

Detergent-induced cell aggregation in

Pseudomonas aeruginosa

Dissertation

Zur Erlangung des Grades eines Doktors der Naturwissenschaften
im Fachbereich Biologie der naturwissenschaftlichen Sektion
der Universität Konstanz

vorgelegt von

Janosch Klebensberger

Konstanz

Februar 2007

Tag der mündlichen Prüfung: 17.04.2007
Prüfungskommission: Prof. Dr. Bernhard Schink
Prof. Dr. Alasdair Cook
Prof. Dr. Daniel Dietrich

Halte dir jeden Tag dreißig Minuten für deine Sorgen frei und mach in dieser Zeit ein Nickerchen

Abraham Lincoln

Danksagung

Die vorliegende Arbeit wurde im Zeitraum von November 2002 bis Februar 2007 am Lehrstuhl für Mikrobielle Ökologie von Prof. Dr. Bernhard Schink unter der Betreuung von Dr. Bodo Philipp angefertigt.

Dabei gilt mein besonderer Dank:

Prof. Dr. Bernhard Schink für die Möglichkeit, die vorliegende Arbeit an seinem Lehrstuhl durchzuführen, sowie für die kritische Durchsicht der Manuskripte als auch der vorliegenden Arbeit.

Dr. Bodo Philipp für die Überlassung des Themas, die stetige Hilfsbereitschaft bei Fragen und Diskussionen, sowie die engagierte Betreuung während der gesamten Zeit der Promotion.

Prof. Dr. Alasdair Cook für die Übernahme des Koreferates und für das Interesse an dieser Arbeit.

Allen Mitarbeitern des Lehrstuhls Schink und der Arbeitsgruppe Cook, die ich während meiner Doktorarbeit kennen gelernt habe, für die stets konstruktive Zusammenarbeit und die angenehme Arbeitsatmosphäre. Stellvertretend seien die ehemaligen Diplomanden Oliver Rui, Eva Fritz und Karin Lautenschlager genannt, deren Ergebnisse ihren Anteil zum Gelingen der vorliegenden Arbeit beigetragen haben.

Christine Dittrich, Daniel Bressler, Dr. Jost Wingender, Dr. Alex Böhm, Dr. Jacob Malone und Prof. Urs Jenal für die fruchtbaren Kooperationen und konstruktiven Diskussionen.

Andrea für ihre Unterstützung und ihren unbegrenzten Optimismus.

Table of contents

1	Zusammenfassung	1
2	Summary	3
3	Introduction	5
4	Aim of this thesis	10
5	Cell aggregation of <i>Pseudomonas aeruginosa</i> strain PAO1 as an energy-dependent stress response during growth with sodium dodecyl sulfate	11
5.1	Abstract	11
5.2	Introduction	12
5.3	Materials and methods	13
5.4	Results	18
5.5	Discussion	26
5.6	Acknowledgments	28
6	Detergent-induced cell aggregation in subpopulations of <i>Pseudomonas aeruginosa</i> as a pre-adaptive survival strategy	29
6.1	Abstract	29
6.2	Introduction	30
6.3	Materials and methods	32
6.4	Results	37
6.5	Discussion	45
6.6	Acknowledgments	48

7	Genes responsible for SDS-induced aggregation: Identification of an potential c-di-GMP signalling pathway which regulates transcription and posttranscriptional modifications of the <i>cupA</i> operon	49
7.1	Abstract	49
7.2	Introduction	50
7.3	Materials and methods	51
7.4	Results	56
7.5	Discussion	66
7.6	Acknowledgments	68
8	Discussion	69
9	Appendix	75
10	References	76

1 Zusammenfassung

Pseudomonas aeruginosa Stamm PAO1 konnte mit dem toxischen anionischen Detergens Natriumdodecylsulfat (SDS) als einziger Kohlenstoff- und Energiequelle wachsen. Während des Wachstums auf oder in Gegenwart von SDS wurde die Bildung makroskopischer Zellaggregate beobachtet. Diese Aggregate bestanden aus beschädigten und unbeschädigten Zellen, welche in eine Matrix aus sauren Polysacchariden und DNA eingebettet waren. Diese Aggregate wurden gebildet, wenn frei suspendierte Zellen bei hoher Energieversorgung mit SDS versetzt wurden. Dabei aggregierte immer nur ein Teil der Gesamtpopulation. Bei stark limitierter Energieversorgung lysierten die Zellen in Gegenwart von SDS ohne die Bildung von Aggregaten. Diese physiologischen Untersuchungen zeigen, dass SDS toxisch für *P. aeruginosa* ist und die Bildung von Zellaggregaten einen aktiven und energieabhängigen Prozess darstellt.

Aus Kulturen, die mit SDS wuchsen, wurde die nicht aggregierende Spontanmutante Stamm N isoliert. Dieser Stamm bildete glatte Kolonien bei Wachstum auf SDS-haltigen Agarplatten, wohingegen der Wildtyp raue und strukturierte Kolonien auf diesen Medien ausbildete. Aggregation in Flüssigkultur und Bildung rauher Kolonien bei Wachstum mit SDS konnten in Stamm N durch die Expression des Gens PA4929, welches eine putative Guanylatzyklase für die Synthese des Signalmoleküls *cyclic di-guanosine monophosphate* (c-di-GMP) codiert, wiederhergestellt werden. Die Expression der Phosphodiesterase CC3396 in Stamm PAO1, welche den Abbau von c-di-GMP katalysiert, führte zu einer stark verminderten Aggregation und einem teilweisen Verlust der rauhen Koloniemorphologie während des Wachstums mit SDS. Die nicht aggregierenden Stämme N und PAO1[CC3396] wiesen unter starker Energielimitierung eine erheblich geringere Überlebensrate bei der Exposition gegenüber SDS auf. Die Überlebensrate dieser nicht-aggregierenden Stämme konnte jedoch durch die Integration von Zellen in die Aggregate von Stamm PAO1 stark erhöht werden. Diese Untersuchungen zeigen, dass die Bildung von Aggregaten keine Voraussetzung für das Wachstum mit SDS ist, jedoch eine erhöhte Überlebensrate unter starker Energielimitierung gewährleistet.

Um Gene zu finden, die an der SDS-induzierten Aggregation beteiligt sind, wurde eine Transposonmutagenese durchgeführt. Bei der Mehrzahl der nicht-aggregierenden Transposonmutanten waren zwei Gencluster, das *psl*- und das *cupA* Operon, betroffen, welche für die Anheftung von *P. aeruginosa* an Oberflächen benötigt werden. Das *psl* Operon kodiert für Proteine, die für die Biosynthese eines extrazellulären Polysaccharids benötigt werden, dessen Monomere vorwiegend aus Mannose und Glucose bestehen. Durch *Confocal Laser Scanning Microscopy* von Aggregaten, die mit einem spezifischen Lektin

gefärbt worden waren, konnten zahlreiche Regionen lokalisiert werden, die Mannose und Glukose enthielten.

Das *cupA* Operon kodiert für Proteine, die für die Bildung adhäsiver Fimbrien benötigt werden. Northern Blot Analysen zeigten eine starke Zunahme des *cupA1* Transkriptes in SDS-gewachsenen Zellen von Stamm PAO1 im Vergleich zu Succinat-gewachsenen Zellen. Eine nicht-aggregierende Transposonmutante mit einem Defekt innerhalb des Gens PA0172 zeigte diese Erhöhung des *cupA1* Transkriptes nicht. Das Gen PA0172 ist Teil eines Clusters (PA0172-PA0169), dessen Funktion bisher unbekannt war. Durch gezielte Mutation der Gene PA0172 und PA0169 konnte gezeigt werden, dass diese Gene Teil eines putativen c-di-GMP-abhängigen Signaltransduktionswegs sind, der an der transkriptionellen Regulation des *cupA* Operons beteiligt ist. Sequenzanalysen legen dabei nahe, dass innerhalb eines solchen Signaltransduktionsweges das Genprodukt von PA0172 ein Rezeptor für einen noch unbekanntem Reiz darstellt. Die konservierte GGDEF-Domäne des Genprodukts von PA0169 lässt vermuten, dass es sich bei diesem Protein potentiell um eine Guanylatzyklase handelt, die diesen Reiz über das intrazelluläre Signal c-di-GMP weiterleitet. Durch Transkriptionsanalysen wurde die Beteiligung dieser Gene an der transkriptionellen Aktivierung des *cupA* Operons nachgewiesen. Auch wurden Hinweise gefunden, dass diese Gene zudem an der posttranskriptionellen Stabilisierung der mRNA des *cupA* Operons beteiligt sind.

Die vorliegende Arbeit beschreibt eine neuartige Stressantwort gegenüber toxischen Detergentien, die sowohl physiologisch als auch molekularbiologisch charakterisiert wurde. Dabei konnten durch Identifizierung eines bisher unbekanntem Signaltransduktionswegs und dessen Beteiligung an der transkriptionellen Regulation von adhäsiven Oberflächenstrukturen neue Erkenntnisse über die komplexen Vorgänge der Aggregation bzw. Biofilmbildung gewonnen werden. Anhand dieser Ergebnisse kann die SDS-induzierte Aggregation einer Teilpopulation als eine adaptive Strategie von *Pseudomonas aeruginosa* aufgefasst werden, die das Überleben der Gesamtpopulation unter variierenden Umweltbedingungen gewährleistet.

2 Summary

Pseudomonas aeruginosa strain PAO1 was able to use the toxic detergent sodium dodecyl sulfate (SDS) as sole source of carbon and energy. Growth of strain PAO1 with and in the presence of SDS was accompanied by the formation of large macroscopic aggregates. Characterization of these aggregates revealed that they contained a mixture of damaged and undamaged cells embedded in a matrix of acidic polysaccharides and DNA as structural components. In experiments with dense cell suspensions, we identified a strictly energy-dependent response of cells towards SDS exposure. Cells completely deprived of energy lysed without the formation of aggregates. Under high-energy supply, a subpopulation of cells responded with aggregate formation in an active, energy-requiring process.

The isolation of the spontaneous mutant strain N growing with SDS without aggregation revealed that SDS-induced aggregation was not essential for growth. Another characteristic of strain N was the formation of smooth colonies on SDS-containing agar, in contrast to the rough and structured colonies formed by the parent strain. Complementation with genes encoding for putative di-guanylate cyclases (DGCs), which are responsible for the biosynthesis of cyclic di-guanosine monophosphate (c-di-GMP), restored aggregation and rough colony morphology in strain N during growth with SDS. This finding implicated strongly that this novel second messenger is involved in SDS-induced aggregation. This hypothesis was further supported by decreased aggregation and partial loss of rough colony morphology in strain PAO1 expressing a specific phosphodiesterase (PDE), responsible for the degradation of c-di-GMP. Although aggregate formation was not essential for growth, we found dramatically decreased survival rates under energy-limited conditions with those strains that did not respond with aggregate formation. However, strain N could reduce this detrimental effect significantly by co-integration into aggregates formed by strain PAO1

To investigate the genetic basis of SDS-induced aggregation, we performed transposon mutagenesis and identified genes essential for the formation of aggregates during growth with SDS. In most of the mutants, the transposon was inserted in two distinct gene clusters responsible for increased adhesiveness of the cells. The *psl* gene cluster is responsible for the biosynthesis of an extracellular polysaccharide mainly consisting of mannose and glucose. With confocal laser scanning microscopy of aggregates stained with a specific lectin for mannose and glucose moieties, we obtained evidence for expression of the *psl* gene cluster in aggregates of strain PAO1 during growth with SDS.

The *cupA* gene cluster encodes proteins responsible for the biosynthesis and assembly of adhesive fimbria. Northern blot analysis demonstrated that SDS triggered increased levels of the *cupA1* transcript in strain PAO1 in a strictly c-di-GMP-dependent

manner. This effect was completely lost in a mutant deficient in the gene PA0172 which is part of a gene cluster (PA0172-0169) with unknown function. With knockout mutants deficient in PA0172 or PA0169, we demonstrated that these genes are part of a potential c-di-GMP signalling pathway which is involved in the regulation of the *cupA* operon. Sequence analyses implicated that gene PA0172 most likely represents a sensor for an environmental signal within this pathway. The GGDEF domain of the protein encoded by PA0169 suggested that this protein could be responsible for downstream signal propagation via synthesis of c-di-GMP. With transcriptional *lacZ*-fusions, we uncovered that these genes are responsible for transcriptional activation of *cupA* during growth with SDS. Furthermore, we obtained evidence that these genes are also involved in the stability of the respective mRNA by a yet uncharacterized posttranscriptional mechanism.

In this thesis, we found a novel stress response of bacteria towards a toxic detergent and characterized this response physiologically and genetically. The identification of a so far unknown signalling pathway and its involvement in the transcriptional regulation of adhesive surface structures led to new findings within the complex events that are responsible for cell aggregation and biofilm development. From these results, SDS-induced aggregation of a subpopulation can be described as an adaptive strategy of *Pseudomonas aeruginosa* to ensure survival of the whole population under varying environmental conditions.

3 Introduction

Detergents

Sodium dodecyl sulfate (SDS) is an anionic detergent which is widely used in household products like toothpaste or shampoos. Detergents are characterized by their amphiphilic structure containing at least one polar, hydrophilic moiety attached to a non-polar, hydrophobic moiety (Fig. 1a). This amphiphilic structure is responsible for their surface activity, which promotes enhanced solubility of non-polar compounds such as organic solvents in aqueous solutions. In general, detergents can be classified into four groups according to the charge of their polar moiety.

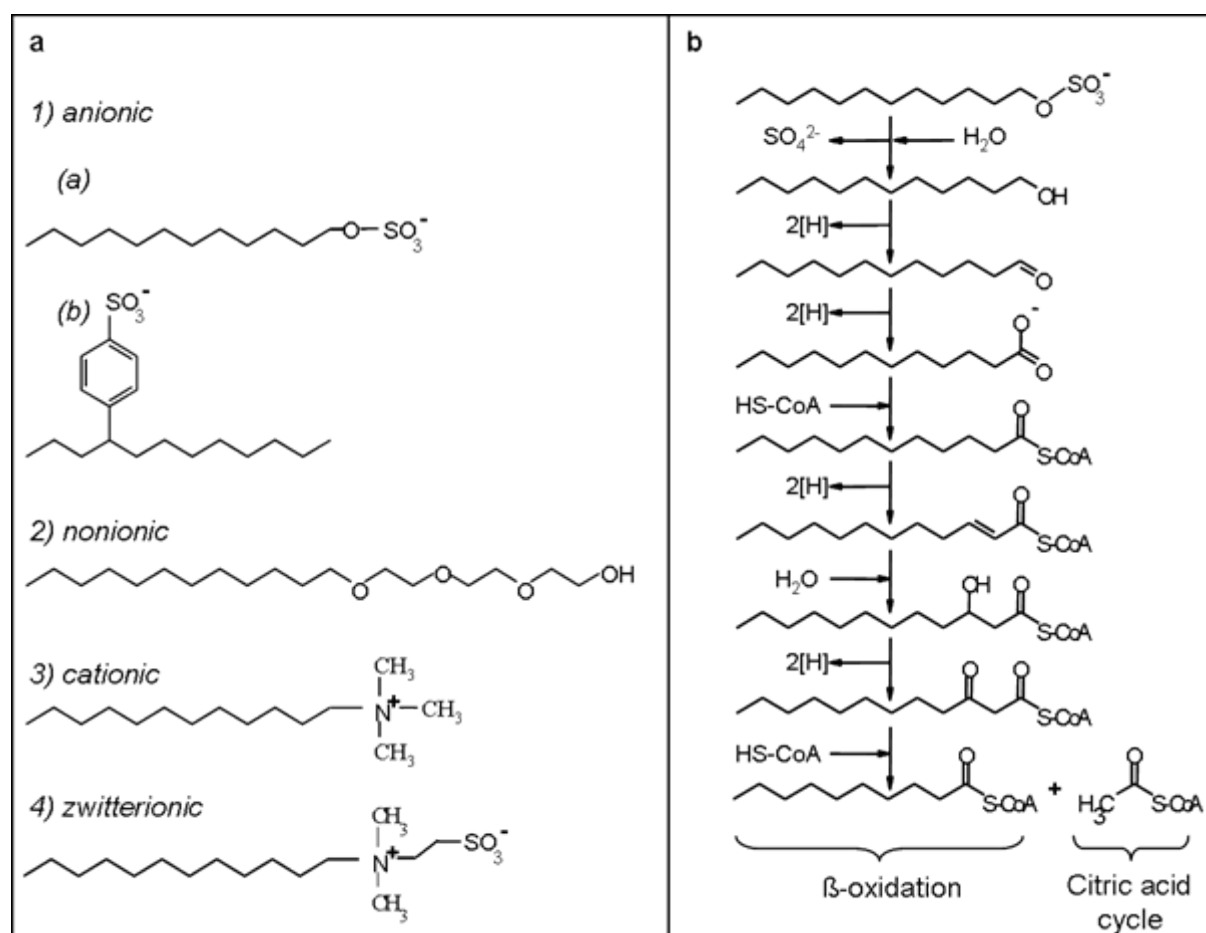


Fig. 1. Classification of detergents (a) and the degradation pathway of the anionic detergent sodium dodecyl sulfate (SDS) (b). **a)** Representatives of the different classes of detergents. 1) Anionic detergents: (a) sodium dodecyl sulfate (SDS), (b) linear alkylbenzenesulfonate (LAS). 2) Nonionic detergents: alkylethoxylate. 3) Cationic detergents: alkyltrimethylammonium. 4) Zwitterionic detergents: alkylsulfobetaine. **b)** Degradation pathway of the anionic detergent SDS (Thomas and White 1989).

Sulfate ester detergents like SDS or linear alkylbenzene sulfonate detergents like LAS are known to be biodegradable (Scott and Jones 2000). Complete degradation of these compounds can be carried out by either single bacterial strains in case of SDS (Ellis *et al.* 2002; Marchesi *et al.* 1994; Payne and Feisal 1963) or in a complex cooperation of specialized bacteria in the case of commercial LAS (Schleheck *et al.* 2004; Schleheck *et al.* 2003). Degradation of SDS is initiated by an alkyl sulfatase which hydrolyzes SDS to sulfate and the corresponding alcohol 1-dodecanol (Hagelueken *et al.* 2006) (Fig. 1b). This primary alcohol is then oxidized to lauric acid, activated with coenzyme A, and further degraded by β -oxidation leading to acetyl-CoA residues which are oxidized in the citric acid cycle (Thomas and White 1989).

Although detergents can be used as growth substrates, their degradation is a major challenge for bacteria because they are generally termed toxic for microorganisms. This is a consequence of its ability to interact with biological membranes, finally leading to cell lysis in a five step process, and to denature proteins by cooperative binding to the polypeptide chain (Helenius and Simons 1975). Several resistance mechanisms are known to protect bacterial cells against the toxic effects of anionic detergents. These mechanisms include changes in the permeability of the outer membrane (Nikaido 2003), multidrug efflux pumps with a broad substrate specificity (Poole 2004), and the presence of Clp-proteases which degrade misfolded proteins (Rajagopal *et al.* 2002). A common feature of all these resistance mechanisms is their energy dependency (Nickerson and Aspedon 1992). Consequently, bacteria utilizing detergents as sole source of carbon and energy have to find a trade-off for energy investment: to generate energy for growth, they have to take up the toxic substrate, thereby risking to be injured. For protection and repair, they have to invest part of this energy which is consequently not available for growth. So far it is not known whether bacteria that utilize detergents as growth substrates require additional strategies to protect themselves.

Pseudomonas aeruginosa and biofilm formation

Cell aggregates that are either freely floating or attached to surfaces are considered to be the predominant form of microbial life in nature (Costerton *et al.* 1995). Biofilms are also involved in most bacterial infections (Costerton *et al.* 1999). This correlation has dramatic consequences because residing in aggregates has been shown to confer increased resistance to bacterial cells to biocides such as antibiotics, disinfectants, and detergents (Drenkard and Ausubel 2002; Fux *et al.* 2005; Gilbert *et al.* 2002; Lewis 2001). Thus, investigations on the formation and development of biofilms have increased extensively over the recent years. In this research area, the opportunistic pathogen *Pseudomonas aeruginosa* has become a model organism for several reasons. One reason is

its metabolic versatility and variability to respond towards environmental signals which promote successful colonization of different habitats and growth under varying environmental conditions (Clarke 1982; Rodrigue *et al.* 2000). Another reason is that *P. aeruginosa* is a threatening organism in clinical settings, because it is a serious cause of infections in burn patients and the predominant cause of lung infections and mortality in patients with cystic fibrosis (Lyczak *et al.* 2002; Tredget *et al.* 2004). The formation of biofilms is characterized by a series of diverse and complex regulatory processes which are not fully understood. Several authors have suggested models for the development of biofilms which can be summarized in a five-stage process (Caiazza and O'Toole 2004; Stoodley *et al.* 2002) (Fig. 2).

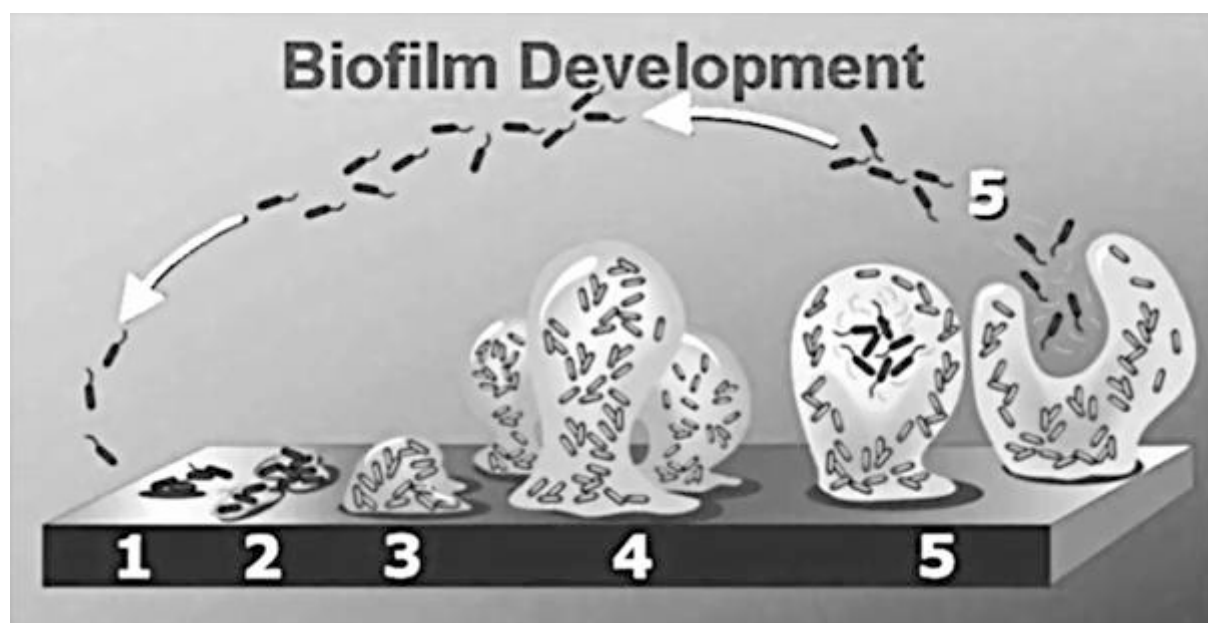


Fig. 2. Model for the development of a biofilm in a five-stage process (adapted from Stoodley *et al.* 2002). Stage 1: initial and reversible attachment of cells to a surface. Stage 2: irreversible attachment of cells. Stage 3: microcolony formation. Stage 4: maturation of the biofilm. Stage 5: dispersion of single cells from the biofilm. Further details of this process are discussed in the text.

