYFRTTDVTGNCELPEGVEMVMPGDNIKMVVTLIAPIAMEDGL
RFAIREGGRTVGAGVVAKIIE

Result P/P blast:
PA4277  [gene=tufB] [prot=elongation factor Tu] [comment=PA4277]   796  0.0

PA4265  [gene=tufA] [prot=elongation factor Tu] [comment=PA4265]   796  0.0
Score =  796 bits (2055), Expect = 0.0
                  Identities = 397/397 (100%), Positives = 397/397 (100%)

                  Query: 1 Sbjct: 1
MAKEKFERNKPHVNVGTIGHVDHGKTTLTAALTKVCSDTWGGSARAFDQIDNAPEEKARG 60
MAKEKFERNKPHVNVGTIGHVDHGKTTLTAALTKVCSDTWGGSARAFDQIDNAPEEKARG
MAKEKFERNKPHVNVGTIGHVDHGKTTLTAALTKVCSDTWGGSARAFDQIDNAPEEKARG 60

                  Query: 61 Sbjct: 61
ITINTSHVEYDSAVRHYAHVDCPGHADYVKNMITGAAQMDGAILVCSAADGPMPQTREHI 120
ITINTSHVEYDSAVRHYAHVDCPGHADYVKNMITGAAQMDGAILVCSAADGPMPQTREHI
ITINTSHVEYDSAVRHYAHVDCPGHADYVKNMITGAAQMDGAILVCSAADGPMPQTREHI 120

                  Query: 121 Sbjct: 121
LLSRQVGVPYIVVFLNKADMVDDAELLELVEMEVRDLLNTYDFPGDDTPIIIGSALMALE 180
LLSRQVGVPYIVVFLNKADMVDDAELLELVEMEVRDLLNTYDFPGDDTPIIIGSALMALE
LLSRQVGVPYIVVFLNKADMVDDAELLELVEMEVRDLLNTYDFPGDDTPIIIGSALMALE 180

                  Query: 181 Sbjct: 181
GKDDNGIGVSAVQKLVETLDSYIPEPVRAIDQPFLMPIEDVFSISGRGTVVTGRVERGII 240
GKDDNGIGVSAVQKLVETLDSYIPEPVRAIDQPFLMPIEDVFSISGRGTVVTGRVERGII
GKDDNGIGVSAVQKLVETLDSYIPEPVRAIDQPFLMPIEDVFSISGRGTVVTGRVERGII 240

Query: 241 Sbjct: 241
KVQEEVEIVGIKATTKTTCTGVEMFRKLLDEGRAGENVGILLRGTKREDVERGQVLAKPG 300
KVQEEVEIVGIKATTKTTCTGVEMFRKLLDEGRAGENVGILLRGTKREDVERGQVLAKPG
KVQEEVEIVGIKATTKTTCTGVEMFRKLLDEGRAGENVGILLRGTKREDVERGQVLAKPG 300

                  Query: 301 Sbjct: 301
TIKPHTKFECEVYVLSKEEGGRHTPFFKGYRPQFYFRTTDVTGNCELPEGVEMVMPGDNI 360
TIKPHTKFECEVYVLSKEEGGRHTPFFKGYRPQFYFRTTDVTGNCELPEGVEMVMPGDNI
TIKPHTKFECEVYVLSKEEGGRHTPFFKGYRPQFYFRTTDVTGNCELPEGVEMVMPGDNI 360

                  Query: 361 KMVVTLIAPIAMEDGLRFAIREGGRTVGAGVVAKIIE 397
                       KMVVTLIAPIAMEDGLRFAIREGGRTVGAGVVAKIIE
                  Sbjct: 361 KMVVTLIAPIAMEDGLRFAIREGGRTVGAGVVAKIIE 397
```

QSMutant 8B10 0 PA 3268

Protein Name : probable TonB-dependent receptor (Class 3)
Function, Class Membrane proteins;Transport of small molecules
Subcellular Localization:
Outer membrane protein (Class 2) Range from 3658151 Range to 3655986
Homology 61% similar to E. coli fecA gene product.