The first step (Stage 1) is the reversible attachment of free-swimming cells to a surface (O'Toole and Kolter 1998). After initial attachment, the cells undergo transition to an irreversible state of attachment (Stage 2). This is most likely caused by altered surface properties and initial production of extracellular polymeric substances (EPS). The EPS represent a major characteristic of biofilms, which provide a highly hydrated and structured matrix for bacteria. In *P. aeruginosa* biofilms, the EPS have been shown to contain DNA (Allesen-Holm *et al.* 2006; Steinberger and Holden 2005; Whitchurch *et al.* 2002) and various polysaccharides such as alginate and a yet uncharacterized mannose and glucose-rich polymer (Friedman and Kolter 2004; Linker and Jones 1964; Tielen *et al.* 2005;

Ude *et al.* 2006). After irreversible attachment, the cells proceed to form microcolonies either by clonal growth or in a type IV pilus-dependent manner (Stage 3) (Klausen *et al.* 2003b). Twitching motility mediated by type IV pili was also found to be involved in the maturation of biofilms, resulting in a complex architecture (Stage 4) (Klausen *et al.* 2003a). During this phase of development, the cells undergo dramatic changes in gene expression and protein formation patterns resulting in physiological differentiation (Sauer *et al.* 2002; Whiteley *et al.* 2001). Finally, cells can also disperse from matured biofilms, which is believed to be a physiologically regulated event (Stage 5). The understanding of dispersal is limited, but recent studies have demonstrated that dispersal is triggered by changes in nutrient concentrations or the accumulation of nitric oxide (NO) from anaerobic respiration (Barraud *et al.* 2006; Morgan *et al.* 2006; Sauer *et al.* 2004).

Intracellular signalling via cyclic di-guanosine monophosphate (c-di-GMP)

As described above, the development of cell aggregates or biofilms is a succession of several complex events, which obviously requires the involvement of regulatory circuits. Only recently, the novel second messenger c-di-GMP has been found to be involved in the regulation of biofilm formation in *P. aeruginosa* and other bacteria (Jenal and Malone 2006; Römling *et al.* 2005). This intracellular signalling molecule was originally found in *Gluconacetobacter xylinus* where it acts as an allosteric regulator of cellulose synthase (Ross *et al.* 1986). The global impact of c-di-GMP as a second messenger was then recognized by identifying conserved domains responsible for c-di-GMP metabolism (Fig. 3) and their wide distribution among prokaryotes (Galperin *et al.* 2001; Tal *et al.* 1998). The biosynthesis of c-di-GMP from 2 GTP is catalyzed by diguanylate cyclases (DGCs) containing a characteristic GG[D/E]EF domain as the active centre (Chan *et al.* 2004; Ryjenkov *et al.* 2005). Hydrolysis of c-di-GMP is catalyzed by specific phosphodiesterases (PDEs) containing either a conserved E[A/V]L or a HD-GYP domain (Christen *et al.* 2005; Ryan *et al.* 2006).

Since its discovery, more and more evidence of c-di-GMP playing a key role in regulating the transition from sessility to motility and vice versa in different species has been obtained. One example represents the occurrence of phenotypic variations with an autoaggregative phenotype during growth in heterogeneous environments (Goymer *et al.* 2006; Rainey and Travisano 1998; Ude *et al.* 2006) and in biofilms, respectively (Deziel *et al.* 2001; Drenkard and Ausubel 2002; Kirisits *et al.* 2005; Webb *et al.* 2004). Several studies demonstrated a direct link between autoaggregation and regulatory systems responsible for c-di-GMP metabolism (D'Argenio *et al.* 2002; Häußler *et al.* 2003; Römling 2005). One such system is the Wsp pathway, responsible for the “wrinkly spreader” phenotype in Pseudomonads (Rainey and Travisano 1998). In this

chemosensory pathway the kinase activity of WspE positively regulates the response regulator WspR, resulting in the biosynthesis of c-di-GMP (Jenal and Malone 2006). Activity of WspE depends on conformational changes of a sensor complex in response to an unknown environmental signal. Further regulation of WspE activity is mediated by the methylesterase WspF, which is involved in a feedback mechanism leading to adaptation to the signal. The Wsp pathway has been shown to regulate adhesive factors such as cellulose production in *P. fluorescens*, and it is needed for the colonization of specific habitats (Spiers *et al.* 2003; Spiers and Rainey 2005). Another well studied example is the life-cycle of the aquatic bacterium *Caulobacter crescentus*. In this organism, the transition from free-swimming to surface-adherent stalked cells is mediated by the response regulator PleD (Aldridge and Jenal 1999; Hecht and Newton 1995). This regulator consists of two N-terminal receiver domains and a C-terminal GGDEF output domain which acts as a DGC, required for stalk formation (Aldridge *et al.* 2003; Paul *et al.* 2004).

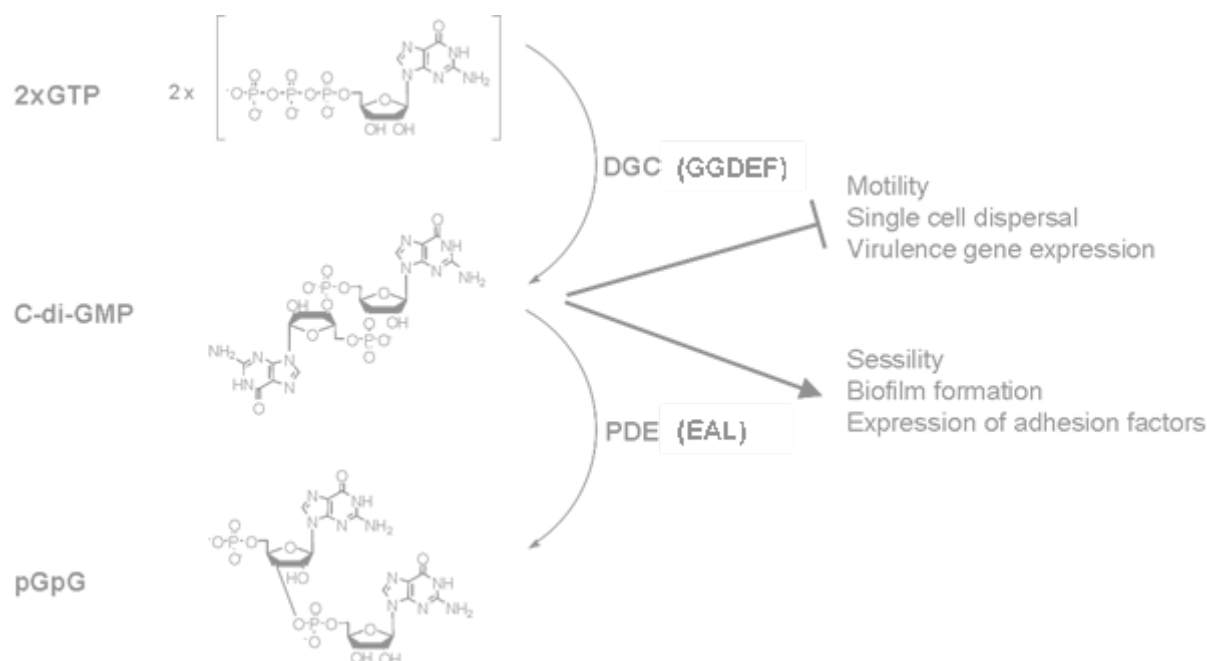


Fig. 3. Scheme of synthesis and hydrolysis of c-di-GMP. Di-guanylate cyclases (DGCs) catalyze the biosynthesis of c-di-GMP from two GTP molecules. Specific phosphodiesterases (PDEs) are responsible for the degradation of c-di-GMP. The motives responsible for catalytic activity of these enzymes and the cellular functions affected by c-di-GMP are shown.

The huge number of different input domains associated with GGDEF and EAL domains and their multiple occurrence in several organisms suggests that plenty of environmental signals can be perceived and transmitted by c-di-GMP signalling pathways (Galperin *et al.* 2001). Some of those include oxygen, antibiotics, and the concentration of nutrients (Chang *et al.* 2001; Gjermansen *et al.* 2005; Hoffman *et al.* 2005;

Morgan *et al.* 2006). However, despite intensive research in this field over the recent years, information about environmental triggers, potential target genes, and regulation circuits of c-di-GMP-dependent signalling is still limited at present.

4 Aim of this thesis

The general interest underlying this thesis was to understand how bacteria can cope to grow with toxic detergents as their sole source of carbon and energy. To investigate this question, we used *Pseudomonas aeruginosa* strain PAO1 growing with the toxic anionic detergent sodium dodecyl sulfate (SDS) as a model substrate.

The starting point for our research was the observation that growth with or in the presence of SDS was characterized by the formation of macroscopic aggregates. The aim of this thesis was to explore the physiological function of aggregate formation during growth with SDS and to identify genes that are involved in this process.

5 Cell aggregation of *Pseudomonas aeruginosa* strain PAO1 as an energy-dependent stress response during growth with sodium dodecyl sulfate

Janosch Klebensberger, Oliver Rui, Eva Fritz, Bernhard Schink, Bodo Philipp

Archives of Microbiology (2006) **185**: 417-427

5.1 □□ Abstract

Pseudomonas aeruginosa strain PAO1 grew with the detergent sodium dodecyl sulfate (SDS). Growth commenced with the formation of macroscopic cell aggregates which consisted of respiring cells embedded in extracellular matrix composed of acidic polysaccharides and DNA. Damaged and uncultivable cells accumulated in these aggregates compared to those cells that remained suspended. We investigated the response of suspended cells to SDS under different conditions. At high energy supply, the cells responded with a decrease in optical density and in viable counts, release of protein and DNA, and formation of macroscopic aggregates. This response was not observed if the energy supply was reduced by inhibiting respiration with KCN, or if cells not induced for SDS degradation were exposed to SDS. Exposure to SDS caused cell lysis without aggregation if cells were completely deprived of energy, either by applying anoxic conditions, by addition of CCCP, or by addition of KCN to a mutant defective in cyanide-insensitive respiration. Aggregated cells showed a more than 100-fold higher survival rate after exposure to SDS plus CCCP than suspended cells. Our results demonstrate that cell aggregation is an energy-dependent response of *P. aeruginosa* to detergent stress which might serve as a survival strategy during growth with SDS.

5.2 □□ Introduction

In their natural environments, bacteria do quite often not occur as freely suspended cells but in cell aggregates that are either freely floating or attached to surfaces as biofilms (Stoodley *et al.* 2002). Such aggregates are stabilized by a matrix of extracellular polymeric substances (EPS) that consist of polysaccharides, proteins, and DNA (Sutherland 2001; Whitchurch *et al.* 2002). The factors that promote aggregation are not understood completely (Bossier and Verstraete 1996b). Chemical stress by toxic compounds is one factor among the possible triggers for active bacterial aggregation. For example, *Pseudomonas putida* strain CP1 forms aggregates during degradation of chlorophenols (Farrell and Quilty 2002) and *Comamonas testosteroni* strain A20 acquires the ability to co-aggregate with yeast cells in response to hydrogen peroxide (Bossier and Verstraete 1996a). Formation of aggregates as a protection mechanism appears to be an attractive concept because bacteria in biofilms are known to be more resistant against biocides than suspended cells (Gilbert *et al.* 2002; Lewis 2001). This increased resistance is based on multiple factors, including that EPS act as a diffusion barrier or that less susceptible physiological states of individual cells are frequently found in biofilms. The resistance of bacteria in aggregates has so far been addressed mainly with regard to antibiotics and disinfectants. Only recently, the induction of biofilm formation as a defensive reaction to the presence of aminoglycoside antibiotics has been shown in *P. aeruginosa* (Hoffman *et al.* 2005).

We are interested in the role of bacterial aggregates in the degradation of toxic chemicals, in particular anionic detergents. Sulfate ester detergents like sodium dodecyl sulfate (SDS) are considered as readily biodegradable (Scott and Jones 2000). Several publications have described SDS degradation by pure cultures of *Pseudomonas* strains (Ellis *et al.* 2002; Marchesi *et al.* 1994; Payne and Feisal 1963; Stavskaia *et al.* 1989). Degradation is initiated by an alkyl sulfatase which hydrolyses SDS to sulfate and 1-dodecanol. This primary alcohol is oxidized to lauric acid and further degraded by β -oxidation to acetyl-CoA residues (Thomas and White 1989). Degradation of SDS is a major challenge for bacteria because this detergent solubilizes biological membranes and denatures proteins (Helenius and Simons 1975). However, no publication dealing with SDS degradation has addressed the toxicity of SDS so far. Several resistance mechanisms against anionic detergents like diffusion barriers (Nikaido and Vaara 1985), multidrug efflux pumps (Poole 2004), or Clp-proteases (Rajagopal *et al.* 2002) have been described. All these resistance mechanisms require energy and have been shown to protect cells which grow in the presence of detergents (Nickerson and Aspedon 1992). Bacteria using detergents for growth face an additional challenge. They have to invest part of their energy into protection while taking an increased risk of damage because they have to take up the

toxic detergents to metabolize them. So far it is not known whether bacteria that utilize detergents as growth substrates require additional strategies to protect themselves. We hypothesize that formation of cell aggregates would be a feasible strategy for this purpose.

To test this hypothesis we isolated an SDS degrading bacterium from a bathroom soap bin that formed large aggregates when growing with SDS. This isolate turned out to be a *Pseudomonas aeruginosa* strain. *P. aeruginosa* is a ubiquitous Gram-negative bacterium of broad metabolic versatility (Alonso *et al.* 1999) and is highly resistant to many biocides like antibiotics or detergents, especially when living in biofilms (Rajagopal *et al.* 2003; Spoering and Lewis 2001). The tendency to form biofilms (Stoodley *et al.* 2002) is important in infections caused by this opportunistic pathogen (Costerton *et al.* 1999; Lyczak *et al.* 2000). The combination of metabolic versatility, biocide resistance, and formation of biofilms render *P. aeruginosa* an appropriate model organism to study the function of aggregation in degradation of toxic compounds. As *P. aeruginosa* strain PAO1 can utilize SDS as a sulfur source (Hummerjohann *et al.* 2000) we tested whether strain PAO1 could use SDS also as a carbon and energy source. Since this was true, we continued our investigations with this well-characterized strain.

5.3 □□ Materials and methods

Cultivation and growth experiments

Pseudomonas aeruginosa strain PAO1 (Holloway Collection) was maintained on solid (1.5% w/v agar) Luria-Bertani (LB) medium. A *rpoS* mutant of the same strain (Diggle *et al.* 2002) and a insertion mutant of *cioB* [32522] derived from strain MPAO1 (Jacobs *et al.* 2003) provided by the Washington genome centre (<http://www.genome.washington.edu/UWGC>) were maintained on solid LB medium containing 50 µg/ml kanamycin or 60 µg/ml tetracycline, respectively. For cultivation in liquid media, LB medium and a modified M9 medium (Sambrook *et al.* 1989) were used. The M9 medium contained the following components (final concentration in mM): Na₂HPO₄ (47.6), KH₂PO₄ (22), NaCl (8.6), NH₄Cl (18.6), MgSO₄ (Kovach *et al.*), CaCl₂ (0.1), FeCl₂ (0.03) and the trace element solution SL10 (Widdel and Pfennig 1981). SDS (3.5 mM) or Na₂-succinate (10 mM) was used as carbon and energy sources. Growth was measured as optical density at 600 nm (OD₆₀₀) in a spectrophotometer. For growth experiments, a test tube with 5 ml LB medium was inoculated with strain PAO1 from a LB-plate and incubated on a rotary shaker (Orbital Incubator S 150; Stuart Scientific) at 150 rpm for 10-14 h at 30°C. This pre-culture was used to inoculate 100 ml of M9 medium in a 500 ml Erlenmeyer flask without baffles at OD₆₀₀ = 0.01. These flasks were then incubated on a rotary shaker at 200 rpm at 30°C. Immediately after inoculation and at regular intervals thereafter, samples

were withdrawn from cultures to measure bacterial growth and substrate degradation. Samples for substrate measurements were centrifuged in plastic tubes at $18.500 \times g$ for 10 min at room temperature. Supernatants were transferred into new plastic tubes and stored at -20°C until further analysis.

Characterization of macroscopic cell aggregates

Macroscopic cell aggregates of strain PAO1 from growing cultures or from SDS shock experiments (see below) were collected and washed twice with M9 medium. These aggregates were treated with DNase I (Type II, stock solution in water; Sigma) or alginate lyase (stock solution in water; Aldrich) in appropriate buffer solutions (50 mM Tris-HCl with 10 mM MgCl_2 at pH 7.2 with DNase I and 50 mM Tris-HCl at pH 7.5 with alginate lyase) with shaking at 50 rpm at 37°C . Aggregates were stained with 0.1% (w/v) Alcian Blue 8GX (dissolved in M9; Fluka) in M9 medium with shaking at 50 rpm at 30°C , or with 2 mM CTC (5-cyano-2,3-ditoly tetrazolium chloride, stock solution in M9 medium; Polysciences) in M9 medium with shaking at 50 rpm at 37°C . LIVE/DEAD staining (BacLight, 2 \times stock solution in M9 medium; Molecular Probes) of aggregates was performed in M9 medium without shaking in the dark. For quantification of cells stained as live or dead after SDS shock (see below) and DNase treatment, coverslips (Thermanox 13 mm; Nunc) were placed into cell suspensions in 24-well microtiter plates for 5 min and washed twice in 1 ml of M9 medium to remove SDS, which otherwise interfered with the fluorescent dyes of the LIVE/DEAD kit. Cells attached to the coverslips were stained, washed again, and counted by epifluorescence microscopy.

Preparation of cell suspensions

Cells were grown as described above and harvested in the late exponential phase by centrifugation in sterile 50 ml plastic tubes at $12.850 \times g$ for 15 min at 20°C . Cells were washed once in 20 ml M9 medium without carbon source. After final resuspension, the cell suspension still contained cell aggregates that were removed by filtration through a sterile polycarbonate membrane filter (25 mm diameter; Nuclepore) with 5 μm pore size. These filtrates containing only freely suspended cells were adjusted to $\text{OD}_{600} = 1$ with M9 medium. For substrate degradation and respiration experiments, cell aggregates remaining after the final resuspension were removed by centrifugation at $50 \times g$ for 5 min at 20°C .

SDS shock experiments

SDS shock experiments were performed with 1 ml filtered cell suspensions in sterile half-micro plastic cuvettes (Greiner) at 30°C and were reproduced in at least three

independent runs. Experiments were started by addition of SDS (3.5 mM) or SDS plus succinate (10 mM). In control experiments, only succinate (10 mM) or water was added. For inhibitor studies, KCN (50 mM stock solution in 20 mM NaOH), NaN_3 (200 mM stock solution in water), or carbonyl cyanide chlorophenylhydrazone (CCCP, 25 mM stock solution in methanol) was added to final concentrations of 2 mM (KCN, NaN_3) or 1 mM (CCCP) before starting the shock experiments. Immediately after starting the experiments and at regular intervals thereafter (15, 30, 45 min), the OD_{600} of the cell suspensions was determined after inverting the cuvettes 3 times. After 45 min incubation, colony forming units (CFU) were counted with the cell suspensions from SDS shock experiments. Aliquots of 20 μl of each cell suspension were diluted in a decimal series in M9 medium. The residual volume of the cell suspensions was filtered through a sterile polycarbonate filter (13 mm diameter; Osmonics) with 5 μm pore size and used for a second decimal dilution series. From each dilution step, three aliquots of 15 μl were used for CFU counts by the drop plate method (Hoben and Somasegaran 1982). Total cell counts were determined with a microscopic Thoma chamber. The mean value of at least 4 individual counts (> 150 cells) from each sample was used for calculation. For DNA and protein quantification in cell-free supernatants of SDS shock experiments, 4 parallel cuvettes for each experimental condition were set up. At different time points, cell suspensions of one cuvette were filter sterilized (FP 30/0.2 CA; Schleicher&Schuell), immediately frozen in liquid nitrogen, and stored at -20°C until further analysis. For anoxic SDS shock experiments, cells were grown aerobically and harvested as described above. Further processing was performed under anoxic conditions. Cells were washed with anoxic M9 medium and adjusted to $\text{OD}_{600} = 1$ in an anoxic chamber under N_2/H_2 atmosphere (95/5 v/v). Shock experiments were performed in sterile glass cuvettes that were filled and sealed with gas-tight butyl rubber stoppers inside the anoxic chamber. SDS, succinate, and inhibitors were added to the cell suspensions with gas-tight syringes (Hamilton) from anoxic sterile stock solutions. CFU counts of anoxic cell suspensions were performed under oxic conditions as described above.

For comparing survival rates of suspended cells and cells within aggregates, SDS shock experiments were modified. Cells growing with SDS were separated into two fractions of aggregates and suspended cells by centrifugation at $80 \times g$ for 5 min. Both fractions were washed twice with M9 medium by centrifugation at either at $80 \times g$ (aggregates) or $10.000 \times g$ (suspended cells), finally suspended in 10 ml M9 medium in 50 ml plastic tubes, and shocked with SDS for 45 min at 30°C with shaking at 50 rpm. After 45 min, 40 ml M9 was added to the tubes. Cells were harvested by centrifugation at $10.000 \times g$ for 10 min, washed twice in M9 medium, and finally suspended in 5 ml DNase buffer. After treatment with 10 U/ml DNase for 30 min at 37°C with shaking at 200 rpm, CFU counts were performed as described above.

Substrate degradation and oxygen uptake experiments

Substrate degradation experiments were performed with 10 ml cell suspensions in 100 ml Erlenmeyer flasks on a rotary shaker at 200 rpm for 4 h at 30°C. Experiments were started by addition of SDS (3.5 mM) or succinate (10 mM). Immediately after starting the experiments and at regular intervals thereafter, samples were withdrawn from the cell suspensions for substrate quantification, and were processed as described above. Oxygen uptake rates of cell suspensions were determined with a Clark type electrode at 30°C. For the measurements, 100 µl of a cell suspension ($OD_{600} = 5$) kept on ice was diluted with preheated (30°C) M9 medium in the reaction chamber. After a constant basic oxygen uptake rate was observed, SDS (3.5 mM), SDS plus succinate (10 mM), succinate, and the inhibitors (2 mM) were added to the cell suspension with syringes to a final volume of 500 µl.

Preparation of cell-free extracts and sulfatase assay

Cells were grown in M9 medium as described above and harvested by centrifugation at $10.000 \times g$ for 10 minutes at 4°C. Cells were washed twice with 50 mM Tris-HCl, pH 7.0 at 4°C, and finally resuspended in a small volume of this buffer. Cells were broken by 3 passages through a pre-cooled French Press (SLM Aminco; SLM Instruments) at 136 MPa. The homogenates were centrifuged at $20.800 \times g$ for 10 min at 4°C. The supernatants (cell-free extract) were transferred to a plastic tube and either used directly for the sulfatase assay or stored at -20°C. Alkyl sulfatase was measured discontinuously by determination of sulfate. Assays were performed in plastic tubes in a final volume of 1 ml at 30°C, containing 50 mM Tris-HCl pH 7.0, cell-free extract (ca. 0.5 mg protein), and were started by the addition of SDS (1 mM). Immediately after start of the assay and at regular intervals thereafter, samples (100 µl) were withdrawn and diluted with 100 µl 1 M NaOH to stop the reaction. These samples were stored at -20°C until further analysis.

Detection of oxidized proteins in cell-free extracts

100 ml suspensions of succinate-grown cells ($OD_{600} = 1$) were supplied with succinate plus SDS or with succinate only and incubated at 30°C with shaking (200 rpm). After 2 h, cell suspensions were harvested and washed twice with M9 medium. From a part of the cell suspensions supplied with SDS plus succinate, cell aggregates were removed from the suspended cells, and both fractions were harvested separately. All cell suspensions were then treated with DNaseI as described above and finally washed with M9 medium. Cell-free extracts were prepared as described above under anoxic conditions. Each extract was diluted with potassium phosphate buffer (3 mM, pH 7.2) to obtain samples containing identical amounts of protein. These samples were blotted (SRC 96 D Minifold I; Schleicher&Schuell) on a nitrocellulose membrane (Hybond-C super; Amersham). The

contents of oxidized proteins of cell-free extracts were detected with the Protein Oxidation Detection Kit (OxyBlot, Chemicon) following the manufacturer instructions.

Protein and DNA quantification

Protein was quantified with the advanced protocol of the BCA Protein Assay Kit (Pierce). DNA was quantified with a Hoefer DyNA Quant 200 (Amersham Pharmacia Biotech) after staining with the fluorescent dye Hoechst H33258. Lambda DNA (MBI Fermentas) was used as a standard. Differences in the AT-content of the standard DNA (50%) and the DNA of the samples (34% for strain PAO1) were considered for evaluation according to the manual. Samples for DNA determination were defrosted at 37°C for 10 min, cooled to room temperature, and incubated for 1 min with 0.1 µg/ml H33258 in 1 × TNE buffer in the dark prior to the measurements. Calibration with 0 and 100 ng/ml of the DNA standard was done regularly after two measurements

SDS quantification

SDS was quantified in culture supernatants with a modified Stains-all assay (Rusconi *et al.* 2001). Nine hundred µl of an adequately diluted sample (triplicates) were mixed with 900 µl Stains-all assay solution and incubated for 2 min in the dark prior to reading the absorption at 438 nm wavelength. New calibration curves (0-20 µM SDS) were acquired with each set of samples. Other compounds in culture supernatants of strain PAO1 grown with SDS or with SDS plus succinate did not interfere with this assay.

Succinate and sulphate quantification

Succinate was measured by ion-exclusion HPLC. The HPLC-system consisted of a high pressure pump (Sykam), an Aminex HPX-87H column (300X 7.8 mm; BioRad) at 40°C, a refraction index detector (ERC-7512, Sykam) and an autoinjector (Gilson 234; Abimed). As the eluent, 5 mM H₂SO₄ at a flow rate of 0.6 ml/min was used. Sulfate was measured by ion chromatography. The HPLC-system (Sykam) consisted of a high pressure pump (S 4260), an ion-exchange column (LCA-A03) at 30°C, a conductivity detector (S 3110) and an autoinjector (S 5200). As the eluent, a solution containing 5 mM Na₂CO₃, 50 mg/l 4-hydroxybenzotrile and 200 ml/l acetonitrile at a flow rate of 1.5 ml/min was used. The column was regenerated intermittently with 0.2 M H₂SO₄.

Microscopy, photography and image processing

For microscopic studies, an epifluorescence microscope (Axiophot; Zeiss) equipped with a cooled CCD camera (Magnafire; Intas) and respective filter sets for fluorescent dyes

(HQ 480/40, Q 505 LP, HQ 527/30 for SYTO9; HQ 545/30, Q 570 LP, HQ 610/75 for propidium iodide) was used. Photographs of microtiter plates and macroscopic aggregates were acquired with a digital camera. All images were processed with the computer software Magnafire and Paint Shop Pro 4.

5.4 □□ Results

Growth with and in the presence of SDS

P. aeruginosa strain PAO1 degraded SDS concomitantly with growth (Fig. 1a). When the maximal turbidity was reached, SDS was not detectable in the culture supernatant. About 2 h after inoculation, we observed an increasing number of whitish aggregates in the culture. Within the next 4 h, some of these aggregates grew larger (up to 1 cm in diameter). After 8 h, the turbidity of the culture increased and suspended cells started to grow exponentially. In this growth phase, biofilms formed at the air-liquid interface and at the bottom of the flask. Obviously, the biomass formed during SDS degradation was not homogeneously distributed. During sampling, we avoided removing large aggregates from the culture, and, thus, OD₆₀₀ does not reflect growth correctly. However, SDS degradation at the highest rate coincided with the increase of OD₆₀₀ during exponential growth. In cell-free extracts of SDS-grown cells, we detected SDS-dependent release of sulfate at a specific activity of 30 mU/mg protein. This alkyl sulfatase activity was not detectable in cell-free extracts of succinate-grown cells and in heat-inactivated extracts of SDS-grown cells.

When strain PAO1 was grown with a mixture of SDS and succinate, the two substrates were degraded sequentially (Fig. 1b). SDS degradation started only after succinate had been consumed completely. The growth rate decreased about 3-fold when SDS degradation commenced. We determined alkyl sulfatase activity also in cultures growing with a mixture of SDS and succinate at different time points. Parallel cultures were harvested after 9 and 15 h, and cell-free extracts were prepared. The specific sulfatase activity was 3.7 mU/mg protein after 9 h and 44 mU/mg protein after 15 h. This 10-fold increase was in accordance with the sequential degradation of succinate and SDS. Thus, strain PAO1 could grow in the presence of SDS indicating that degradation is not a prerequisite to survive exposure to SDS. Nevertheless, aggregates formed after 2 h in the same manner as with SDS as single substrate. During growth with succinate as sole substrate, no aggregate formation was observed.

P. aeruginosa could also grow with 1-dodecanol or lauric acid, the assumed first intermediates of SDS degradation (not shown). During growth with these compounds, no macroscopic aggregates were observed.

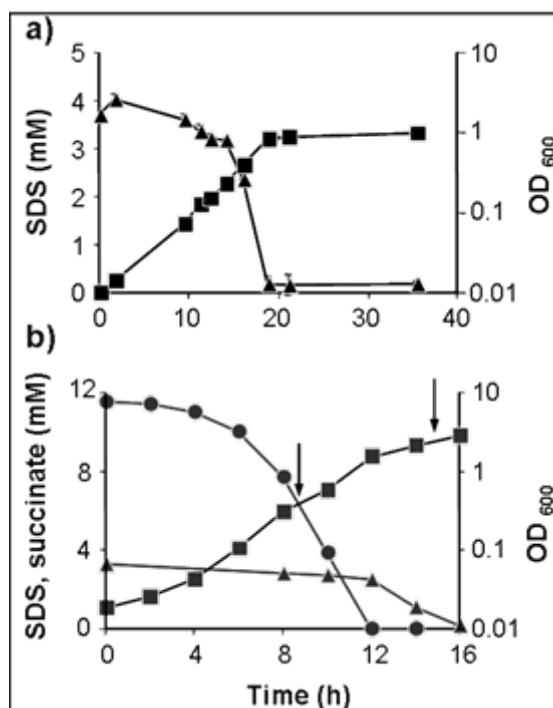


Fig. 1 Growth of *P. aeruginosa* strain PAO1 with SDS (▲) (a) or with a mixture of SDS (▲) and succinate (●) (b). Growth was measured as OD₆₀₀ (■). Arrows indicate the time points when samples were taken for determination of alkyl sulfatase activity.

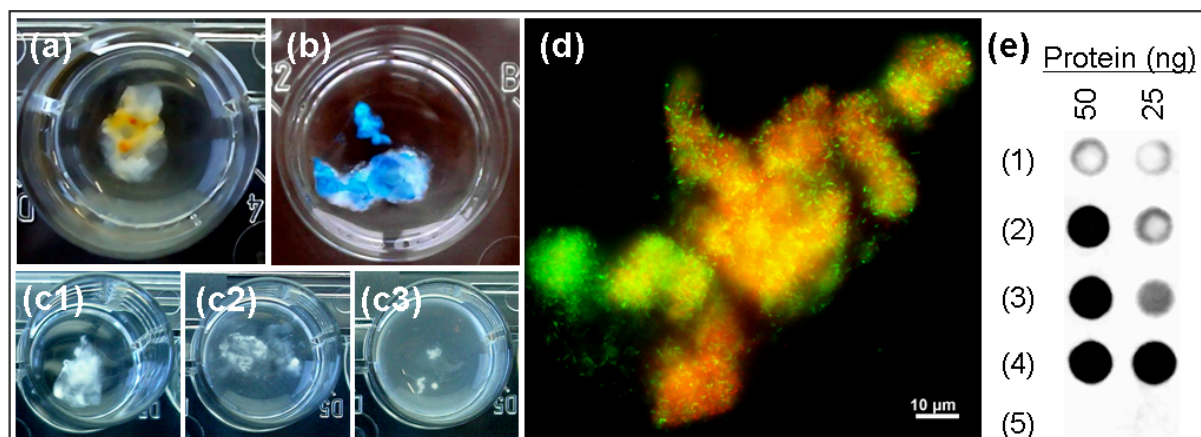


Fig. 2 Characterization of cell aggregates of *P. aeruginosa* strain PAO1 formed in SDS shock experiments. (a) Staining with 5-cyano-2,3-ditolyly tetrazolium chloride (CTC). (b) Staining with a 0.1% (w/v) Alcian Blue solution. (c) Incubation with DNase (10 U/ml). Pictures were taken after 0 min (c1), 10 min (c2), and 20 min (c3). (d) LIVE/DEAD-staining of microscopic aggregates. Damaged cells and free DNA are indicated by red fluorescence. Non-damaged cells are indicated by green fluorescence. (e) Dot-blot immunodetection of proteins oxidized by reactive oxygen species in cell-free extracts of succinate-grown cells with the Protein Oxidation Detection Kit (OxyBlot, Chemicon). Cell-free extracts were prepared from cell suspensions supplied with: (1) succinate, (Kovach *et al.*) succinate plus SDS (suspended plus aggregated cells), (3) succinate plus SDS (suspended cells), (4) succinate plus SDS (aggregated cells), (5) succinate plus SDS (non-derivatized negative control). Identical amounts of protein (50 ng and 25 ng) of each extract were blotted.

Characterization of macroscopic aggregates

Macroscopic aggregates that had formed after 6-8 h of growth with or in the presence of SDS were removed from growing cultures and subjected to staining and enzymatic treatments. During incubation with CTC, a major part of the aggregate turned reddish, indicating the presence of respiring cells (Fig. 2a). Parts of the aggregates were stained with Alcian Blue, indicating the presence of acidic polysaccharides (Fig. 2b). Treatment with alginate lyase could not disintegrate the aggregates. Upon treatment with DNase, the aggregates became smaller and the surrounding liquid became turbid within 20 min (Fig. 2c1-c3). Release of cells from the aggregate was shown by CFU counts that increased by about one order of magnitude from 10^7 to 10^8 CFU/ml during 30 min of incubation with DNase.

SDS shock experiments

A first step to elucidate the function of these cell aggregates during degradation of SDS was to investigate the sensitivity of *P. aeruginosa* to SDS. We tested how suspensions of SDS- and succinate-grown cells responded to the addition of SDS (3.5 mM) under different conditions. SDS caused a decrease of OD₆₀₀ from 1 to about 0.2 within 30 min to suspensions of SDS-grown cells during static incubations (Fig. 3a, panel 1). The OD₆₀₀ in the control suspension without SDS remained constant. During the experiment, the SDS-treated cell suspensions became more viscous. DNA (Fig. 3b, panel 1) and protein (Fig. 3c, panel 1) concentrations in the supernatant increased over time to about 2 µg/ml and 200 µg/ml, respectively. In control suspensions without SDS, the DNA concentration was below the detection limit (0.01 µg/ml), and the protein concentration remained constant below 10 µg/ml. After 45 min, cells were plated to determine CFU counts. SDS caused about 80% reduction of CFU compared to the control without SDS (Fig. 4a). Microscopic examination of cell suspensions after SDS shock revealed the formation of cell aggregates. Removing these aggregates by passing the cell suspensions through a filter with 5 µm pore size reduced the CFU counts of SDS-treated cells further while the control suspension without SDS was not affected by this treatment (Fig. 4a). If SDS-grown cell suspensions shocked with SDS were shaken (150 rpm), macroscopic aggregates formed that looked very similar to the aforementioned aggregates in growing cultures (Fig. 5a). After 60 min of incubation, a large aggregate formed, and the turbidity of the surrounding liquid decreased strongly. In controls without SDS, no such aggregation or clearance was observed.

To succinate-grown cell suspensions, SDS caused no or only a slight decrease in OD₆₀₀ (Fig. 3a, panel 2) and reduction in CFU/ml (Fig. 4b). The free DNA concentration in the SDS-treated cell suspensions was constant around 0.3-0.4 µg/ml while it was below the detection limit in the control without SDS (Fig. 3b, panel 2). The free protein concentration

(30 $\mu\text{g/ml}$) was higher than in the control without SDS (below 10 $\mu\text{g/ml}$), but remained constant over time (Fig. 3c, panel 2). If SDS was added together with succinate the cells reacted in a similar manner as SDS-grown cells reacted to addition of SDS (Fig. 3a-c, panel 2; Fig. 4b), including aggregation. The SDS-induced reduction of OD_{600} was observed at SDS concentrations of 3.4 and 1.7 mM for both SDS- and succinate-grown cells (in the presence of succinate), but not at 0.34 mM SDS. With 0.34 mM SDS, the cells did also not aggregate.

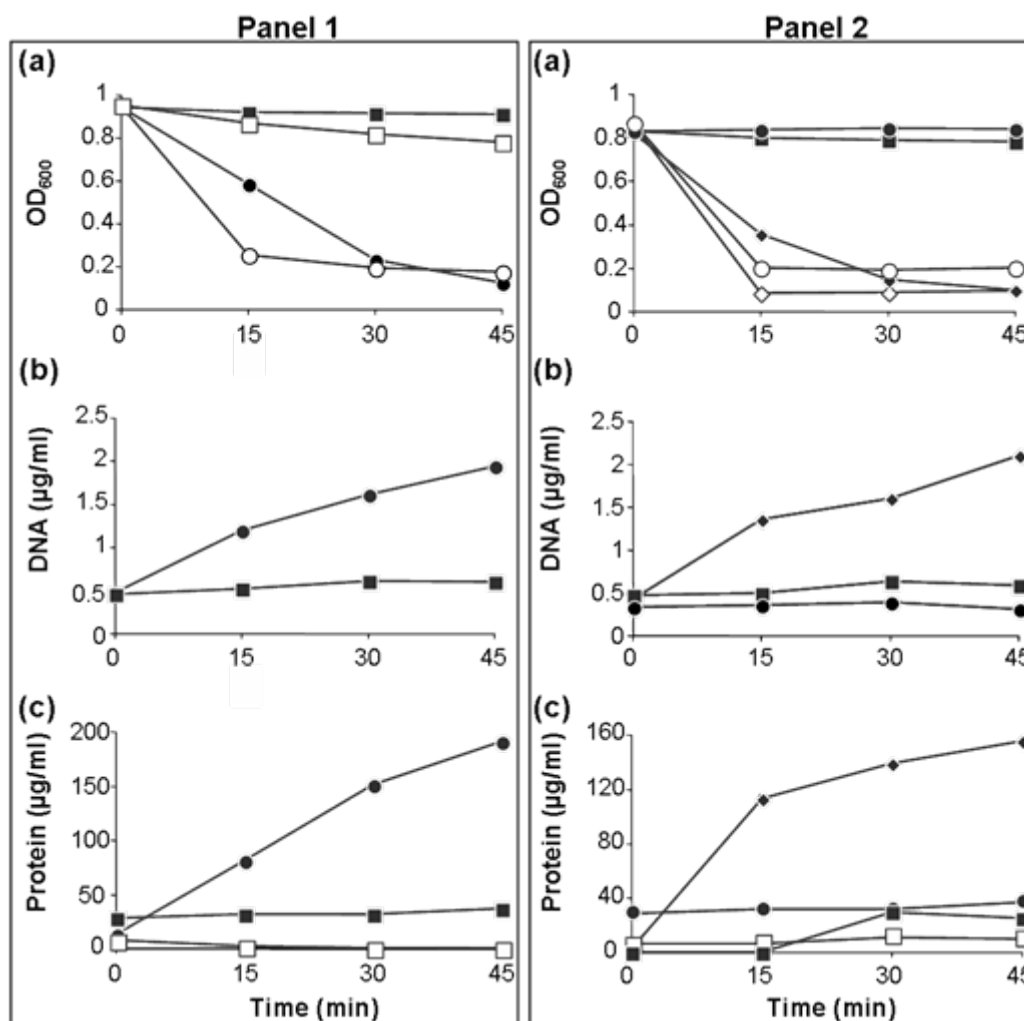


Fig. 3 Effect of SDS on optical density (a), DNA release (b) and protein release (c) of cell suspensions of *P. aeruginosa* strain PAO1 in the presence or absence of inhibitors.

Panel 1: Suspensions of SDS-grown cells were supplied with SDS in the absence (●) or in the presence of KCN (■) or CCCP (○). Controls (□) did not contain SDS or inhibitors.

Panel 2: Suspensions of succinate-grown cell were supplied with SDS (●), SDS in the presence of CCCP (◇), SDS plus succinate in the absence (◆), or in the presence of KCN (■) or CCCP (○). Controls (□) did not contain SDS or inhibitors. Concentrations of SDS, succinate, and inhibitors are given under Materials and Methods. In the absence of SDS, inhibitors and their respective solvents did not affect OD_{600} and release of DNA or protein

Characterization of cells from aggregates

Cell aggregates formed in SDS shock experiments could be stained and disintegrated in the same way as the aggregates from growing cultures described above. The cultivation efficiency (CFU/total cell counts) of cells released from aggregates by DNase treatment was 20% while the cultivation efficiency of those cells that remained in suspension after SDS shock was 100% and did not differ from the value before SDS shock. Staining with the LIVE/DEAD BacLight system (Fig. 2d) showed that the aggregates contained a mixture of intact cells (indicated by green fluorescence) and cells with damaged membranes (indicated by red fluorescence). 25% of the cells released from aggregates by DNase treatment stained red while only 1-3% of those cells that remained in suspension after SDS shock stained red. This value did not differ from the percentage of red cells found in cell suspensions before SDS shock. As an indication of damaged proteins, we determined the content of proteins oxidized by reactive oxygen species (ROS) because it has been postulated that proteins damaged by denaturing agents are more easily attacked by ROS (Nystrom 2005). The signal for such oxidized proteins was much higher in cell-free extracts of cells shocked with succinate plus SDS compared to cell-free extracts of cells supplied with succinate only (Fig. 2e, 1-2). In addition, we compared cells released from aggregates with those cells that had remained in suspension within the same experiment. Very clearly, the extracts of cells from the aggregates contained more oxidized proteins than the extracts of suspended cells (Fig. 2e, 3-4).

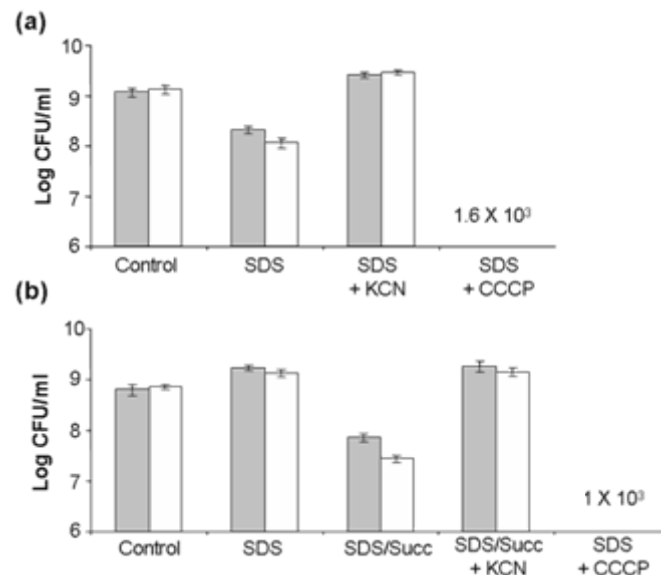


Fig. 4 Effect of SDS on CFU counts of cell suspensions of *P. aeruginosa* strain PAO1 in the presence or absence of inhibitors. After 45 min incubation, cell suspensions were either plated directly (grey bars) or after filtration through a polycarbonate membrane filter of 5 μm pore size to remove microscopic aggregates (white bars). Error bars indicate standard deviation ($n = 6$). (a) SDS-grown cells. (b) Succinate-grown cells. In the absence of SDS, inhibitors and their respective solvents did not affect the CFU counts compared to the control

SDS shock experiments in the presence of inhibitors and under anoxic conditions

In order to investigate the influence of energy supply on the response of strain PAO1 to SDS, we performed SDS shock experiments in the presence of inhibitors of respiratory ATP synthesis. In suspensions of SDS-grown cells exposed to SDS and in suspensions of succinate-grown cells exposed to SDS plus succinate, the presence of KCN (2 mM) prevented all SDS-dependent effects, namely the decrease in OD₆₀₀, the release of DNA and protein over time (Fig. 3a-c, panels 1-2), and the decrease in CFU/ml (Fig. 4a, b). Importantly, KCN also inhibited the formation of microscopic and macroscopic aggregates (Fig. 5b). NaN₃ (2 mM) had a similar but weaker effect in SDS shock experiments (not shown). To verify that both inhibitors really interfered with the energy metabolism we tested their effects on respiration and substrate degradation. NaN₃ decreased the substrate-dependent oxygen consumption of SDS-grown and succinate-grown cells by about 50% whereas KCN reduced the substrate-dependent oxygen uptake to a basal level that was measured in the absence of substrates (Table 1). Both inhibitors also reduced degradation of the respective substrates to the same extent as they inhibited respiration (not shown). To account for the influence of cyanide-insensitive respiration (Cunningham *et al.* 1997), we investigated a *cioB* mutant which is defective in the cyanide-insensitive cytochrome c oxidase Cio (Cooper *et al.* 2003). KCN inhibited the substrate-dependent oxygen uptake in this mutant completely (Table 1). In SDS shock experiments with this mutant strain, suspensions of SDS-grown cells responded with a decrease in OD₆₀₀ and reduction of CFU in a similar manner as the parental strain (Fig. 6a, b). Addition of KCN in the presence of SDS caused a rapid decrease in OD₆₀₀ and in CFU counts without aggregation (Fig. 6a, b; Fig. 5c). In the absence of SDS, KCN had no effect on OD₆₀₀ or CFU counts of this mutant.