```
>X132_8B10Tn2_a1.fasta 736 bp
CTCAACCAGGCGTTCCTCGGCGGGACCGCCGACAACGGCCTGGGCGTGGCGCTGCTGTAT
TCCGGGGTGAAGGGCGCCGACTACCGCGACGGCAACAACGACAATGATATCGACGACGTG
CTGCTCAAGACCCATTGGCAGCTCACCGACAGCGACCAGTTGGCGGCCAACTTCCACTAC
TACGACGCCTACGCCGATATGCCGGGCGGCCTGACCCAGGCGCAGTACGACGACGATCCC
TTCCAGTCGGTACGCGACTGGGACAATTTCCGTGGTCGGCGCAAGGACTTCTCGCTGAAG
TACACCCGCCAGGTCGACGACCTCACCCAGTTCGAGGTGCTCACCTACTACAGCGACAGT
TTCCGCGGCAGCAGCATCGCCGCGCGCAACCTCAGGACCATCACCTCGTACCCGCGCGAC
```

```
TACCATGTGTTCGCGGTGGAGCCGCGGGTCTCGCGGATCTTCTTCGCCGGCCCGACCACC
CAGGAGGTCGGCATCGGCTACCGCTACCTGAAGGAAGCGATGAACGAGCGCGCCAGCCAG
CTGGCCCTGGTCGACAATGTGCCGACCGTGCGTCCCGGCTCGGACGGCCATACCTACCAG
GACCTGACTCTTATACACAAGTGCGGCCGCACTAGTCTAGATTTAAATTAATTAAGAGCT
CGAATTGACATAAGCCTGTTCGGTTCGTAAACTGTAATGCAAGTAGCGTATGCGCTCACG
CAACTGGTCCAGAACC

>X132_8B10Tn2_a2.fasta 720 bp
CGAACCGAACAGGCTTATGTCAATTCGAGCTCTTAATTAATTTAAATCTAGACTAGTGCG
GCCGCACTTGTGTATAAGAGTCAGGTCCTGGTAGGTATGGCCGTCCGAGCCGGGACGCAC
GGTCGGCACATTGTCGACCAGGGCCAGCTGGCTGGCGCGCTCGTTCATCGCTTCCTTCAG
GTAGCGGTAGCCGATGCCGACCTCCTGGGTGGTCGGGCCGGCGAAGAAGATCCGCGAGAC
CCGCGGCTCCACCGCGAACACATGGTAGTCGCGCGGGTACGAGGTGATGGTCCTGAGGTT
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GCCCGGCATATCGGCGTAGGCGTCGTAGTAGTGGAAGTTGGCCGCCAACTGGTCGCTGTC
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Product a1 transposon alignment:
Query: 605 Sbjct: 1755
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tgactcttatacacaagtgcggccgcactagtctagatttaaattaattaagagctcgaa 1696

Query: 665 Sbjct: 1695
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ttgacataagcctgttcggttcgtaaactgtaatgcaagtagcgtatgcgctcacgcaac 1636

Query: 725  tggtccagaacc 736
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Sbjct: 1635 tggtccagaacc 1624

Product a2 transposon alignment:
Query: 1    cgaaccgaacaggcttatgtcaattcgagctcttaattaatttaaatctagactagtgcg 60
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Sbjct: 1673 cgaaccgaacaggcttatgtcaattcgagctcttaattaatttaaatctagactagtgcg 1732
Query: 61   gccgcacttgtgtataagagtca 83
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Protein BLAST:
Query: 1 Sbjct: 195
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Query: 181 Sbjct: 255 YDAYADMPGGLTQAQYDDDPFQSVRDWDNFRGRRKDFSLKYTRQVDDLTQFEVLTYYSDS 360
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Query: 361 Sbjct: 315
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Query: 541 Sbjct: 375
LALVDNVPTVRPGSDGHTYQ 600
LALVDNVPTVRPGSDGHTYQ
LALVDNVPTVRPGSDGHTYQ 394

Nucleotide blast:
Score = 1189 bits (600), Expect = 0.0
                  Identities = 603/604 (99%)
                  Strand = Plus / Minus
Query: 1 Sbjct: 3657569      ctcaaccaggcgttcctcggcgggaccgccgacaacggcctgggcgtggcgctgctgtat 60
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Query: 601 Sbjct: 3656969
gacc 604
||||
gacc 3656966
QSMutant 10A12 No hits in PAOI, but hits in P.aeruginosa strain C plasmid!

>X132_10A12Tn2_a1.fasta 371 bp
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TTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGCACGAACCCAGTGGACATAAGCCT
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>X132_10A12Tn2_a2.fasta 377 bp
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CAAGGTTTCGGTCTCCACGCATCGTCAGGCATTGGCGGCCTTGCTGTTCTTCTACGGCAA
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Alignment with transposon, a1:
62 basepairs missing from transposon end!!!
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LOCUS       AY257539               23061 bp    DNA     linear   BCT 08-JAN-2004
DEFINITION  Pseudomonas aeruginosa strain C plasmid pKLC102 transposon TNCP23,
            complete sequence.
ACCESSION   AY257539
VERSION     AY257539.1  GI:37955767
KEYWORDS    .
SOURCE      Pseudomonas aeruginosa
  ORGANISM  Pseudomonas aeruginosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 23061)
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  AUTHORS   Klockgether,J., Reva,O., Larbig,K. and Tummler,B.
  TITLE     Sequence Analysis of the Mobile Genome Island pKLC102 of
            Pseudomonas aeruginosa C
  JOURNAL   J. Bacteriol. 186 (2), 518-534 (2004)
   PUBMED   14702321
REFERENCE   2  (bases 1 to 23061)
  AUTHORS   Klockgether,J., Reva,O.N., Larbig,K.D. and Tummler,B.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-MAR-2003) Klinische Forschergruppe OE6711,
            Medizinische Hochschule Hannover, Carl-Neuberg-Strasse 1, Hannover
            D-30625, Germany
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /strain="C"
                     /isolation_source="airways of a cystic fibrosis patient"
                     /db_xref="taxon:287"
                     /plasmid="pKLC102"
     repeat_region   complement(1..>857)
                     /insertion_seq="IS6100"
     gene            complement(63..857)