Table 1. Oxygen uptake rates of cell suspensions (OD₆₀₀ = 1) of *P. aeruginosa* strain PAO1 and a *cioB* insertion mutant [32522] derived from strain MPAO1 measured with a Clark electrode. Cells were incubated with SDS (3.5 mM) or succinate (10 mM) at 30°C. KCN was added to a final concentration of 2 mM; ± indicates standard deviation (n = 3).

Strain	Growth substrate	Incubation substrate	Inhibitor	Oxygen uptake [μmol (L min) ⁻¹]
PAO1 WT	SDS	-	-	15.8 ± 5.9
	SDS	SDS	-	56.2 ± 5.9
	SDS	SDS	KCN	20.1 ± 5.7
PAO1 WT	Succinate	-	-	12.8 ± 2.3
	Succinate	SDS	-	9.6 ± 1.5
	Succinate	Succinate	-	102.6 ± 9.2
MPAO1 [32522]	Succinate	Succinate	KCN	23.3 ± 2.0
	SDS	-	-	11.0 ± 0.7
	SDS	SDS	-	51.2 ± 7.2
	SDS	SDS	KCN	0.7 ± 0.8

In the presence of CCCP (1 mM), SDS caused a rapid decrease in OD₆₀₀ and a dramatic decrease in CFU/ml to SDS-grown cells of strain PAO1 (Fig. 3a, panel 1; Fig. 4a). Succinate-grown cells were affected by CCCP and SDS in the same way, independent of the presence of succinate (Fig. 3a, panel 2). In CFU counts, no colonies could be detected in dilution steps higher than 10⁻². No macroscopic aggregates were formed when cell suspensions shocked with SDS in the presence of CCCP were shaken. The effect of CCCP could not be prevented by addition of KCN or NaN₃. In the absence of SDS, all inhibitors had no effect on OD₆₀₀ or CFU counts.

Under anoxic conditions, SDS caused a fast decrease in OD₆₀₀ and strong reduction of CFU counts to cells grown aerobically with SDS (Fig. 6c, d). Compared to oxic conditions, these effects were stronger and could not be prevented by the addition of KCN. Oxic control experiments with the same cells indicated that the increased sensitivity to SDS was not caused by the anoxic treatment. Macroscopic aggregates were never formed under anoxic conditions. Succinate-grown cells reacted with a strong decrease of OD₆₀₀ and CFU counts, independent of the presence of succinate (not shown).

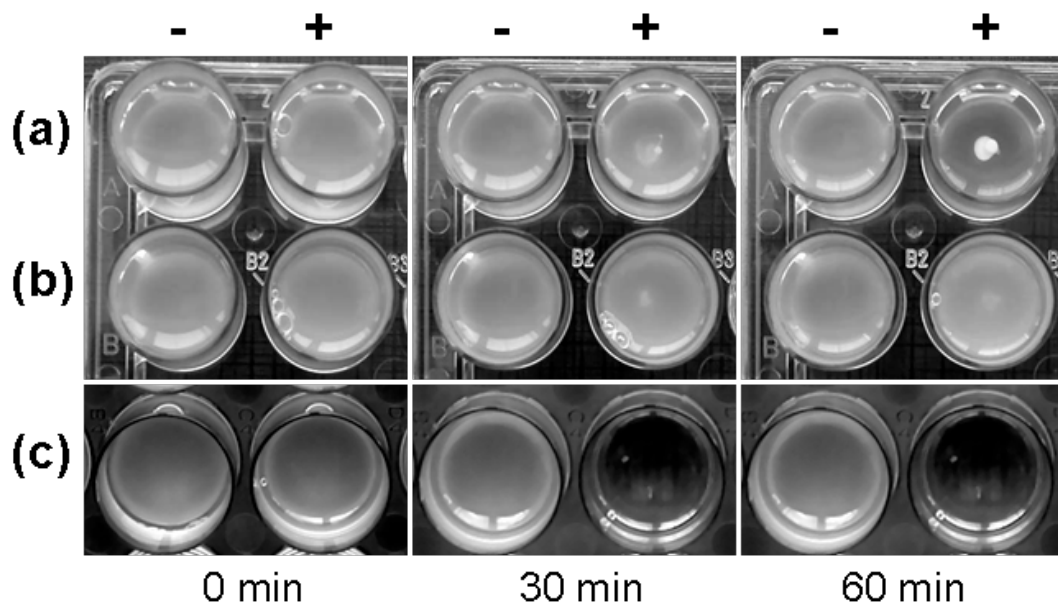


Fig. 5. Formation of macroscopic cell aggregates by SDS-grown cells of *P. aeruginosa* strain PAO1 (a-b) and a *cioB* mutant [32522] (c) derived from strain MPAO1 after SDS shock with shaking (150 rpm) in 24-well microtiter plates (15 mm in diameter; Nunc). Cell suspensions were supplied with SDS (+) in the absence (a) or in the presence of KCN (b, c). Controls did not contain SDS (-).

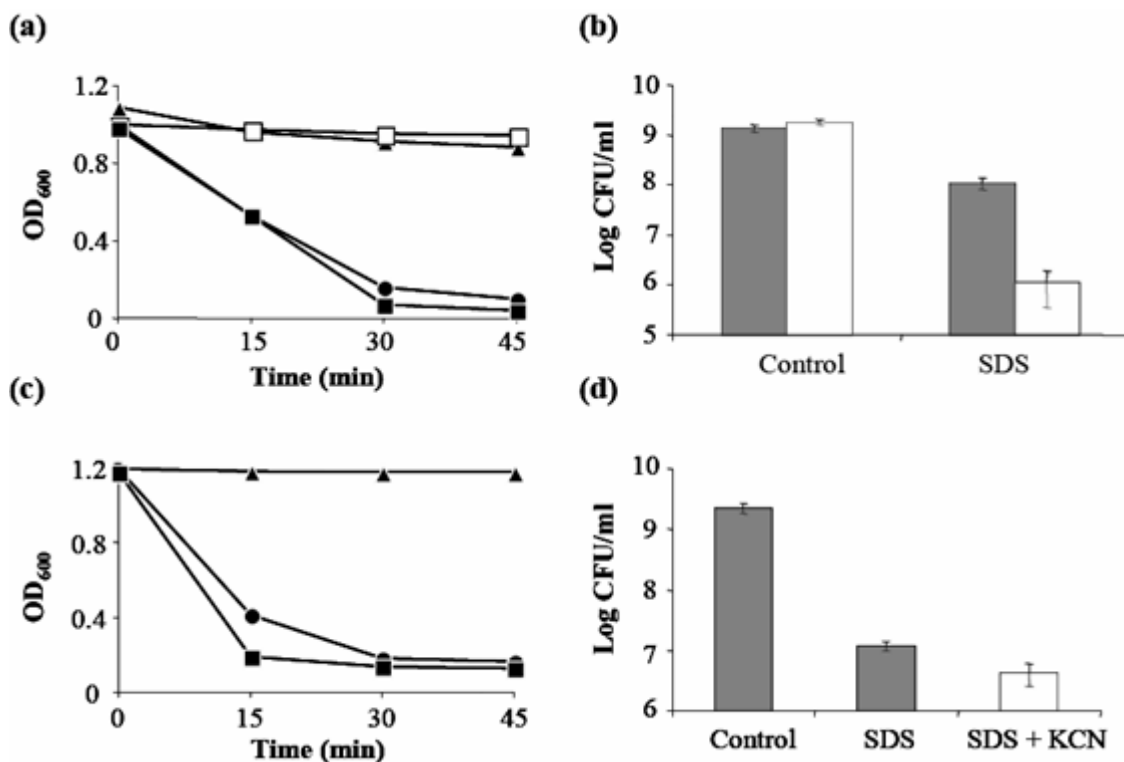


Fig. 6. Effects of SDS on suspensions of SDS-grown cells of a *cioB* insertion mutant [32522] derived from *P. aeruginosa* strain MPAO1 under oxic conditions (a-b), and of *P. aeruginosa* strain PAO1 under anoxic conditions (c-d). (a, c) OD₆₀₀: Cell suspensions were supplied with SDS (●) or SDS in the presence of KCN (■). Controls did not contain SDS in the absence (▲) or presence of KCN (□). (b, d) CFU counts after 45 min of incubations in the absence (grey bars) or in the presence of KCN (white bars). Error bars indicate standard deviation (n = 6).

Determination of survival rates of suspended cells and cells within aggregates after SDS shock in the presence of CCCP

As described above, suspended cells of strain PAO1 were readily killed during exposure to SDS in the presence of CCCP. To investigate whether cells in aggregates have a higher survival rate than cells that remained in suspension, we separated suspended cells and cells within aggregates from cultures grown with SDS and submitted them to a modified SDS shock experiment (see Materials and methods). Aggregates and suspended cells were exposed to SDS plus CCCP for 45 min. After removal of SDS and CCCP, aggregates and suspended cells were equally treated with DNase to obtain cell suspensions for CFU counts. The survival rate of suspended cells decreased by 3 orders of magnitude after exposure to SDS plus CCCP compared to the control that contained SDS only (Fig. 7). The survival rate of cells within aggregates decreased by less than one order of magnitude compared to the control. Thus, the survival rate of cells within aggregates treated with SDS plus CCCP was more than 100-fold higher than the survival rate of suspended cells.

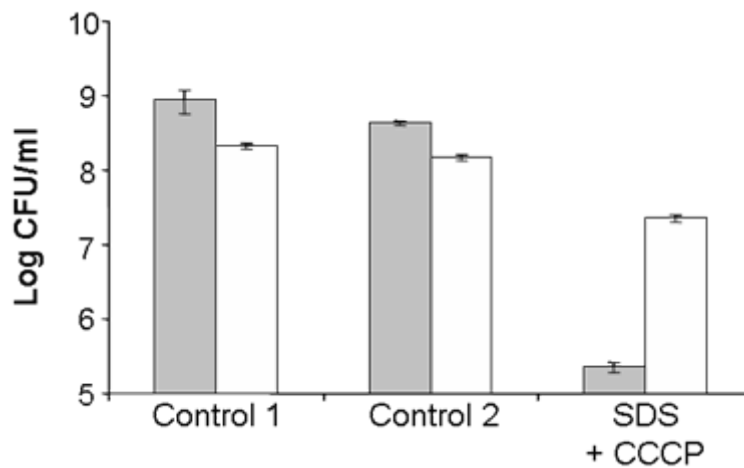


Fig. 7. Survival of suspended cells (grey bars) or cells within aggregates (white bars) of *P. aeruginosa* strain PAO1 after exposure to SDS (3.5 mM) plus CCCP (1 mM). Suspended and aggregated SDS-grown cells were submitted to a modified SDS shock experiment as described under Materials and methods. Controls contained methanol (solvent of CCCP) in the absence (Control 1) or presence (Control 2) of SDS. Error bars indicate standard deviation (n = 3).

5.5 Discussion

The aim of our study was to investigate the function of macroscopic cell aggregates that were formed by *Pseudomonas aeruginosa* strain PAO1 during growth with or in the presence of the toxic detergent SDS. Our hypothesis was that aggregation is an active process in response to the toxic effects of SDS. The key to test this hypothesis was to study aggregation of *P. aeruginosa* under defined conditions with cell suspension experiments. We found that the formation of these aggregates was strictly energy-dependent. *P. aeruginosa* formed aggregates only under conditions of high energy supply, but not at intermediate energy supply or when cells were completely deprived of energy. The latter situation was created by exposing aerobically grown cells of *P. aeruginosa* to SDS in the absence of oxygen or any other alternative electron acceptors like nitrate. In these experiments, the majority of the cells lysed indicated by a rapid drop of OD₆₀₀ and a strong decrease of CFU. This effect demonstrated the actual toxicity of SDS for *P. aeruginosa* and the requirement of energy-dependent resistance mechanisms to survive exposure to SDS. Rapid and strong lysis by SDS occurred also under oxic conditions if the uncoupling agent CCCP was present. This effect indicated the importance of the proton motive force and supported earlier observations that proton-dependent multidrug efflux pumps are involved in SDS resistance (Poole 2004). It must be emphasized that the formation of macroscopic aggregates was never observed with completely lysed cells because it demonstrates that these structures are not simply formed by cell debris agglutinated by cytoplasmatic DNA.

Conditions of intermediate energy supply were created by exposing succinate-grown cells, which were not induced for SDS-utilization, to SDS. Succinate-grown cells did not

aggregate upon SDS-exposure, and they were also no indications of cell lysis. According to our oxygen uptake measurements, these cells were capable of basal respiration which, obviously, generated sufficient energy for operating resistance mechanisms against SDS. This conclusion is supported by the fact that succinate-grown cells lysed in the presence of SDS when they were completely deprived of energy.

Conditions of high energy supply were given if SDS-grown cells were exposed to SDS or succinate-grown cells were exposed to SDS plus succinate. In both cases, the cells formed the same kind of macroscopic aggregates that were also observed in growing cultures. Obviously, the formation of aggregates was energy-dependent. This conclusion is supported by the fact that KCN inhibited aggregation. In the presence of this inhibitor, the cells respired at a low rate comparable to the level of intermediate energy supply described above. This basal respiration rate in the presence of cyanide was not observed in a *cioB* mutant indicating that respiration by cyanide-insensitive oxidases generated sufficient energy to survive exposure to SDS. Accordingly, the *cioB* mutant could not survive exposure to SDS when KCN was present.

The same conditions that caused aggregation rendered the cells also sensitive to SDS. In particular, the cells showed a response indicative of partial cell lysis (decrease of optical density and CFU, release of DNA and protein), and this response could also be inhibited by KCN. The coincidence of aggregation and increased sensitivity forces the question how they are linked. Both responses occurred under growth permitting conditions. Growing cells are likely to be more vulnerable because cell division involves re-arrangements of surface structures that may render sensitive parts of the cell more accessible to SDS. As outlined in the introduction, SDS causes damage to cells by interference with membranes and protein (Helenius and Simons 1975). We have clearly demonstrated that membrane-damaged cells, cells containing proteins oxidized by ROS, and cells with reduced cultivability strongly accumulated within the aggregate. Damage caused by SDS could be the trigger for the synthesis and the release of DNA and acidic polysaccharides which we detected in the EPS of the aggregates. Upon release of EPS, damaged cells formed microscopic aggregates that assembled to macroscopic aggregates. Such a scenario would be in agreement with the energy dependency of aggregation.

DNA has been found in EPS of *P. aeruginosa* before but its origin has not been clarified so far (Matsukawa and Greenberg 2004; Whitchurch *et al.* 2002). Delivery of DNA outside the cell could occur via the formation of membrane vesicles (Beveridge 1999; Kadurugamuwa and Beveridge 1995), and in electron micrographs of cells from macroscopic aggregates we actually observed such membrane vesicles (unpublished data). In addition, the DNA could originate from lysis or autolysis induced by stress (Webb *et al.* 2003). The acidic polysaccharides that we detected in the EPS were most

probably not alginate because the aggregates could not be disintegrated by alginate lyase. Recently, it has been shown that *P. aeruginosa* strains are capable of producing other polysaccharides than alginate (Friedman and Kolter 2004; Matsukawa and Greenberg 2004; Wozniak *et al.* 2003).

The energy-dependent formation of aggregates in which damaged cells accumulate suggest that aggregation is an active stress response of *P. aeruginosa* to toxic effects of SDS. A physiological function of this response could be to increase the chance of survival because cells within an aggregate are better protected against biocides (Gilbert *et al.* 2002; Lewis 2001). This interpretation is strongly supported by the observation that cells within aggregates were much less affected by exposure to SDS plus CCCP than suspended cells. Apparently, residing in aggregates conferred protection for cells against a treatment which was detrimental for suspended cells. Therefore, formation of aggregates could be a survival strategy of *P. aeruginosa* for growth with the toxic detergent SDS, particularly at the beginning of growth when the SDS concentration was still high. This strategy may also explain the formation of aggregates by another detergent-degrading bacterium, strain DS1, which commenced to grow with anionic detergents only in aggregates that formed around a solid support (Schleheck *et al.* 2000).

To verify our physiological results on the genetic level, we are in the process of identifying genes involved in SDS-induced aggregation. The global stress regulator RpoS (Joergensen *et al.* 1999; Suh *et al.* 1999) was apparently not involved in this response because a *rpoS* mutant did not differ from the parental strain in our SDS shock experiments (not shown).

5.6 □□ Acknowledgments

The authors appreciate experimental support from S. Weinitschke and valuable discussions with A. Cook and D. Schleheck. This study was supported by a grant of the Deutsche Forschungsgemeinschaft, (Bonn) to B.P. (PH71/2-1).

6 Detergent-induced cell aggregation in subpopulations of *Pseudomonas aeruginosa* as a pre-adaptive survival strategy

Janosch Klebensberger, Karin Lautenschlager, Daniel Bressler, Jost Wingender, Bodo Philipp

Environmental Microbiology, accepted for publication

6.1 Abstract

During growth of *Pseudomonas aeruginosa* strain PAO1 with the toxic detergent sodium dodecylsulphate (SDS), a part of the population actively formed macroscopic cell aggregates while the other part grew as freely suspended cells. The physiological function of aggregation for growth with SDS was investigated. Three mutants growing with SDS without aggregation were isolated: the spontaneous mutant strain N and two mutants with transposon insertions in the *psl* operon for exopolysaccharide synthesis. SDS-induced aggregation in strain N but not in a *pslJ* mutant was restored by complementation with two genes encoding diguanylate cyclases responsible for synthesis of cyclic-di-guanosine monophosphate (c-di-GMP). By expressing a c-di-GMP specific phosphodiesterase SDS-induced aggregation of strain PAO1 was reduced. Upon exposure to SDS in the presence of the uncoupler CCCP, the aggregating strains had a ca. 500-fold higher survival rates than the non-aggregating strains. Co-incubation experiments revealed that strain N could integrate into aggregates of strain PAO1 and thereby increase its survival rate more than 1000-fold. These results showed that SDS-induced aggregation involved c-di-GMP signalling with the *psl* operon as a possible target. Cell aggregation could serve as a pre-adaptive strategy ensuring survival and growth of *P. aeruginosa* populations in environments with multiple toxic chemicals.

6.2 Introduction

The ubiquitous bacterium *Pseudomonas aeruginosa* is characterized by a great metabolic versatility (Clarke 1982) and a high adaptability to cope with different environmental stress factors (Rodrigue *et al.* 2000). These traits support its successful colonization of hostile anthropogenic environments where it encounters toxic organic substances, such as detergents and disinfectants, some of which can be utilized as growth substrates. Examples for such environments are industrial wastewaters and healthcare settings where *P. aeruginosa* is a major cause for hospital infections (Bjarnsholt and Givskov 2006). Sinks for disposal of disinfectants and detergents have been repeatedly identified as environmental sources of several *P. aeruginosa* outbreaks in clinical settings (Muscarella 2004). Understanding survival and growth strategies of *P. aeruginosa* is thus a feasible approach for developing effective measures to prevent the establishment of this opportunistic pathogen in anthropogenic environments.

Recently, we have reported that *P. aeruginosa* strain PAO1 can grow with the anionic detergent SDS (Na-dodecylsulphate) as sole source of carbon and energy (Klebensberger *et al.* 2006). SDS is a common ingredient of many household products and very toxic to bacteria because it damages membranes and proteins (Helenius and Simons 1975). Bacteria require energy-dependent resistance mechanisms such as efflux pumps and Clp-proteases for growing in the presence of detergents (Poole 2004; Rajagopal *et al.* 2002). We have shown that SDS was also toxic for strain PAO1 and that surviving of SDS exposure required energy (Klebensberger *et al.* 2006). If SDS is used for growth, the cells have to find a trade-off for energy investment: to generate energy for growth, they have to uptake SDS, thereby risking to be injured. For protection and repair, they have to invest part of this energy which is consequently not available for growth. This dilemma calls attention to further adaptive strategies that allow growth with a toxic compound as sole source of carbon and energy. We reported that strain PAO1 formed macroscopic cell aggregates as an active, energy-requiring stress response to SDS. The formation of cell aggregates or biofilms is an important adaptive strategy of *P. aeruginosa* and other bacteria to colonize adverse environments because residing in such structures confers increased resistance to biocides such as antibiotics, disinfectants, and detergents (Drenkard and Ausubel 2002; Fux *et al.* 2005; Gilbert *et al.* 2002; Lewis 2001).

The formation of cell aggregates or biofilms requires the production of extracellular polymeric substances (EPS). The EPS of *P. aeruginosa* biofilms have been shown to contain DNA (Allesen-Holm *et al.* 2006; Steinberger and Holden 2005; Whitchurch *et al.* 2002) and different polysaccharides (Friedman and Kolter 2004; Linker and Jones 1964; Tielen *et al.* 2005; Ude *et al.* 2006). The EPS of the macroscopic aggregates formed by

strain PAO1 during growth with SDS contained acidic polysaccharides and DNA (Klebensberger *et al.* 2006). By treatment with DNase, these aggregates were disintegrated and viable cells were released. The formation of cell aggregates and biofilms in *P. aeruginosa* and other bacteria has been shown to involve cyclic-di-guanosine monophosphate (c-di-GMP) signalling (Jenal and Malone 2006; Römling *et al.* 2005). This intracellular second messenger molecule was originally found in *Gluconacetobacter xylinus* where it acts as an allosteric regulator of cellulose synthase (Ross *et al.* 1986). C-di-GMP biosynthesis from 2 GTP is catalyzed by diguanylate cyclases (DGCs) containing a characteristic GGDEF-domain as the active center (Chan *et al.* 2004; Ryjenkov *et al.* 2005). The hydrolysis of c-di-GMP is catalyzed by specific phosphodiesterases (PDEs) containing either an EAL or a HD-GYP domain (Christen *et al.* 2005; Ryan *et al.* 2006). *P. aeruginosa* harbors 17 genes with a GGDEF-domain, 6 genes with an EAL-domain, and 14 genes containing both domains. A function in cell aggregation or biofilm formation has been demonstrated only for a few of them so far (D'Argenio *et al.* 2002; Drenkard and Ausubel 2002; Hickman *et al.* 2005; Hoffman *et al.* 2005).

We proposed that aggregate formation of strain PAO1 is a survival strategy for growth with the toxic detergent SDS. This hypothesis was supported by the fact that aggregated cells of strain PAO1 had a more than 100-fold higher survival rate than freely suspended cells if exposed to SDS in the presence of a second toxic compound, carbonyl cyanide chlorophenylhydrazone (CCCP), which deprives the cells of energy by dissipating the proton motive force (Klebensberger *et al.* 2006). However, in the cultures growing with SDS, the cells were not homogeneously distributed as they grew in aggregates and as freely suspended cells. This observation forces the question for the exact physiological role of cell aggregation during growth with SDS. As aggregate formation strictly preceded growth of suspended cells, we hypothesized that cell aggregation is important for initiating growth of strain PAO1 with SDS. If cell aggregation would not be essential for growth with SDS, it should be readily lost because it is energy-requiring. In this case non-aggregating mutants would arise and successfully compete against cells that maintain the aggregative phenotype. If cell aggregation is essential such non-aggregating mutants should not arise unless they are more resistant to SDS. Attempts to isolate spontaneous non-aggregating mutants of *P. aeruginosa* strain PAO1 were the starting point of our study to define the physiological role of cell aggregation during growth with the toxic detergent SDS.

6.3 □□ Materials and methods

Table 1. Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source or reference
<i>P. aeruginosa</i>		
PAO1	Wild-type of strain PAO1, (RH)*	Holloway collection
N	Spontaneous mutant of strain PAO1, (SH) [†]	This study
PAO1-Tn7- <i>yfp</i>	PAO1 with Tn7 chromosomal insertion of <i>yfp</i> , (RH)*	This study
N-Tn7- <i>cfp</i>	N with Tn7 chromosomal insertion of <i>cfp</i> , (SH) [†]	This study
PAO1-D1	Mariner transposon mutant of strain PAO1, insertion at position 596 of gene <i>pslF</i> (1178 bp), (SH) [†]	This study
PAO1-D4	Mariner transposon mutant of strain PAO1, insertion at position 1118 of gene <i>pslJ</i> (1437 bp), (SH) [†]	This study
PAO1-KO[1107]	PAO1, PA1107::pKO[1107], (RH)*	This study
PAO1-KO[1727]	PAO1, PA1727::pKO[1727], (RH)*	This study
PAO1-KO[4929]	PAO1, PA4929::pKO[4929], (RH)*	This study
<i>E. coli</i>		
JM109	<i>endA1 recA1 gyrA96 thi hsd R17 (r_K⁻, m_K⁺), relA1 supE44 Δ(lac-proAB) [F' traD36 proAB⁺ lacI^q lacZΔM15]</i>	Promega
SM10::λpir	<i>thi1 thr1 leuB6 supE44 tonA21 lacY1 recA::RP4-2-Tc::Mu Km^r λpir</i>	(Miller and Mekalanos 1988)
HB101	<i>thi-1 hsd S20 (r_B⁻ m_B⁻) supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20 (str^r) xyl-5 mtl-1 galK2</i>	Promega
S17-1	<i>thi pro hsdR hsdM⁺ recA RP4-2-Tc::Mu-Km::Tn7</i>	(Simon 1983)
Plasmids		
pUCP18	<i>Escherichia-Pseudomonas</i> shuttle vector, (Ap ^r)	(West <i>et al.</i> 1994)
pKnockout-G	Suicide vector used for gene inactivation, (Ap ^r , Gm ^r)	(Windgassen <i>et al.</i> 2000)
pALMAR3	Plasmid with mariner transposon (Tet ^r)	Jenal lab
pBK-miniTn7(gm) P _{A1/04/03} - <i>eyfp-a</i>	Plasmid for chromosomal integration of <i>yfp</i> , (Ap ^r , Gm ^r , Cm ^r)	(Klausen <i>et al.</i> 2003b)
pBK-miniTn7(gm) P _{A1/04/03} - <i>ecfp-a</i>	Plasmid for chromosomal integration of <i>cfp</i> , (Ap ^r , Gm ^r , Cm ^r)	(Klausen <i>et al.</i> 2003b)
pBBR1MSC-5	Broad-host-range cloning vector, (Gm ^r)	(Kovach <i>et al.</i> 1995)
pBBR[CC3396]	pBBR1MSC-5 containing CC3396 from <i>C. crescentus</i>	Jenal lab
pUCP18[1107]	pUCP18 harboring a SmaI fragment (2008 bp) encoding PA1107	This study
pUCP18[1727]	pUCP18 harboring a EcoRI-HindIII fragment (2476 bp) encoding PA1727	This study
pUCP18[4929]	pUCP18 harboring a Sall fragment (2426 bp) encoding PA4929	This study
pKO[1107]	pKnockout-G harboring an internal EcoRI fragment (883 bp) of PA1107	This study
pKO[1727]	pKnockout-G harboring an internal HincII fragment (702 bp) of PA1727	This study
pKO[4929]	pKnockout-G harboring an internal BamHI-PstI fragment (908 bp) of PA4929	This study
pUX-BF13	Plasmid providing the Tn7 transposase genes, (Ap ^r)	(Bao <i>et al.</i> 1991)
pRK 600	<i>ori ColE1 RK2-Mob⁺ RK2-Tra⁺</i> , (Cm ^r)	(Kessler <i>et al.</i> 1992)

* (RH) indicates rough colony morphology on SDS-containing agar

[†] (SH) indicates smooth colony morphology on SDS-containing agar

Bacterial strains and growth media

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultivated in Luria Bertani medium or in a modified M9 mineral medium supplied with 3.5 mM SDS or 10 mM Na-succinate as carbon and energy sources as described previously (Klebensberger *et al.* 2006). Colony morphologies for each *Pseudomonas aeruginosa* strain are indicated in Table 1 as rough (RH) or smooth (SH). Plasmid harboring *Escherichia coli* strains were selected and maintained on LB plates containing 100 µg/ml ampicillin (Fluka), 15 µg/ml gentamycin, 10 µg/ml tetracycline (Fluka), or 10 µg/ml kanamycin (Fluka). Plasmid-harboring *P. aeruginosa* strains were selected on Pseudomonas isolation agar (PIA; Difco) containing 200 µg/ml carbenicillin (Sigma), 120 µg/ml gentamycin, or 160 µg/ml tetracycline. In liquid M9 medium, the concentrations of carbenicillin and gentamycin were decreased to 50 µg/ml and 10 µg/ml, respectively.

Growth experiments and swarming assay

Growth experiments with *P. aeruginosa* were performed as described previously (Klebensberger *et al.* 2006). For determination of molar growth yields (Y) with SDS, cultures were incubated until onset of the stationary phase. Cells were harvested immediately by centrifugation at $15.000 \times g$ for 10 min at 7°C. To obtain the complete biomass, biofilms attached to the glass surface were scraped off and added to the fraction of suspended cells. The cells were washed once with 30 ml distilled water, resuspended in a small volume of distilled water, transferred to weight-constant test tubes, and centrifuged at $2057 \times g$ for 10 min at 7°C. Supernatant were decanted and cell pellets were completely dried at 70°C. After cooling the tubes in a desiccator, the dry weights were determined. SDS concentrations at the beginning and at the end of growth were determined as described previously (Klebensberger *et al.* 2006).

Swarming motility was determined as described previously (D'Argenio *et al.* 2002).

Gene library construction and complementation of mutants

Genomic DNA of strain PAO1 was purified (Puregene DNA Isolation Kit; Gentra) and partially digested with *Sau3A*I. DNA fragments between 1 and 8 kb were extracted and purified (E.Z.N.A Gel Extraction Kit, Peqlab) from agarose gels and ligated into the *Bam*HI restriction site of pUCP18 (West *et al.* 1994). Competent *E. coli* JM109 cells (Promega) were transformed with these plasmids and submitted to ampicillin selection and blue-white screening. 6000 positive clones were transferred into 96 well plates by pooling 5 clones per well in 200 µl LB with ampicillin and grown with shaking at 200 rpm for 24 h at 37°C. All pools of transformants were combined at equal volumes, washed twice in LB medium, and frozen as glycerol stocks (40% v/v) in liquid nitrogen. The plasmids from this gene library were

extracted (peqGold Plasmid Miniprep Kit I; Peqlab) and transformed into *P. aeruginosa* strain N (Irani and Rowe 1997). Transformants were screened on M9 agar plates containing 0.15% SDS and 125 µg/ml carbenicillin. Clones with rough colony morphology were further tested for SDS-induced aggregation in 3 ml M9 medium containing 0.1% SDS in small petridishes (3.5 cm in diameter; Nunc) on a rotary shaker at 120 rpm (Orbital Incubator S150, Stuart Scientific) for 18 h at 30°C. Plasmids that restored aggregation of strain N in liquid media were isolated; the chromosomal fragments were sequenced and identified by comparison with the *Pseudomonas* Genome Project database (<http://v2.pseudomonas.com/>).

Construction of plasmids and insertional mutants

To construct plasmids pUCP18[1107], pUCP18[1727], and pUCP18[4929], the genes PA1107, PA1727, and PA4929 were excised as fragments from the inserts of complementing plasmids as *Sma*I, *Eco*RI-*Hind*III, and *Sal*I fragments, respectively, and cloned into pUCP18 digested with the respective enzymes. To construct the plasmids pKO[1107], pKO[1727], and pKO[4929] internal fragments (indicated in Table 1) of PA1107, PA1727, and PA4929 were cloned into the respective restriction sites of the suicide vector pKnockout-G (Windgassen *et al.* 2000). The resulting plasmids were transferred in strain PAO1 by bi-parental mating (see below) with *E. coli* strain S17-1 as donor. Correct chromosomal insertion of the vectors was confirmed by PCR with appropriate primers (Table 2).

Transposon mutagenesis

For random transposon mutagenesis of strain PAO1 the vector pALMAR3 carrying a mariner transposon (Lampe *et al.* 1999) with a tetracycline resistance gene was used (kindly provided by Urs Jenal). For bi-parental matings, *E. coli* S17-1 harboring pALMAR3 (donor) was grown in LB medium with shaking at 150 rpm at 37°C, while strain PAO1 (recipient) was grown in LB medium with shaking at 50 rpm at 42°C. After incubation overnight, 5×10^8 cells of the donor and 1×10^9 cells of the recipient were harvested by centrifugation at 10,000 rpm for 1.5 min (5415 D; Eppendorf), washed twice in 2 ml pre-warmed LB medium, and finally resuspended in 50 µl LB medium. Donor and recipient were carefully mixed by pipetting and spread onto sterile membrane filters (OE66 0.2 µm 25 mm diameter; Schleicher&Schuell) that were placed on pre-warmed LB agar plates. After incubation for 6 h at 37°C, the filters were transferred to a 50 ml plastic tube (Greiner) containing 2 ml NaCl (0.9%). After vortexing, aliquots of the cell suspensions were spread on PIA agar plates containing 160 µg/ml tetracycline to select for transposon mutants of strain PAO1. After incubation for 48 h at 37°C, all colonies (~ 20,000) were scraped off, transferred into a plastic

tube containing 25 ml NaCl (0.9%, w/v), washed twice, and finally resuspended in 4 ml LB medium.

Transposon mutants with smooth colony morphology on M9 agar plates containing 0.15% SDS and 80 µg/ml tetracycline were further screened for SDS-induced aggregation in liquid culture. In non-aggregating mutants, the exact position of the transposon insertion was identified by inverse PCR with primers indicated in Table 2 in cooperation with Trenzyme GmbH (Konstanz). Transposon insertion sites were identified by comparison with the *Pseudomonas* Genome Project database. Strains PAO1-Tn7-yfp and N-Tn7-cfp were constructed through site-directed transposon insertion into the chromosome via four-parental mating as described previously (Klausen *et al.* 2003b).

SDS shock experiments

SDS shock experiments with cell suspensions of single cultures of different *P. aeruginosa* strains were performed as described previously (Klebensberger *et al.* 2006). In SDS-shock experiments with mixed cell suspensions of different *P. aeruginosa* strains, 500 µl from cell suspensions (OD₆₀₀ = 1.5) of two different strains were combined in one well of a 24-well microtiter plate (Nunc Surface; Nunc), supplied with SDS (3.5 mM), and incubated on a rotary shaker at 200 rpm and 30°C for 45 min. After macroscopic aggregates had formed, they were separated from the suspended cells. For this, aggregates were transferred into 30 ml of DNase buffer (50 mM Tris-HCl with 10 mM MgCl₂ at pH 7.2) with a pipette, harvested by centrifugation at 80 × g for 5 min, washed twice, and finally resuspended in 3 ml DNase buffer. Aggregates were disintegrated by treatment with DNase-I (Type II, stock solution in water; Sigma) for 30 min at 37°C. Cells released from aggregates and cells that had remained in suspension during SDS shock were quantified by CFU counts as described previously (Klebensberger *et al.* 2006). As controls, single cultures of the individual strains were submitted to the same procedure in parallel.

To determine the survival rates of strains in single or mixed culture after exposure to SDS plus CCCP (Sigma), SDS shock experiments were set up as described above. After 45 min, cell suspensions were supplied with CCCP (1 mM) and incubated for additional 60 min with shaking at 75 rpm. Then, the complete cell suspensions (Klebensberger *et al.*) were transferred into 30 ml DNase-buffer and harvested by centrifugation at 10.000 × g for 8 min at room temperature. After washing, the cells were resuspended in DNase-buffer in a final volume of 3 ml and treated with DNaseI for 30 min at 37°C. CFU counts were determined as described above. Cell suspensions with methanol were used as controls.

Single colonies of the individual strains from experiments with mixed cultures were identified by their colony morphology or by their fluorescence. To identify strains by their colony morphology, 50 µl aliquots of appropriately diluted cell suspensions were spread onto

agar plates containing M9 medium containing 0.15% SDS. After incubation for 2 days at 37°C rough and smooth colonies could be unambiguously differentiated and counted. To identify YFP and CFP-tagged strains, 50 µl aliquots of appropriately diluted cell suspensions were spread onto LB agar plates containing the respective antibiotics. At least 60 single colonies were picked and grown in 200 µl LB medium in 96 well plates with shaking at 200 rpm at 37°C. After 14 h of incubation, the OD595 and the relative fluorescence (RFU) at 535 nm (excitation at 485 nm) were measured with a microtiterplate reader (GENIOS; Tecan). The strains could be unambiguously differentiated by their ratio of RFU/OD595 because YFP-tagged strains showed a significantly higher RFU/OD595 than CFP-tagged strains. The RFU/OD595 ratio of the latter was in the same range as that of untagged strains.

All SDS shock experiments were performed in triplicates and reproduced in at least two independent runs.

Other analytical methods

Determination of sulfatase activity and protein concentration in cell-free extracts were performed as described previously (Klebensberger *et al.* 2006).

For pyocyanine determination, aliquots (5 ml) of supernatants from SDS-grown cultures from early stationary phase were extracted with chloroform (3 ml) in a plastic tube (Sarstaedt). After centrifugation at 2057 × g for 2 min, 2 ml of the chloroform phase was transferred to a new tube and subsequently mixed with 1 ml of 0.2 M HCl. Pyocyanine was determined by measuring the absorbance of the aqueous phase at 520 nm (A520).

Confocal laser scanning microscopy and macroscopic images

Macroscopic aggregates of *P. aeruginosa* cells labelled with CFP and YFP were placed on glass slides and fixed by embedding them in polyacrylamide (13%). Microscopic investigation was performed either directly or after staining of the bacterial aggregates with the DNA-binding fluorochrome SYTO 9 (Molecular Probes) and the lectin concanavalin A labelled with the fluorescent dye tetramethyl rhodamine isothiocyanate (TRITC-ConA; Sigma). Samples were stained by layering a solution containing TRITC-ConA (10 µg/ml) and SYTO 9 (1.5 µl/ml) in deionized water on top of the aggregates, incubating them statically for 20 min in the dark, and washing them twice in deionized water. Microscopic examination was performed with a LSM 510 confocal laser scanning microscope (Zeiss, Germany), consisting of a laser scanning module that was mounted on an Axiovert 100 M BP inverted microscope (Zeiss) equipped with the following objectives (Zeiss): Plan-Neofluar 10×/0.30, LD-Achroplan 40×/0.60 Korr, and LD-Achroplan 63×/0.75 Korr Ph2. An argon laser and a helium-neon laser were applied for the analysis of the samples. Images of CFP-labelled cells were recorded at

an excitation wavelength of 458 nm and an emission wavelength of 475-515 nm; images of YFP-labelled cells were recorded at an excitation wavelength of 488 nm and emission wavelength > 515 nm, using a LP 505 nm long-pass detection filter. Aggregates stained with SYTO 9 and TRITC-ConA were visualized at an excitation wavelength of 488 nm and an emission wavelength range of 505-550 nm provided by a BP 505-550 band-pass filter (SYTO 9) and at an excitation wavelength of 543 nm and an emission wavelength > 570 nm using an LP 560 long-pass filter (TRITC-ConA). Image recording of optical thin sections was performed with the LSM software (version 3.2 SP2, Zeiss). Image analysis including data processing and three-dimensional reconstruction was performed using the AxioVision software version 3.1 (Zeiss). Macroscopic images of colonies, SDS-induced aggregation, and swarming plates were taken with a Canon Powershot G6 camera. Images were processed with Paint Shop Pro 4.

Table 2. Primer used in this study.

Primer	Sequence
Chromosomal insertion analysis	
pKO-G	5'-GCGCGTTGGCCGATTCATTA-3'
PA1107-Check-R	5'-CTGGTCGGCGGCGCTGTAGAG-3'
PA1727-Check-R	5'-CCGGCAGGCGTTTGTAGATACAG-3'
PA4929-Check-R	5'-CTGGTCGGCGGCCTTGTAGAGTTT-3'
Tn7-GlmS	5'-AATCTGGCCCAAGTCGGTGAC-3'
Tn7R109	5'-CAGCATAACTGGACTGATTCAG-3'
Transposon insertion analysis	
Marseq-F	5'-TGAATGCGCAAACCAACCCTTGGC-3'
Marseq-R	5'-GGAAACAGCTATGACCATGATTACGCC-3'
Sequence analysis	
PA1107-F	5'-GACGCGCCGGAGCCCTGTTCG-3'
PA1107-R	5'-GAGCGCCACGGACCATCTTA-3'
PA1727-F	5'-GGGAATTCCATGAGACGCACCTCCTGT-3'
PA1727-R	5'-GCGATGGGCGAATCTGAAGCTTCTGAC-3'
PA4929-F	5'-GAAGGGCGCCGGACCGAAACACTC-3'
PA4929-R	5'-TGCGGCCGGGAATCATGCTCTACG-3'

6.4 □□ Results

Isolation and characterization of strain N

To investigate whether spontaneous non-aggregative mutants of strain PAO1 would enrich in liquid cultures, freely suspended cells from cultures growing with SDS in the early logarithmic phase were repeatedly transferred into fresh medium. After 15 transfers we obtained a culture that contained only few and small aggregates. From this culture, aliquots were spread on solid M9 medium with SDS as carbon and energy source. We observed

different colony morphologies, namely rough and highly structured colonies as well as smooth, soft, and unstructured colonies. The parental strain PAO1 formed rough colonies on SDS-containing agar plates (Fig. 1b1) and cell aggregates during growth with SDS in liquid culture (Fig. 1c1). With succinate as carbon and energy source, strain PAO1 formed smooth colonies on agar plates (Fig. 1a1) and did not aggregate in liquid cultures (not shown). One strain forming smooth colonies on SDS-containing agar plates, strain N, was characterized further. Strain N formed smooth colonies irrespective of the substrate (Fig. 1ab2) and did not aggregate in liquid cultures with SDS (Fig. 1c2). This phenotype was stable without the appearance of revertants after several passages in media without SDS. Therefore, we consider strain N as a spontaneous mutant of strain PAO1 that does not aggregate during growth with SDS.

Strain N reached a higher OD600 than the suspended cells of strain PAO1 (Fig. 2a) but their molar growth yields did not differ significantly ($Y = 163 \pm 16$ g dry mass/mol SDS for strain N and $Y = 196 \pm 30$ g dry mass/mol SDS for strain PAO1). The growth rate of strain N with SDS ($\mu = 0.395 \text{ h}^{-1}$) was higher than the growth rate of those cells of strain PAO1 that grew in suspension ($\mu = 0.3081 \text{ h}^{-1}$). To test whether the increased growth rate of strain N was caused by a higher specific activity of enzymes involved in SDS degradation we determined the activity of SDS-alkyl sulfatase which catalyses the first step of SDS degradation (Hagelueken *et al.* 2006). The specific activities of SDS alkyl sulfatase in cell-free extracts of both strains were not significantly different ($25 \pm 3 \text{ mU/mg}$ protein for strain N and $31 \pm 3 \text{ mU/mg}$ protein for strain PAO1).

During growth with SDS, strain PAO1 produced about 50% more pyocanine than strain N (Fig. 2b). As a further difference, strain N had a higher swarming motility than strain PAO1 (Fig. 1d1-2) whereas no differences were observed in swimming motility (not shown).

To investigate whether strain N was more resistant to SDS, we submitted it to the same shock experiments as described previously for strain PAO1 (Klebensberger *et al.* 2006). In cell suspensions of strain N, SDS (3.5 mM) caused a decrease in OD600 and CFU counts as well as a release of DNA and protein into the medium (not shown). These responses to SDS were indistinguishable from the responses of suspended cells of strain PAO1. As also observed earlier with strain PAO1, energy limitation by addition of KCN (2 mM) prevented all responses to SDS (not shown) while complete deprivation of energy by the addition of CCCP (1 mM) in the presence of SDS caused a rapid and complete lysis of the cells (Fig. 3). These results gave no indication that strain N was more resistant to SDS than suspended cells of strain PAO1. The only striking difference in these SDS shock experiments was that strain PAO1 formed macroscopic aggregates while strain N did not.

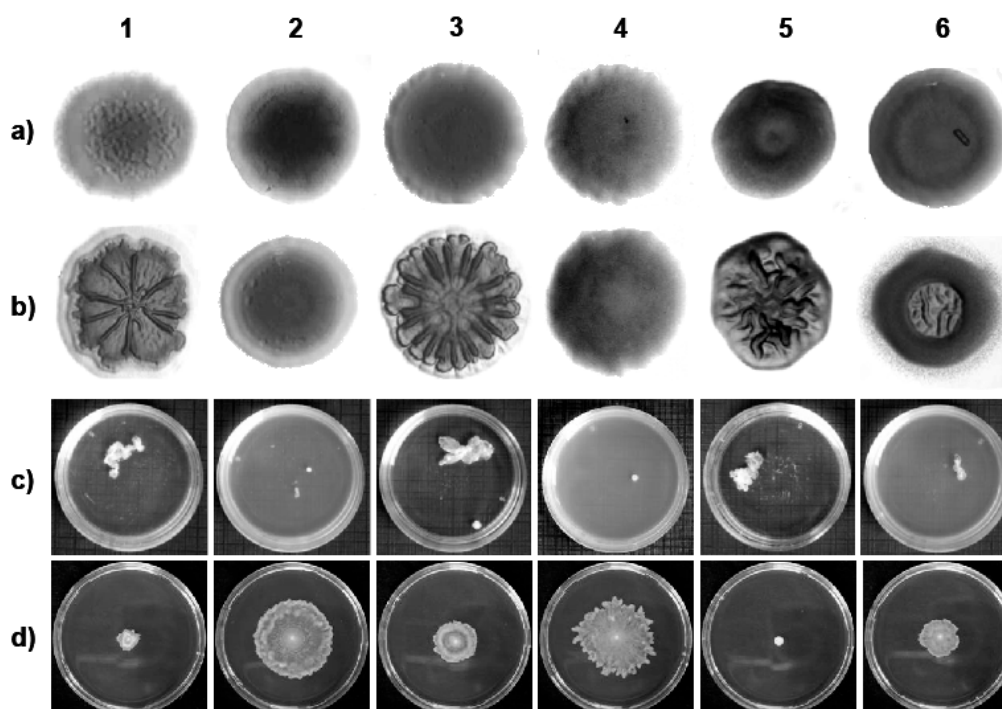


Fig. 1. Characterization of different *Pseudomonas aeruginosa* strains. a) Colony morphology on M9 containing 10 mM succinate after incubation for 1 d at 37°C. b) Colony morphology on M9 containing 0.15% SDS after incubation for 3 d at 37°C. c) Growth in liquid M9 medium containing 0.1% SDS after incubation for 18 h at 30°C with shaking at 120 rpm. e) Swarming motility on semi solid agar after incubation for 24 h at 37°C. The different strains are indicated by the following numeration: 1) PAO1 pUCP18, 2) N pUCP18, 3) N pUCP18[4929], 4) PAO1-D4 pUCP18, 5) PAO1 pBBR, 6) PAO1 pBBR[CC3396].