Sequences producing significant alignments:                      (bits) Value

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gi|38455225|gb|AY456904.1|  Binary vector pRE1, complete seq...   636   e-180
gi|9836704|gb|AF164956.1|  Corynebacterium glutamicum strain...   636   e-180
gi|31540471|gb|AY260546.2|  Escherichia coli plasmid pAK33 i...   636   e-180
gi|32350768|gb|AY224185.1|  Escherichia coli isolate Ec1484R...   636   e-180
gi|30349182|gb|AY259086.1|  Escherichia coli plasmid pHSH2 e...   636   e-180
gi|30349167|gb|AY259085.1|  Escherichia coli plasmid pHSH1 e...   636   e-180
gi|42563726|gb|AY444814.1|  Pseudomonas aeruginosa integron ...   636   e-180
gi|18092639|gb|AF458081.1|  Citrobacter freundii isolate CF-...   636   e-180
gi|46802186|emb|BX323601.6|  Zebrafish DNA sequence from clo...   636   e-180
gi|46559085|emb|AJ586617.2|  Pseudomonas aeruginosa partial ...   636   e-180
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gi|19919175|gb|AF347074.1|  Pseudomonas aeruginosa class I i...   636   e-180
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gi|4210822|emb|AJ223604.1|PAE223604  Pseudomonas aeruginosa ...   636   e-180
gi|17383994|emb|AJ310778.1|STY310778  Salmonella typhimurium...   636   e-180
gi|12719011|gb|AF261825.2|  Salmonella enterica subsp. enter...   636   e-180
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gi|7530137|emb|AJ277027.1|KPN277027  Klebsiella pneumoniae p...   636   e-180
gi|1435171|emb|X98393.1|PACATAAC6  Pseudomonas aeruginosa pl...   636   e-180
gi|48205|emb|X12870.1|TN21AADA  Transposon Tn21 (plasmid R6-...   636   e-180
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gi|7381447|gb|AF191564.1|AF191564  Pseudomonas aeruginosa in...   636   e-180
gi|13272354|gb|AF302086.1|AF302086  Pseudomonas aeruginosa p...   636   e-180
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gi|10185689|gb|AF188331.1|AF188331  Shigella flexneri transp...   636   e-180
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GCCTGGGCTTCGGCGGGCAGGCGGTAGCTGGGCGCCACGGCGTCCGCGGCGAGCACCTCG
TCCACCGCGATATCGCCGTCGGCGCGCTCGAACAGTGCGTGGCCGCGGCTGCAATAGAGC
TGCGCGTGCTCGTCATACAGCGGCAGGTACTCGAGCCCCGACAGCGGACTGATCAACGGC
ACCACGCCGACATGCAGGTGGCCGTCGAGCACGCCCAGTTCGATCTCGTTGGGGGTGGTC
ATGCCGATGTTGATCCGCACCTGCGGTCCCTGCCCCTTGAGCGCGCTGAGAGCGTGGGTG
ATGCGCATCTGCGGCAGGGTCACCAGGTTGTTGATGATGCCGATGTTGAGTTCGCCGCGC
AGGTGCTGGTGCAGGTCGTTGACCTCGGCGCGGAACCCCT

Mutant 23B4 0 PA 5291 (no transposon alignment!) identical with 2 A 12!!
Protein Name
probable choline transporter (Class 3) betT2
Function / Class: Membrane proteins;Transport of small molecules
Subcellular Localization:
Inner membrane protein (Class 2)
Range From 5955180 to 5957165
Homology: 64% similar to betT gene product of *Escherichia coli*

Left primer, larger PCR product
>II116_23B4oben_links.fasta
TGCCTGGGCGATCTACGCCATCGTCGCGCTGATCCTGGCCTTCTTCAGCTATCGCCACGG
CCTGCCGCTGACCCTGCGCTCGGCGCTGTATCCGCTGATCGGCGAACGTATCTACGGCCC
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GCTCGGCTACGGCGTGCTGCAGATCAACAGCGGCCTGCACCACCTGTTCGGCTGGCCGGT
GAACCAGACCGTGCAGATCGCCCTGATCGCCGCCACCTGCGGCCTCGCCACGCTCTCGGT
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GATCCTGCTGCTCTTCGTGCTGGTCCTCGGGCCGACCGTGTTCCTCCTGCAGACCTACGT
GCAGAACACCGGGGCCTACCTGTCCGACATCGTCAACAAGACCTTCAACCTGTACGCCTA
CGAGCCCACCGACTGGATCGGCGGCTGGACCCTGCTGTACTGGGGCCTGGTGGCTGTCCT
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Right primer
>II116_23B4oben_rechts.fasta
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GCAGGATCACCGCCAGGCTCAGGTTCAGCTCGGACAGGATGCGGATGCCGCGGTCCAGTC
CGCTGGCCACCGAGAGCGTGGCGAGGCCGCAAGTGGCGGCGATCAGGGCGATCTGCACGG
TCT

BLAST results for left primer:
Query: 1 Sbjct: 5955620
tgcctgggcgatctacgccatcgtcgcgctgatcctggccttcttcagctatcgccacgg 60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tgcctgggcgatctacgccatcgtcgcgctgatcctggccttcttcagctatcgccacgg 5955679