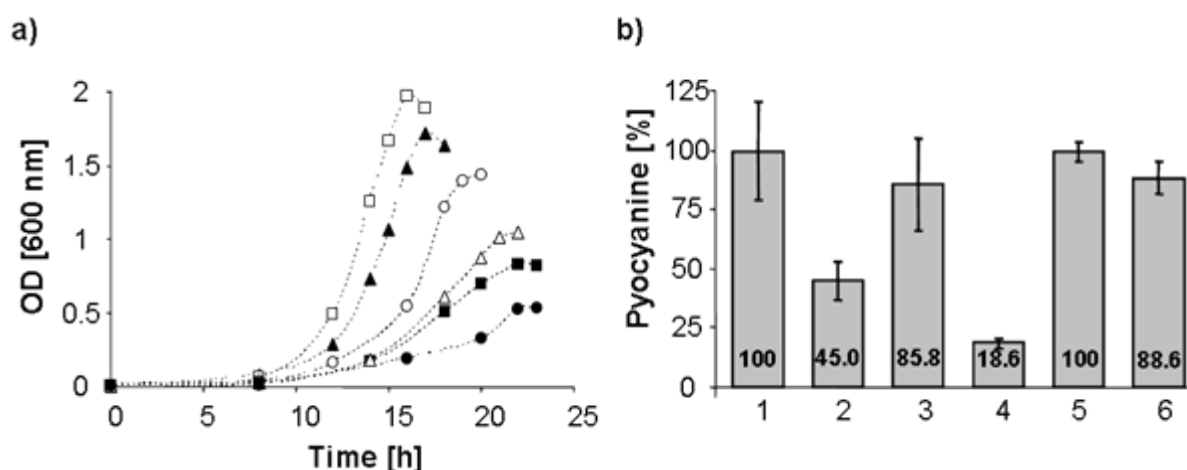


Fig. 2. Growth and pyocyanine production of different *Pseudomonas aeruginosa* strains in M9 medium containing 3.5 mM SDS at 30°C with shaking at 200 rpm. a) OD₆₀₀ of strains PAO1 pUCP18 (■), N pUCP18 (▲), N pUCP18[4929] (Δ), PAO1-D4 pUCP18 (□), PAO1 pBBR (●), and PAO1 pBBR[CC3396] (○). b) Percentages of pyocyanine produced by the individual strains normalized to pyocyanine production of strain PAO1 pUCP18 (100%). The different strains are indicated by the following numeration: 1) PAO1 pUCP18, 2) N pUCP18, 3) N pUCP18[4929], 4) PAO1-D4 pUCP18, 5) PAO1 pBBR, 6) PAO1 pBBR[CC3396]. Error bars indicate standard deviation (n = 3).

Restoration of SDS-induced aggregation in strain N by complementation

To investigate whether SDS-induced aggregation could be genetically restored in strain N, we transformed strain N with a genomic library of strain PAO1 in pUCP18 and screened for clones with rough colony morphology on SDS containing agar plates. Among 6000 transformants we found 28 rough colonies. These were further tested for SDS-induced aggregation in liquid culture. Ten of these clones formed macroscopic aggregates during growth with SDS. Sequence analysis revealed that 6 out of 10 complementing plasmids harboured genomic fragments encoding genes with a GGDEF motif, namely PA1107, PA1727, and PA4929. Subcloning of these as single genes revealed that they sufficed for complementation in strain N.

To investigate whether the formation of macroscopic aggregates by these complemented clones of strain N was SDS-dependent or a constitutive phenotype caused by overexpression of DGCs, we compared their phenotypes during growth with SDS and with succinate. Strains N pUCP18[1107] (not shown) and N pUCP18[4929] formed rough colonies on agar plates and macroscopic aggregates in liquid medium only with SDS but not with succinate as carbon and energy source (Fig. 1a3-c3). In contrast, strain N pUCP18[1727] formed rough colonies and macroscopic aggregates also during growth with succinate (not shown). Swarming motility, growth curves, and pyocyanine production of strain N pUCP18[1107] (not shown) and of strain N pUCP18[4929] resembled those of the parental strain PAO1 (Fig. 1d3, Fig. 2ab). The macroscopic aggregates formed by strain N complemented with pUCP18[1107] or pUCP18[4929] were indistinguishable from aggregates of strain PAO1.

To check if strain N carried mutations in the genes PA1107, PA1727, or PA4929 we amplified them with the primers indicated in Table 2 and sequenced them. No mutation could be detected compared to the corresponding sequences in *Pseudomonas* Genome Project database. We also constructed insertion mutants of strain PAO1 in each of the three genes. These insertional inactivations did neither change the SDS-dependent formation of rough colonies on agar plates nor the SDS-induced cell aggregation in liquid culture.

*Expression of a PDE from *Caulobacter crescentus* in strain PAO1*

The restoration of SDS-dependent cell aggregation in strain N by complementation with DGCs strongly suggested that this process involved the second messenger c-di-GMP. To test this hypothesis we transformed strain PAO1 with the plasmid pBBR[CC3396] containing the gene for the known PDE CC3396 from *Caulobacter crescentus* (Christen *et al.* 2005). In strain PAO1 pBBR[CC3396] the roughness of colonies on SDS-containing agar plates (Fig. 1b6) and the formation of macroscopic aggregates in SDS-containing liquid culture (Fig. 1c6) were strongly reduced compared to the vector

control strain PAO1 pBBR. Furthermore, strain PAO1 pBBR[CC3396] showed increased swarming motility (Fig. 1d6) and a higher growth rate with SDS (Fig. 2a) than the suspended cells of the vector control (Fig. 1d5; 2a). Pyocyanine production was only slightly decreased (Fig. 2b).

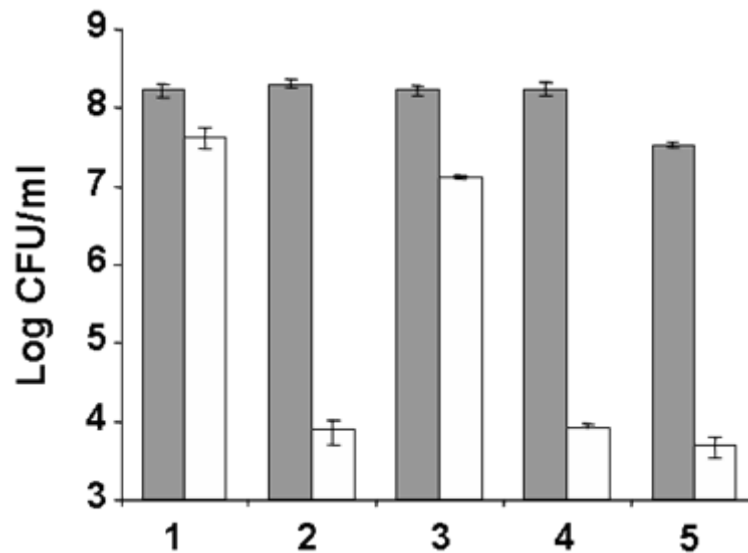


Fig. 3. Survival of different *Pseudomonas aeruginosa* strains after exposure to 3.5 mM SDS in the absence (grey bars) or in the presence (white bars) of 1 mM CCCP determined as CFU counts. The different strains are indicated by the following numeration: 1) PAO1 pUCP18, 2) N pUCP18, 3) N pUCP18[4929], 4) PAO1-D4 pUCP18, 5) PAO1 pBBR[CC3396]. Error bars indicate standard deviation (n = 3).

SDS shock experiments with single and mixed cultures

To compare the survival rates of the aggregating and non-aggregating *P. aeruginosa* strains we submitted them to SDS-shock experiments in the presence and absence of CCCP. The individual strains were incubated with SDS alone before CCCP was added, thereby allowing those strains capable of aggregation to form aggregates. CFU counts of strains PAO1 and N pUCP18[4929] decreased by about 1 order of magnitude after the exposure to CCCP compared to the solvent control (Fig. 3). In contrast, strains N and PAO1 pBBR[CC3396] suffered a dramatic decrease in CFU counts by more than 4 orders of magnitude compared to the solvent controls. These results clearly demonstrated that those strains capable of SDS-induced aggregation had a ca. 500-fold higher survival rate than those strains incapable of SDS-induced aggregation.

In the next step, we investigated whether the non-aggregating strains could be integrated into aggregates of strain PAO1 and, if so, whether they would benefit from this integration during exposure to SDS in the presence of CCCP. For this, we set up SDS shock experiments in which we mixed strain PAO1 with strains N, N pUCP18[4929], and PAO1 pBBR[CC3396] at equal cell numbers prior to addition of SDS, as described under

Experimental Procedures. In all combinations, macroscopic cell aggregates were formed. Strain N accounted for 35.7 % of the CFUs released from aggregates formed with strain PAO1 (Fig. 4a1). Thus, although strain N was incapable of SDS-induced aggregation it could be integrated into cell aggregates of the parental strain. This integration was confirmed by confocal laser scanning microscopy analysis of aggregates containing strains PAO1-Tn7-cfp and N-Tn7-yfp (Fig. 5a). Aggregates consisting mainly of strain PAO1 cells were interspersed with single cells and cell clusters of strain N. When mixed cell suspensions with strains PAO1 and N were exposed to SDS in the presence of CCCP after strain N had been integrated into the aggregates, the percentage of strain N decreased to 14% (Fig. 4a3). Considering the total CFU counts in these experiments (mean value of 4.33×10^7 CFU ml⁻¹), this percentage equals 6×10^6 CFU ml⁻¹. The CFUs of strain N in the absence of strain PAO1 were 7.5×10^3 after exposure to SDS in the presence of CCCP (Fig. 3). Taking into account that twice as much cells of strain N were used in the single strain experiment, the survival rate of strain N was more than 1000-fold higher in the presence of strain PAO1 than in cell suspensions of strain N alone. If strain N was complemented with pUCP18[4929], its percentage of CFUs released from aggregates increased to about 50% (Fig. 4b1). Complementation with pUCP18[4929] also increased the survival rate of strain N after addition of CCCP more than 2-fold (Fig. 4b3). If strain PAO1 harboured pBBR[CC3396] its percentage of CFUs released from aggregates was slightly reduced to 44.3% (Fig. 4c1). This value was further decreased to 32.6% if the cells were exposed to SDS in the presence of CCCP (Fig. 4c3).

Co-cultivation of strains PAO1 and N

To investigate whether strain N would also integrate into aggregates of strain PAO1 during growth with SDS, strains PAO1-Tn7-yfp and N-Tn7-cfp were co-inoculated at a ratio of 5:1 and incubated with SDS as sole carbon and energy source. The final OD600 in these cultures was between the values for strain N and strain PAO1 (not shown). Freely suspended cells and cells released from aggregates were quantified by CFU counts and identified by their fluorescence. Strain N-Tn7-cfp accounted for 72% ($\pm 2\%$) of the freely suspended cells and for 39% ($\pm 5\%$) of cells released from aggregates. Thus, strain N was also integrated into aggregates of strain PAO1 during growth with SDS.

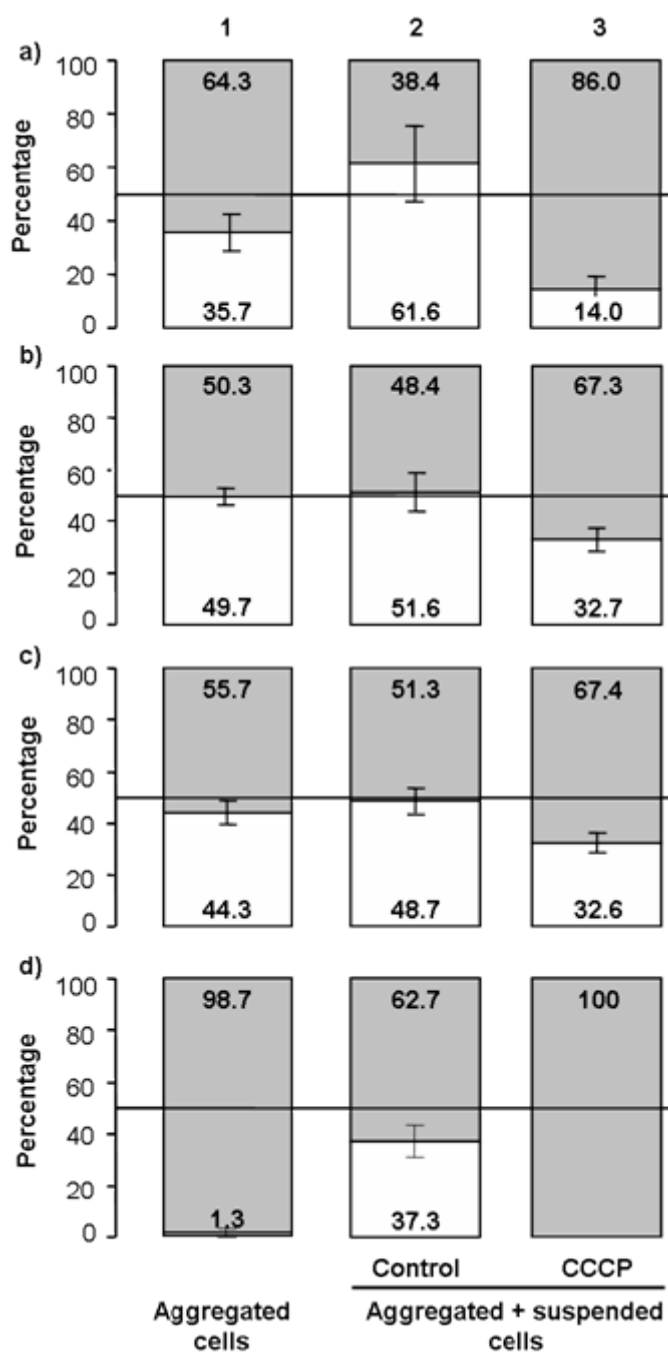


Fig. 4. SDS-shock experiments with mixed cell suspensions of different *Pseudomonas aeruginosa* strains. Grey bars indicate percentages of total CFU counts of strains PAO1 pUCP18 (a, b, d) or PAO1 pBBR (c). White bars indicate percentages of total CFU counts of strains N pUCP18 (a), N pUCP18[4929] (b), PAO1 pBBR[CC3396] (c), and PAO1-D4 pUCP18 (d). Percentages were calculated after differentiating single colonies either by their colony morphology on SDS containing agar plates (a, c, d) or by the fluorescence of YFP- and CFP-tagged strains (b). (1) Percentages of individual strains in macroscopic cell aggregates formed after incubation of mixed cell suspensions with SDS for 45 min. (2-3) Percentages of individual strains in whole cell suspensions (aggregated plus suspended cells) after incubation with SDS for 45 min and additional incubation with methanol as solvent control (Kovach *et al.*) or CCCP (3) for 60 min. Cell aggregates and whole cell suspensions were treated with DNase prior to CFU determination as described in Experimental Procedures. *Error bars* indicate standard deviation (n = 3).

Characterisation of the transposon mutant strain PAO1-D4

The discovery that smooth colony morphology on SDS-containing agar plates correlated with a non-aggregating phenotype during growth with SDS in liquid culture enabled us to conveniently screen for non-aggregating transposon mutants of strain PAO1. We created a mariner transposon mutant library of strain PAO1 and searched for smooth colonies on SDS-containing agar plates. From a first screening we isolated the transposon mutants PAO1-D1 and PAO1-D4. Sequence analysis revealed that the mariner transposons were inserted in the genes *psIF* and *psIJ*, respectively. These genes encode hypothetical proteins with homology to a putative glycosyltransferase (*psIF*) and polysaccharide polymerase (*psIJ*). They are part of an operon (*psIA-O*) which is responsible for exopolysaccharide biosynthesis and biofilm formation in *P. aeruginosa* (Friedman and Kolter 2004; Jackson *et al.* 2004; Ma *et al.* 2006; Matsukawa and Greenberg 2004). Both strains did not aggregate in liquid cultures with SDS. A role for *psIF* in biofilm formation had already been described (Friedman and Kolter 2004). We characterized strain the *psIJ* mutant strain PAO1-D4 further. Its physiological properties resembled those of strain N, namely smooth colony morphology on SDS-containing agar plates (Fig. 1a4), no aggregation and a higher growth rate with SDS in liquid culture (Fig. 1b4; 2a), increased swarming motility (Fig. 1d4), and strongly decreased pyocyanine production (Fig. 2b). As an important difference to strain N, SDS-induced aggregation could not be restored in strain PAO1-D4 by transformation with pUCP18[4929].

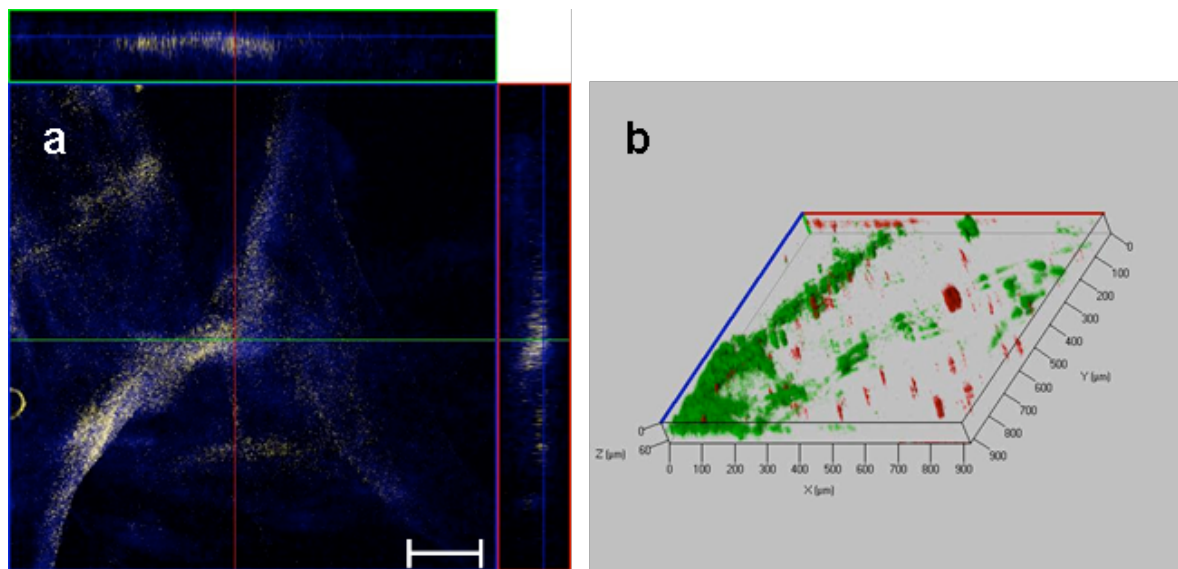


Fig. 5. Confocal laser scanning microscopy of cell aggregates formed by different *Pseudomonas aeruginosa* strains. (a) Top-down view (central picture) and side views (flanking pictures) of an optical section through a cell aggregate formed by strain PAO1-Tn7-cfp (*blue cells*) and strain N-Tn7-yfp (*yellow cells*); the white bar equals 146.2 μm (b) Side-view projection of an aggregate formed by strain PAO1-Tn7-cfp stained with Syto9 (*green colour*) and TRITC-ConA (*red colour*).

In SDS shock experiments strain PAO1-D4 resembled strains N and PAO1 pBBR[CC3396] as treatment with SDS and CCCP caused a drop of CFU counts by more than 4 orders of magnitude compared to the solvent control (Fig. 3). In cell aggregates formed with strain PAO1 we found that strain PAO1-D4 accounted only for 1.3% of the CFUs released from aggregates (Fig. 4d1). If these cell suspensions were supplied with CCCP the CFUs of strain PAO1-D4 were below the detection limit of 0.85% (Fig 4d3).

The impaired ability of strain PAO1-D4 to aggregate and to integrate into aggregates of the wild type suggested an important role of the *psl*-dependent exopolysaccharides for cohesion of aggregates. The structure of this exopolysaccharide is not known but it was shown that it contains among other sugars mannose and glucose (Friedman and Kolter 2004). By CLSM, we detected regions stainable with fluorescently labelled lectin TRITC-ConA which is specific for α -glucose and α -mannose (Fig. 5b). These regions were interspersed into regions stained with Syto9 as well as in areas that were not stained with this dye.

6.5 Discussion

The goal of our study was to define the physiological role of macroscopic cell aggregates formed by *Pseudomonas aeruginosa* strain PAO1 during growth with the detergent SDS. Our investigation started with attempts to isolate mutants that were incapable of SDS-induced aggregation. We could isolate the non-aggregating spontaneous mutant strain N and two non-aggregating transposon mutants in which two genes of the *psl* operon (*pslJ* and *pslF*) were inactivated. Strain N and the *psl* mutants were not impaired in growth with SDS but showed even higher growth rates than the parental strain PAO1. The successful selection for the loss-of-function mutant strain N in cultures growing with SDS and the growth advantage of the *psl* mutants clearly signified SDS-induced cell aggregation of strain PAO1 a dispensable property under our cultivation conditions.

This conclusion raises the question why a part of the population of strain PAO1 did aggregate during growth with SDS. We have demonstrated that cells of *P. aeruginosa* in aggregates had a strongly increased survival rate when they were challenged with two toxic substances simultaneously, namely SDS plus CCCP. Under these conditions, the fast-growing, non-aggregating mutants of *P. aeruginosa* had a detrimental disadvantage. Encountering different toxic substances at the same time represents a realistic scenario that *P. aeruginosa* will face in hostile anthropogenic environments. Thus, our results force the hypothesis that cell aggregation is a pre-adaptive measure that is actively promoted by a subpopulation of *P. aeruginosa* to ensure survival under potentially worsening environmental conditions. The fact that *P. aeruginosa* cultures growing with SDS were divided into

aggregating and non-aggregating subpopulations calls attention to the molecular basis of this phenotypic variation.

Cell aggregation in liquid cultures and formation of rough and structured colonies on agar plates during growth with SDS were reminiscent of two autoaggregative phenotypes frequently found among pseudomonads, the wrinkly spreader (Rainey and Rainey 2003) and the small colony variants (Häußler 2004). So far, both phenotypes have been described as constitutive phenotypic variations that regularly arise during growth in heterogeneous environments (Goymer *et al.* 2006; Spiers *et al.* 2002; Ude *et al.* 2006) and in biofilms, respectively (Deziel *et al.* 2001; Drenkard and Ausubel 2002; Kirisits *et al.* 2005; Webb *et al.* 2004). Our results signify the autoaggregative phenotype as a facultative response that can be specifically induced by exposure to SDS. Mutational origins of autoaggregative phenotypes have been repeatedly found in genes involved in c-di-GMP metabolism (D'Argenio *et al.* 2002; Drenkard and Ausubel 2002; Goymer *et al.* 2006; Hickman *et al.* 2005). We have obtained indirect but clear evidence of c-di-GMP being involved in SDS-induced aggregation. First, we could restore SDS-induced aggregation in strain N by complementation with 3 different DGCs, namely PA1107, PA1727, and PA4929. DGC activity of PA1107 and PA1727 in *P. aeruginosa* has been demonstrated (Kulasekara *et al.* 2006). Second, SDS-induced aggregation could be reduced in strain PAO1 by expression of the known PDE CC3396 from *C. crescentus* (Christen *et al.* 2005).

Complementation with the genes PA1107 and PA4929 restored the autoaggregative phenotype in strain N in a SDS-dependent manner. However, as both genes were not mutated in strain N and their inactivation did not cause a phenotype in strain PAO1 a signalling pathway for SDS-induced aggregation cannot rely on these genes only. Rather, their complementing effect may rely on increasing the intracellular c-di-GMP concentration to a level required for SDS-induced aggregation. This possibility is supported by the effect of the PDE CC3396 because this heterologous enzyme should unspecifically reduce c-di-GMP levels in *P. aeruginosa*. However, despite various extraction methods all our attempts to detect c-di-GMP by HPLC in SDS-grown cells of our strains failed so far (not shown). Several studies have provided evidence of c-di-GMP signalling being strictly regulated in space and time (Jenal and Malone 2006) and acting in microcompartments (Weber *et al.* 2006). Considering that c-di-GMP synthesis is costly and growth with SDS is an energetical challenge, it would be feasible if c-di-GMP synthesis in SDS-grown cells of *P. aeruginosa* was strictly regulated, thereby rendering its intracellular pool below the detection limit. The fact that c-di-GMP biosynthesis in *P. aeruginosa* could be detected by HPLC analysis only upon overexpression of certain DGCs during growth in rich medium indicates that the levels of this second messenger are generally low in this organism (Kulasekara *et al.* 2006).

Recently, two operons involved in synthesis of exopolysaccharides, namely the *psl* (Jackson *et al.* 2004; Matsukawa and Greenberg 2004) and the *pel* operon (Friedman and Kolter 2004), have been identified as possible targets of c-di-GMP signalling in *P. aeruginosa* (Hickman *et al.* 2005). Our results with the non-aggregating *pslF* and *pslJ* mutants clearly assigned an essential function for the *psl* gene cluster in SDS-induced aggregation. Staining with fluorescently-labeled ConA lectins indicated that the mannose- and glucose-rich *psl*-dependent polysaccharide could be a part of the EPS in SDS-induced aggregates. It has been shown that *pslA* was expressed in confined areas of biofilms (Overhage *et al.* 2005). A localized production of the *psl*-dependent exopolysaccharide would be in agreement with the patchy distribution of the ConA signals within the aggregates. In conclusion, we have obtained evidence that SDS-induced aggregation involves a c-di-GMP signalling pathway that eventually activates synthesis of the *psl*-dependent exopolysaccharide. Our previous finding that aggregates contained a high proportion of damaged cells suggests that this signalling pathway could be triggered by so far unknown environmental or intracellular signals that indicate cell damage by SDS.

We do currently not know which mutation is responsible for the stable non-aggregating phenotype of strain N. Our SDS shock experiments gave no indication that strain N was more resistant to SDS than the parental strain PAO1. As the molar growth yields of both strains were equal they could invest equal amounts of energy into biomass production although they must have channelled part of their carbon source into different biosynthetic pathways because strain N did not form aggregates. However, as strain N complemented with DGCs was able to form cell aggregates indistinguishable from those of strain PAO1, it cannot be mutated in a gene required for EPS synthesis. The effective integration of strain N -in contrast to the *pslJ* mutant strain PAO1-D4- into aggregates of strain PAO1 suggested that strain N produced a basal level of adhesive EPS material. As the defect of strain N could be bypassed by expression of DGCs we hypothesize that strain N is mutated in gene upstream of c-di-GMP signalling. The respective gene product could be involved in transducing SDS-induced stress to the postulated c-di-GMP signalling pathway.

We have shown that integration into aggregates of strain PAO1 conferred a substantial survival advantage for strain N upon exposure to SDS in the presence of CCCP. In this respect, cell aggregation could ensure genotypic and phenotypic diversity of *P. aeruginosa* populations which supports survival and growth in environments with fluctuating loads of multiple toxic chemicals, such as the aforementioned industrial wastewaters or clinical settings. Aggregating variants could increase population's survival chances under strongly adverse conditions while non-aggregating and fast-growing variants could increase the population's chances for rapid colonization of a new habitat when conditions improve. Pyocyanine production, which accompanied cell aggregation in our

experiments, would be a means to inhibit competing microorganisms. To understand this survival strategy of *P. aeruginosa* in more detail, we are currently investigating the presumptive stress signals, the signal transduction pathways, and the molecular targets involved in SDS-induced aggregation.

6.6 □□ Acknowledgments

The authors like to thank Urs Jenal, Jakob Malone, and Alexander Boehm from the Biozentrum Basel for the gift of pALMAR3 and pBBR[CC3396] as well as for helpful discussions. Technical assistance from Antje Karst and Oliver Popp and continuous support from Bernhard Schink is acknowledged. This work was funded by a grant from the *Deutsche Forschungsgemeinschaft* to BP (PH71/2-1).

7 Genes responsible for SDS-induced aggregation: Identification of a potential c-di-GMP signalling pathway which regulates transcription and posttranscriptional modifications of the *cupA* operon

Janosch Klebensberger, Karin Lautenschlager, Bodo Philipp

7.1 □□ Abstract

Recently, we demonstrated that SDS-induced aggregation of *Pseudomonas aeruginosa* is an active, but dispensable response of a subpopulation. However, we also found that SDS-induced aggregation dramatically increased survival rates under energy limited conditions, and demonstrated that the *psl* gene cluster is involved in aggregate formation. In this study, we found that the *cupA* operon, and a novel putative c-di-GMP signalling pathway (PA0172-PA0169), were required for SDS-induced aggregation. With mutants deficient in the genes PA0172 and the putative di-guanylate cyclase (DGC) PA0169, we uncovered their involvement in regulation of the *cupA* operon. We found that SDS triggered increased levels of *cupA1* transcripts, in a strictly c-di-GMP-dependent process. Transcriptional *lacZ*-fusions revealed that activation of the *cupA* operon was dependent on the presence of the putative DGC PA0169. Determination of mRNA stability implicated further regulation of the *cup* operon by a yet unknown posttranscriptional mechanism. Our results clearly demonstrate that SDS-induced aggregate formation is regulated by a so far uncharacterized signalling transduction system, which modulates the expression of adhesive surface structures, most likely via the second messenger c-di-GMP. Thus, this study provides further insights in the complex regulation of aggregate formation, phase variable gene expression, and c-di-GMP signal transduction systems.

7.2 □□ Introduction

Metabolic versatility and variability of physiological responses towards environmental signals are important factors for bacteria, and promote successful colonization of different habitats and growth under varying environmental conditions. In environments where bacteria encounter toxic substances, such as industrial wastewaters or healthcare settings, mechanisms ensuring survival are essential. The formation of cell aggregates or biofilms appears feasible as a protection mechanism, because residing in such structures confers increased resistance to biocides such as antibiotics or detergents (Drenkard and Ausubel 2002; Fux *et al.* 2005; Gilbert *et al.* 2002; Lewis 2001). Triggering formation of biofilms by toxic substances has been shown recently for the opportunistic pathogen *Pseudomonas aeruginosa* (Hoffman *et al.* 2005). In this study the induction of biofilms as a defense mechanism towards sublethal concentration of aminoglycoside antibiotics was demonstrated.

Previously, we reported about aggregation of *P. aeruginosa* strain PAO1 as an active and energy-requiring response towards exposure to the toxic detergent sodium dodecyl sulphate (SDS) (Klebensberger *et al.* 2006). We found that SDS triggered rough colony morphology on solid medium and an aggregative phenotype of a subpopulation in liquid medium of strain PAO1. Phenotypic variants with an autoaggregative phenotype and wrinkled colony morphology have previously been shown to appear regularly during growth, especially during long-term incubations or in biofilms (Drenkard and Ausubel 2002; Kirisits *et al.* 2005; Webb *et al.* 2004). These phenotypes are often linked to a regulatory system responsible for the turnover of the intracellular second messenger cyclic di-guanosine monophosphate (c-di-GMP) (D'Argenio *et al.* 2002; Häußler 2004; Römling 2005; Spiers *et al.* 2003). This second messenger is widespread among prokaryotes and has been shown to play a key role in regulating the transition from sessility to motility and vice versa in different species (Jenal and Malone 2006; Römling *et al.* 2005).

Recently, we demonstrated that aggregation was not essential for survival during growth with SDS by the isolation of a non-aggregative spontaneous mutant (Klebensberger *et al.* 2007). However, we found that SDS-induced aggregation strongly increased survival rates of cells during exposure to SDS plus the uncoupler carbonyl cyanide chlorophenylhydrazone (CCCP). Furthermore, we demonstrated that SDS-induced aggregation and as a consequence the survival rates during exposure towards SDS plus CCCP could be strongly influenced by the expression of genes responsible for the turnover of the second messenger c-di-GMP. From these results we concluded that SDS-induced aggregation is a pre-adaptive strategy of *Pseudomonas aeruginosa* to ensure survival under

varying environmental conditions, and postulated that this process is regulated by a c-di-GMP signalling pathway.

In the present study we screened a transposon mutant library to identify genes involved in the SDS-induced aggregation. We found a putative c-di-GMP signalling pathway responsible for SDS-induced aggregation and uncovered its role in regulation of the *cupA* Operon.

7.3 □□ Materials and methods

Bacterial strains, media, and growth experiments

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultivated in Luria Bertani (LB) medium or in a modified M9 mineral medium supplied with 3.5 mM SDS or 10 mM Na₂-succinate as carbon and energy source as described previously (Klebensberger *et al.* 2006). Selection of transformants and insertion mutants was carried out on LB agar plates (1.5%, w/v) containing 100 µg/ml ampicillin (Fluka), 15 µg/ml gentamycin (Sigma), 50 µg/ml tetracycline (Fluka), and 10 µg/ml kanamycin (Fluka) with *Escherichia coli*, and Pseudomonas isolation agar (Difco) containing 200 µg/ml carbenicillin (Sigma), 120 µg/ml gentamycin, 160 µg/ml tetracycline with *P. aeruginosa*. In liquid M9 medium, the concentrations of carbenicillin, gentamycin, and tetracycline were decreased to 50 µg/ml, 10 µg/ml, and 20 µg/ml, respectively. For mRNA stability assays, 300 µg/ml rifampicin was used.

Unless noted otherwise, growth experiments with *P. aeruginosa* were performed as described previously (Klebensberger *et al.* 2006). Colony morphology was evaluated on solid M9 medium containing 0.15% SDS or 10 mM Na₂-succinate after incubation for 3 days at 30°C. SDS-induced aggregation was tested in 3 ml M9 medium containing 0.1% SDS in small Petri dishes (3.5 cm in diameter; Nunc) on a rotary shaker at 120 rpm (Orbital Incubator S150, Stuart Scientific) for 18 h at 30°C.

Transposon mutagenesis and screening for non-aggregating mutants

The generation of random transposon mutants of *Pseudomonas aeruginosa* with the mariner transposon pALMAR3 was described earlier (Klebensberger *et al.* 2007). Approximately 20.000 transposon mutants were generated and pooled to search for mutants with smooth colony morphology on M9 agar plates containing 0.15% SDS and 80 µg/ml tetracycline. In total, 106 mutants with smooth colony morphology were further screened for SDS-induced aggregation in liquid culture. In 8 out of 22 non-aggregating mutants, the exact position of the transposon insertion was identified by inverse PCR as described previously (Klebensberger *et al.* 2007).

Table 1. Strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics	Source or reference
<i>P. aeruginosa</i>		
PAO1	Wild-type of strain PAO1	Holloway collection
PAO1-Tn7- <i>yfp</i>	PAO1 with Tn7 chromosomal insertion of <i>yfp</i>	(Klebensberger <i>et al.</i> 2007)
PAO1-B1	<i>cupA1::Mariner</i> mutant of strain PAO1, Tet ^r	This study
PAO1-F5	PA0172::Mariner mutant of strain PAO1, Tet ^r	This study
KO0172	Deletion mutant of strain PAO1, deletion of an 1165 bp fragment (position 485-1623) of PA0172 (1992 bp)	This study
KO0169	Insertion mutant of strain PAO1, insertion of a resolvase site at position 368 of PA0169 (708 bp)	This study
PAO-P47	<i>mvaT</i> deletion mutant of strain PAO1	(Diggle <i>et al.</i> 2002)
PAO-P47-0169	Insertion mutant of strain PAO-P47, insertion of a <i>res-cat-res</i> cassette at position 368 in PA0169 (708 bp), Cm ^r	This study
<i>E. coli</i>		
JM109	<i>endA1 recA1 gyrA96 thi hsd R17 (r_K⁻, m_K⁺), relA1 supE44 Δ(lac-proAB) [F' traD36 proAB⁺ lacI^q lacZΔM15]</i>	Promega
HB101	<i>thi-1 hsd S20 (r_B⁻ m_B⁻) supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20 (str^r) xyl-5 mtl-1 galK2</i>	Promega
CC118	<i>araD139 Δ(ara leu)7697 ΔlacX74 phoAΔ20 galE thi rpsB argE_{am} recA1</i>	(Manoil and Beckwith 1985)
Plasmids		
pALMAR3	Plasmid harbouring a mariner transposon used for transposon mutagenesis, Tet ^r	Jenal lab
pUCP18	<i>Escherichia-Pseudomonas</i> shuttle vector, Ap ^r	(West <i>et al.</i> 1994)
pUCP18[0169]	Plasmid pUCP18 harboring a <i>XbaI-HindIII</i> fragment (1439 bp) encoding PA0169	This study
pUCP18[0172]	Plasmid pUCP18 harboring a <i>BamHI</i> fragment (2708 bp) encoding PA0172	This study
pUCP18[1107]	pUCP18 harboring a <i>SmaI</i> fragment (2008 Bp) encoding PA1107	(Klebensberger <i>et al.</i> 2007)
pUCP18[4929]	pUCP18 harboring a <i>SaII</i> fragment (2426 Bp) encoding PA4929	(Klebensberger <i>et al.</i> 2007)
pBBR1MSC-5	Broad-host-range cloning vector, (Gm ^r)	Kovach <i>et al.</i> 1995
pBBR[CC3396]	pBBR1MSC-5 containing gene CC3396 from <i>C. crescentus</i>	Jenal lab
pEX18AP	Gene replacement vector, Ap ^r	(Hoang <i>et al.</i> 1998)
pKO2b	pUC18Sfi containing <i>res-cat-res</i> , Ap ^r , Cm ^r	(Smits unpublished)
pUCPParA	parA as EcoRI-HindIII fragment in pUCP24	(Smits <i>et al.</i> 2002)
pMP220 [<i>cupA1LlacZ</i>]	<i>cupA1L-lacZ</i> transcriptional fusion in pMP220	(Vallet <i>et al.</i> 2004)
pRK 600	<i>ori</i> ColE1 RK2-Mob ⁺ RK2-Tra ⁺ , (Cm ^r), helper strain in tri-parental matings	(Kessler <i>et al.</i> 1992)

Construction of mutants and complementing plasmids

For construction of a PA0172 deletion mutant, a 3466 bp fragment containing the gene PA0172 was amplified by PCR (TripleMaster PCR System, Eppendorf) from purified genomic DNA (Puregene DNA Isolation Kit, Gentra) using the primers KOPA0172-F (5'-CAACCTGCTCGCCGGCCTGCTCAC-3') and KOPA0172-R (5'-GTTTCGCGGCTCATCGTCGGCTACTCGT-3'). For construction of a PA0169 insertion mutant, a 1326 bp fragment containing the gene PA0169 was amplified using the primers KO-PA0169-F (5'-GGACCTGCGCCTGCTGTACCTGAA-3') and KO-PA0169-R (5'-GCCTCGCCCGCGCCTATGG-3'). Both amplicons were cloned into the vector Topo PCR2.1 (TA cloning Kit, invitrogen), and transformed into competent cells of *E. coli* JM109 (Promega) following the manufacturer's instructions. The resulting plasmid TopoKO0172 was digested with *Sall* to remove an internal fragment (1165 bp) from gene PA0172 and TopoKO0169 was linearized with *SmaI*. After purification (PCR purification kit, peqlab) the linearized plasmids were blunt ended with T4 DNA polymerase (NEB), purified, and dephosphorylated using Shrimp alkaline phosphatase (Promega). The plasmids were ligated to a blunt ended *res-cat-res* cassette obtained from plasmid pKO2a (kindly provided by Theo Smits), resulting in the plasmids TopoKO0172[Cm] and TopoKO0169[Cm]. Finally, the fragments were subcloned in the blunt ended suicide vector pEX18Ap (Hoang *et al.* 1998) digested with *EcoRI-HindIII*. Therefore, TopoKO0172[Cm] was digested with *NotI*, and TopoKO0169[Cm] was digested with *XbaI-HindIII* and subsequently treated with T4 DNA polymerase. The resulting plasmids pEXKO0172 and pEXKO0169 were transformed into *E. coli* CC118 and transferred into *P. aeruginosa* by tri-parental mating. Clones with chloramphenicol resistance were selected on LB plates containing 300 µg/ml chloramphenicol and 7% sucrose. Clones with chloramphenicol resistance which were sensitive towards carbenicillin were transformed with pUCP24[ParA] to remove the chloramphenicol resistance as described elsewhere (Smits *et al.* 2002). Clones with gentamycin resistance which were sensitive towards chloramphenicol were checked for removal of the chloramphenicol cassette by PCR and positive clones were transferred on LB agar plates without antibiotics several times. Finally, clones sensitive towards chloramphenicol and gentamycin were obtained and designated KO0172 and KO0169.

The *mvaT* deletion mutant PAO-P47 (Diggle *et al.* 2002) was kindly provided by Steve Diggle. The double mutant PAO-P47-0169 was constructed by insertion of the *res-cat-res* cassette in the gene PA0169 into the PAO-P47 mutant. For this, the plasmid pEXKO0169 was introduced into strain PAO-P47 by tri-parental mating as described above. Clones with chloramphenicol and gentamycin resistance were selected on LB plates containing 300 µg/ml chloramphenicol and 7% sucrose. After three transfers, a clone with chloramphenicol resistance which was sensitive towards gentamycin was obtained, checked

for the chromosomal insertion of the *res-cat-res* cassette in gene PA0169 by PCR, and designated PAO-P47-0169.

To construct plasmid pUCP18[PA0169], the gene PA0169 was excised as *XbaI-HindIII* fragment (1439 bp) from TopoKO0169, treated with T4 DNA polymerase, and cloned into a T4 DNA polymerase treated vector pUCP18 (West *et al.* 1994) digested with *EcoRI-HindIII*. To construct the plasmid pUCP[0172], a 2905 bp fragment containing the gene PA0172 was amplified from genomic DNA by PCR using the primer KOPA0172-F (see above) and PA0172-R (5'-CGGGCGGCGTAGCTGCTCCTTGTA-3'), and cloned into the vector Topo PCR2.1 resulting in the plasmid Topo0172. A *BamHI* fragment (2708 Bp) containing the gene PA0172 was finally subcloned into the respective restriction site of the plasmid pUCP18 to obtain the plasmid pUCP[0172]. Correct orientation for expressing of PA0169 and PA0172 from the *lac*-promoter of pUCP18 was confirmed by sequencing.

SDS shock experiments

Unless noted otherwise, SDS shock experiments with cell suspensions of single or mixed cultures of different *P. aeruginosa* strains were performed as described previously (Klebensberger *et al.* 2007). Individual strains in mixed cell suspension experiments were identified by their colony morphology or by their fluorescence as described previously (Klebensberger *et al.* 2007).

RNA isolation

Suspensions (OD₆₀₀ = 1.5) of succinate-grown cells or suspensions of SDS-grown cells were supplied with their respective substrate (10 mM succinate or 3.5 mM SDS) in triplicate in small Petri dishes (3.5 cm in diameter, Nunc) in a final volume of 3 ml. After incubation with shaking at 120 rpm at 30°C for 60 min, these triplicates were combined in a plastic tube (Greiner) filled with 30 ml ice-cold DNase buffer. Cells were harvested by centrifugation at 15.000 × g at 4°C for 1 min, and RNA was extracted from the cells with the Purescript RNA Isolation Kit (Gentra Systems) according to the manufacturer's instructions. RNA from 3 independent experiments was combined, and contaminating DNA was removed with an off-column RNase-free DNase I treatment (QIAGEN) according to the manufacturers instructions. After repurification with an RNeasy column (Quiagen), the samples were quantified spectrophotometrically and stored at -60°C until further analysis.

For RNA stability studies, suspensions (OD = 1.5) of succinate-grown or SDS-grown cells from three independent cultures were mixed before the experiment. Aliquots of 9 ml of these mixed cell suspensions were then distributed into five small Petri dishes (5.5 cm in diameter, Nunc). After 20 min of incubation with their respective substrate (10 mM succinate or 3.5 mM SDS) with shaking (120 rpm) at 30°C, rifampicin was added to a final

concentration of 300 µg/ml. Immediately after the addition and at regular intervals thereafter (4, 8, 12, 24 min), the total RNA of cells from a single Petri dish was extracted and purified as described above.

Northern blot analysis

For Northern blot hybridization, 1% agarose gels containing 3.5% formaldehyde (w/v) were cast and run in 1 × MOPS buffer (20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) for size fractionation of RNA samples. The loading dye for denaturation of t RNA samples contained 50% formamide, 6% formaldehyde, 1 × MOPS buffer, 0.01% bromophenol blue, and 0.2% ethidium bromide.

For Northern blot analysis, 10 µg and for RNA stability studies 5 µg of total RNA were used. Total RNA was transferred to positively charged nylon membranes (Roche) overnight with a Turboblotter (Schleicher & Schuell) using 20 × SSC solution (3 M sodium chloride, 0.3 M sodium citrate, pH 7). After UV-cross linking and washing with 2 × SSC solution for 1 h, the membranes were prehybridized with high-SDS-concentration buffer (7% SDS [w/v] containing 50% formamide [v/v], 5 × SSC, 2% blocking reagent [Roche], 50 mM sodium phosphate, 0.1% N-laurylsarcosine [w/v], pH 7.0) for 2 h at 50°C. A digoxigenin (DIG)-labeled DNA probe for *cupA1* (438 bp) was generated with the PCR DIG Probe synthesis kit (Roche) using the primers *cupA1-S-F* (5'-GCGAAGTGACCGACCAGAC-3') and *cupA1-S-R* (5'-CCCCAGCGGCCGACAGAGGTCGTATT-3'). Hybridization was performed overnight at 50°C with 15 ng DIG-labeled probe per ml of high-SDS-concentration buffer. The membranes were washed twice with 2 × SSC solution with 0.1% SDS for 15 min at room temperature, and subsequently twice with 0.2 × SSC solution with 0.1% SDS for 15 min at 65°C. Blocking and developing of the blots were performed with the DIG luminescence detection kit (Roche) following the manufacturer's instructions. Autoradiography was performed with RX films (Fuji) using a Hypercasette (Amersham), and developed films were scanned using a FX-molecular scanner (Biorad) for further analysis. Signal intensities of the autoradiography as well as ethidium bromide fluorescence intensities of the 23S- and 16S RNA from the respective agarose gel was quantified using GelScan5™ software (BioSciTec). All intensities in the autoradiography were normalized to the total RNA of the respective sample (combined intensities of the 23S- and 16S RNA signal).