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Query: 61 Sbjct: 5955680
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Query: 121 Sbjct: 5955740
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Query: 181 Sbjct: 5955800
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Query: 301 Sbjct: 5955920 ggccagcggactggaccgcggcatccgcatcctgtccgagctgaacctgagcctggcggt 360
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Query: 361 Sbjct: 5955980     gatcctgctgctcttcgtgctggtcctcgggccgaccgtgttcctcctgcagacctacgt 420
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Query: 661 Sbjct: 5956278
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Mutant 23B6

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Middle PCR product, left primer
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AGGAGGCCTACGCCTTCCCGCTGGCGCACATCGAGCGCATGTGCGAGCTGGAAGCGGAGG
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TCTCGGCTGCGCAACTGCTGCAGCGGCCCGAGAGCAGCCGCACCGAAGGGGCCATCCCGG
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AGCGTACCCTGGTGGTGATGCCTCTCGATCCGCGCCTGGGCAAGGTGCGCGACGTTTCCG
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No similarities with transposon
Right primer
>II116_23B6mitte_rechts.fasta
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GACTTGCTCGCGCATGTGTCCCCGTACATC

Small PCR product
Left primer
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TTTCCAGCAGGGCGCGCTCGACGATCGGGTCCAACTGCGGTTCGTCGGGGAACTTCAAGG
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CGAGGCGCTCGGTGGGCAGCGGGTCGTCGAGGCCGGGCTTGAAGAGGCTCTGGCAGGCGC
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GCCGCGATAGGATGGCGCGAAACCCACATACCCGAGTGAAGTGAGTCGAGGATGTCCGAA
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AAA
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Right primer

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AGGTTCAGGCCCATAGCCCACGCACCTCGGCCCGGTTCAGGGCCAGCCACTGCAGGGCGA
TGATGCTCGCCGCGTTGTTGATCCGCCCGTCGCGCACGGCCTGCAGCGCATCTTCCAACG
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Score = 1281 bits (646), Expect = 0.0
                  Identities = 652/654 (99%)
                  Strand = Plus / Plus

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Query: 210 Sbjct: 5580127
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Alignment with transposon:
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                 Strand = Plus / Minus
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Query: 60   Sbjct: 1667
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Query: 120  agtgcggccgcacttgtgtataagagtca 148
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Sbjct: 1727 agtgcggccgcacttgtgtataagagtca 1755



Query: 802 Sbjct: 1
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WPITQYLPSPGGTDEVVHLFVGRCDSEGVGGVHGLPEEGEDIRVHVWPLEDALQAVRDGR 263
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QS-Mutant 22B11

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Score =  749 bits (378), Expect = 0.0
                 Identities = 390/395 (98%)
                 Strand = Plus / Minus
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gcatgccggccgagcgtccgatcaggcgcccatgc 5816005
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Quorum Sensing mutant 28B11

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PA 2360

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GGCGGAGCTGATCAATGCGGACTCGGAGGCGCGCCGGGTCTGGTCGCTGCTTGGCGTACTGCCCTAG

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Abschluß des Studiums der Biochemie im Oktober 1999 mit der Note sehr gut;
Durchführung der Diplomarbeit in der Arbeitsgruppe von Prof. Tümmler an der MHH.
Am **01.12.1999** Beginn der Promotion in der Arbeitsgruppe von Prof. Tümmler.
Am **14.12. 2004** Promotion zum Dr. rer. nat. mit der Note sehr gut

WISSENSCHAFTLICHE PUBLIKATIONEN:

Juhas M, Wiehlmann L, Huber B, Jordan D, Lauber J, Salunkhe P, Limpert A S v.Götz F, Steinmetz I, Eberl L, Tümmler B:

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Limpert A S, Wiehlmann L, Brandes G, Salunkhe P, Miethke N, Faudry E , Meyer A, Strüßmann A, Fauvarque M-O, Attree I, Tümmler B:

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Mitarbeit an folgenden Publikationen:

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