β-Galactosidase assays and protein quantification

Suspensions (OD₆₀₀ = 1.5) of succinate-grown cells or suspensions of SDS-grown cells were supplied with their respective substrate (10 mM succinate or 3.5 mM SDS) in small Petri dishes (3.5 cm in diameter, Nunc) in a final volume of 3 ml in triplicate. After incubation with shaking at 120 rpm at 30°C for 90 min, these triplicates were combined in a

plastic tube (Greiner) filled with 30 ml ice-cold DNase buffer. Cells were harvested by centrifugation (Centrifuge 5804R, Eppendorf) for 1 min at $15.000 \times g$ at 20°C , immediately frozen in liquid nitrogen, and stored at -20°C until further analysis. Cells from at least 2 independent experiments were resuspended and combined in Z- buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgCl_2 , pH 7.0) in a final volume of 2 ml. Cells were broken by three passages through a precooled French press (SLM Aminco; SLM Instruments) at 136 MPa. The homogenates were centrifuged at $20.800 \times g$ for 10 min at 4°C . The supernatant (cell-free extract) was transferred into a new plastic tube and stored at -20°C until further analysis. β -Galactosidase activities in cell-free extracts were measured according to the Miller method, based on *o*-nitrophenyl- β -D-galactopyranoside hydrolysis (Sambrook *et al.* 1989). Protein content of the cell-free extracts was determined with the BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions.

Photography and image processing

Macroscopic images of colonies and SDS-induced aggregation were taken with a Canon Powershot G6 camera. Images were processed with Paint Shop Pro 4.

7.4 □□ Results

Characterization of transposon mutants

To investigate genes that are involved in the SDS-induced aggregation, we screened a transposon mutant library constructed with a mariner transposon for smooth colony morphology on SDS-containing agar plates and for a non-aggregative phenotype during growth with SDS in liquid medium. We isolated 22 clones that satisfied these criteria, and from 8 of these clones the genomic sequence flanking the insertion site was identified (Fig. 1). Two mutants, strains PAO1-D1 and PAO1-D4 harbouring the insertion in the genes *pslF* and *pslJ*, have been described already in a previous study (Klebensberger *et al.* 2007). Both genes are part of the *psl* operon, which has been described recently to be responsible for the synthesis of a yet unknown extracellular polysaccharide in *Pseudomonas aeruginosa* (Friedman and Kolter 2004; Jackson *et al.* 2004).

Five mutants were found to harbour the transposon insertion in the *cupA* operon, which encodes components involved in the biogenesis of adhesive fimbriae via the chaperone-usher pathway (Vallet *et al.* 2001). In the mutant PAO1-B1 the mariner transposon was inserted into in the *cupA1* gene which encodes for the fimbrial subunit, and into all other mutants the transposon was inserted in the *cupA3* gene which encodes for the "usher" protein of this pathway.

In mutant PAO1-F5 the transposon was inserted in gene PA0172 which encodes a protein of unknown function with two predicted transmembrane helices. Domain and sequence analysis of the protein encoded by this ORF with the SMART software tool (<http://smart.embl-heidelberg.de/>) revealed two conserved domains, namely, a sigma factor PP2C-like phosphatase and a HAMP domain (Appleman *et al.* 2003; Aravind and Ponting 1999) which both are predicted to be involved in signal transduction and/or transcription. From the *Pseudomonas* genome database, PA0172 is predicted to be co-transcribed with at least two other genes encoding proteins of unknown function, namely, PA0171 and PA0170. The gene downstream of this cluster (PA0169) encodes for a protein with a GGDEF domain, which is known to represent the catalytic site of di-guanylate cyclases (DGCs), that are responsible for the synthesis of the bacterial second messenger cyclic di-guanosine monophosphate (c-di-GMP) (Galperin *et al.* 2001; Jenal and Malone 2006). Upstream of this cluster, several genes (PA0173-PA0181) are located that are likely to be involved in a chemotaxis-like two-component system.

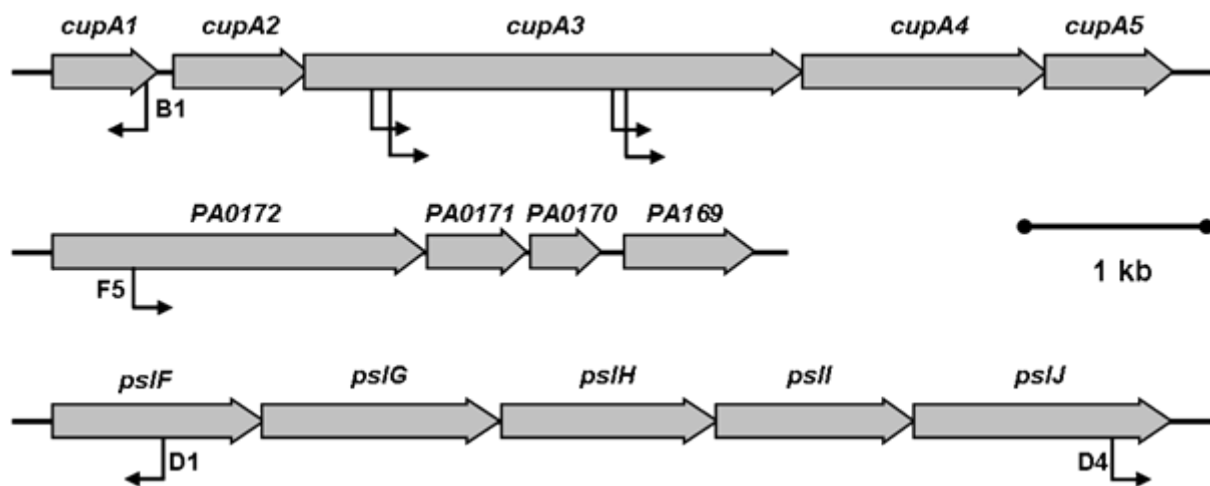


Fig. 1. Map of inactivated genes found in transposon mutants of *Pseudomonas aeruginosa* with a non-aggregative phenotype. Arrows indicate the insertion site of the Mariner transposon. The direction of the arrowhead indicates the orientation of the promoter of the tetracycline resistance gene. Transposon mutants used in this study (B1, F5) or in a previous study (D1, D4) are indicated.

All transposon mutants mentioned above showed a similar phenotype during growth with SDS. On SDS-containing agar plates, these mutants were characterized by smooth colony morphology, as shown for the mutants PAO1-B1 (2a2) and PAO1-F5 (2a4) in Fig. 2, in contrast to the rough and structured colony morphology of strain PAO1. In liquid medium, the mutants did not form macroscopic aggregates during growth with SDS (Fig. 2b2, 2b4), had a higher growth rate, and reached higher final optical densities

compared to strain PAO1 (not shown). These findings confirmed our previous results, that SDS-induced aggregation is not essential growth with SDS (Klebensberger *et al.* 2007).

In a previous study, we demonstrated that genes encoding for a known DGC (PA1107) and a potential DGC (PA4929) could restore the SDS-induced aggregation in a non-aggregating spontaneous mutant of strain PAO1 (strain N), but not in the mutant PAO1-D4 deficient in *psJ* (Klebensberger *et al.* 2007). From these results we concluded that a c-di-GMP dependent signalling pathway is involved in SDS-induced aggregation, and strain N is most likely deficient in signal-sensing or signal-transduction. To investigate whether the mutants deficient in CupA1, CupA3, or PA0172 could be restored in SDS-induced aggregation by the expression of the potential DGC PA4929, we expressed PA4929 from the shuttle vector pUCP18 in these mutants and evaluated their colony morphology and aggregation during growth with SDS.

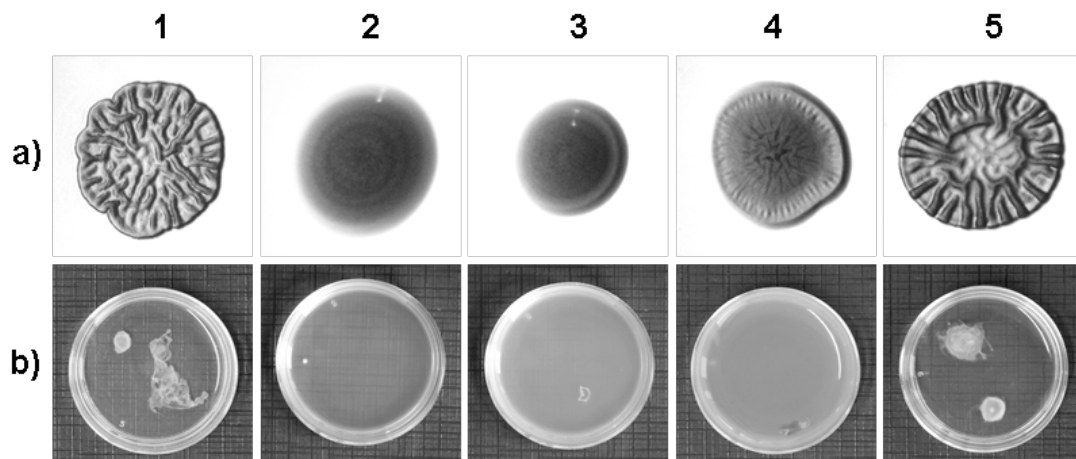


Fig. 2. Complementation of transposon mutants PAO-B1 and PAO-F5 with pUCP[4929]. a) Colony morphology on M9 agar containing 0.15% SDS after incubation for 3 d at 37°C. b) Growth in liquid M9 medium containing 0.1% SDS after incubation for 18 h at 30°C with shaking at 120 rpm. Strains are indicated by the following numeration: 1) PAO1 pUCP18, 2) PAO-B1 pUCP18, 3) PAO-B1 pUCP18[4929], 4) PAO-F5 pUCP18, 5) PAO-F5 pUCP18[4929].

We found that none of the mutants deficient in the *cupA* gene cluster could be restored in rough colony morphology and aggregate formation during growth with SDS, as shown for the mutant PA-B1 in Fig. 2a3 and Fig. 2b3. In contrast, expression of pUCP18[4929] in the mutant PAO1-F5 restored rough colony morphology and aggregate formation during growth with SDS (Fig. 2a5, 2b5).

Characterization of mutants KO0172 and KO0169

The results of complementation suggested that the gene PA0172 could be part of a c-di-GMP signalling pathway which is responsible for aggregate formation during growth with SDS. Given that this hypothesis is true, the proximity of PA0169 suggests that this gene

might encode the DGC responsible for synthesis of the signalling molecule. To test this hypothesis, we constructed mutants in both genes by deletion of a major part of the gene PA0172 (KO0172) and insertion of a non-polar *res*-site in gene PA0169 (KO0169).

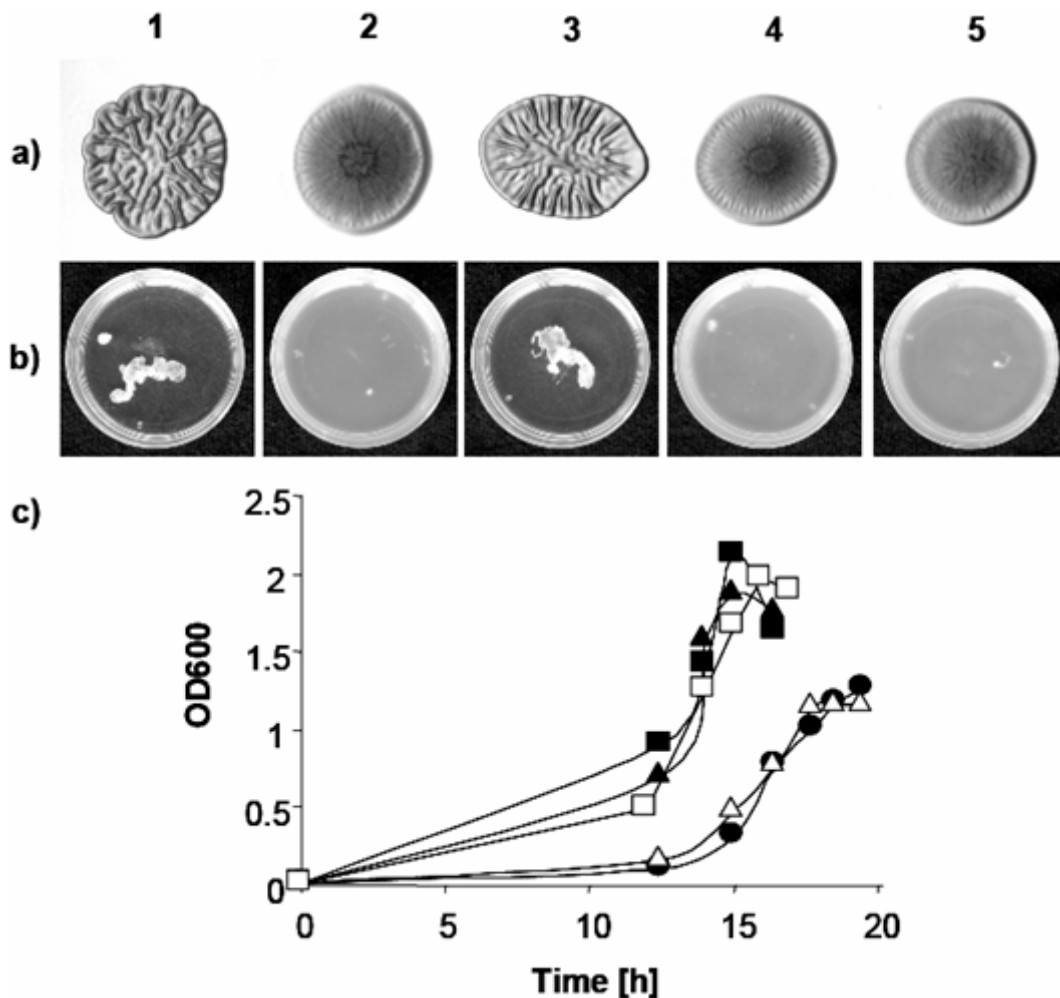


Fig. 3 Complementation of strains KO0169 and KO0172 with pUCP18[0169]. a) Colony morphology on M9 agar containing 0.15% SDS after incubation for 3 d at 37°C. b) Growth in liquid M9 medium containing 0.1% SDS after incubation for 18 h at 30°C with shaking at 120 rpm. c) OD600 in liquid M9 medium containing 0.1% SDS at 30°C with shaking at 200 rpm. The different strains are indicated by the following symbols and numeration: 1) PAO1 pUCP18 (●), 2) KO0169 pUCP18 (▲), 3) KO0169 pUCP18[0169] (△), 4) KO0172 pUCP18 (■), 5) KO0172 pUCP18[0169] (□).

During growth on SDS-containing solid medium, strains KO0169 and KO0172 formed smooth and unstructured colonies (Fig. 3a2, 3a4). During cultivation of strains KO0169 and KO0172 in SDS-containing liquid medium they did not aggregate (Fig. 3c2, 3c4), had a higher growth rate, and reached a higher final optical density compared to strain PAO1 (Fig. 3c2, 3c4). To investigate whether SDS-induced aggregation could be restored genetically in strains KO0169 and KO0172, we expressed the respective genes on the plasmid pUCP18. The expression of pUCP18[0172] had no effect on colony morphology or aggregate formation during growth with SDS in strains KO0172 and KO0169 (not shown).

Expression of pUCP18[0169] specifically restored the formation of rough colonies and aggregates during growth with SDS in strain KO0169 (Fig. 3b3, 3c3), but had no effect on strain KO0172 (Fig. 3b5, 3c5). In addition, the expression of PA1107 and PA4929 restored the wrinkled colony morphology and the formation of macroscopic aggregates during growth with SDS in strains KO0169 or KO0172 (not shown).

SDS shock experiments with single and mixed cultures

From a previous study, we knew that aggregate formation could act as an adaptive survival strategy under conditions which are detrimental for freely suspended cells (Klebensberger *et al.* 2007). In these survival experiments, the individual strains were incubated with SDS alone before CCCP was added, thereby allowing those strains capable of aggregation to form aggregates.

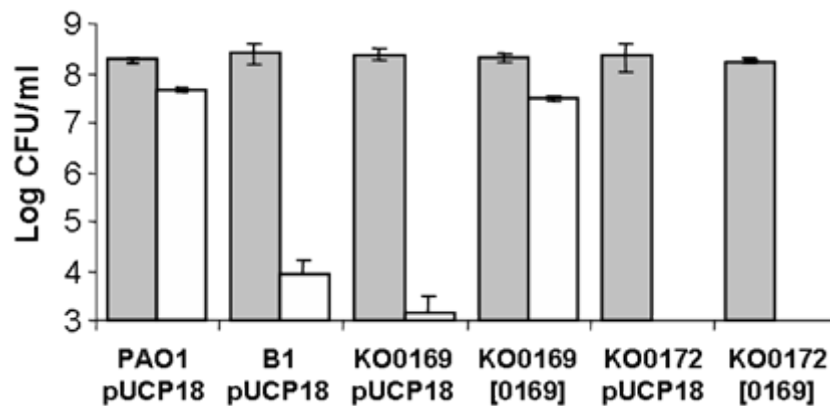


Fig. 4. CFU counts of different *Pseudomonas aeruginosa* strains after 45 min of exposure to 3.5 mM SDS and a subsequent incubation for an additional 60 min in the presence of 1 mM CCCP (white bars) or methanol as a solvent control (grey bars).

When we tested the strains KO0169 and KO0172 in these survival experiments we found similar results. For strains that were not capable to form aggregates during exposure to SDS, namely PAO-B1, KO0169, and KO0172, the addition of CCCP caused a dramatic drop of the survival rates determined as CFU counts by about 4 orders of magnitude compared to strain PAO1 (Fig. 4). This detrimental effect could be reduced to wildtype levels in strain KO0169 complemented with pUCP18[0169] (Fig. 4), and strains KO0169 and KO0172 complemented with pUCP18[4929] (not shown). In contrast, expression of pUCP18[0172] in strains KO0169 and KO0172, pUCP18[0169] in strain KO0172, and pUCP18[4929] in strain PAO-B1 could not increase the survival rates of the individual strains (not shown). These results clearly demonstrated that strains with the ability to form aggregates during growth with SDS had a more than 500 to 1000-fold increased survival rate under these conditions.

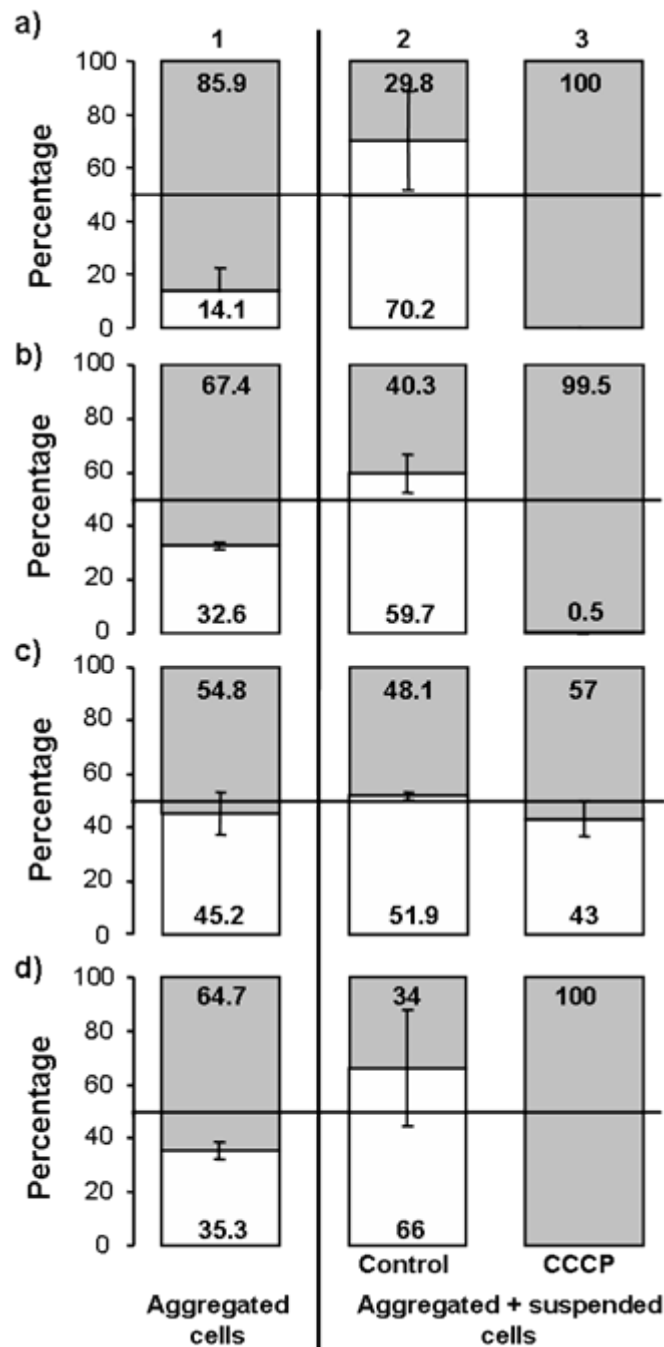


Fig. 5. SDS shock experiments with mixed cell suspensions of different *Pseudomonas aeruginosa* strains. Grey bars indicate percentages of total CFU counts of strains PAO1 pUCP18. White bars indicate percentages of total CFU counts of strains PAO-B1 pUCP18 (a), KO0169 pUCP18 (b), KO0169 pUCP18[0169] (c), KO0172 pUCP18 (d). Percentages were calculated after differentiating single colonies either by their colony morphology on SDS containing agar plates (a, b, d) or by the fluorescence of the YFP-tagged strain PAO1 (c). (1) Percentages of individual strains in macroscopic cell aggregates formed after incubation of mixed cell suspensions with SDS for 45 min. (2-3) Percentages of individual strains in whole cell suspensions (aggregated plus suspended cells) after incubation with SDS for 45 min and additional incubation with methanol as solvent control (Kovach *et al.*) or CCCP (3) for 60 min. Cell aggregates and intact cell suspensions were treated with DNase prior to CFU determination as described previously (Klebensberger *et al.* 2007). Error bars indicate standard deviation (n = 3)

We tested whether strains were able to integrate into aggregates formed by strain PAO1 and, if so, whether they would benefit from this integration during exposure to SDS in the presence of CCCP. Therefore, we started mixed cell suspension experiments as described previously (Klebensberger *et al.* 2007). We found that strain PAO-B1 accounted for 14.1 % of the total CFUs released from aggregates formed by strain PAO1 (Fig. 5a1). The percentage determined with strains KO0169 (32.6%) and KO0172 (35.3%) were more than twice as high as with strain PAO-B1 under these conditions (Fig. 5b1, 5d1). These results demonstrated that, although strain B1, KO0169, and KO0172 were incapable of aggregate formation during exposure to SDS, they could integrate into cell aggregates of the parent strain but with different efficiencies. However, when mixed cell suspensions were exposed to SDS in the presence of CCCP, the percentage of CFUs accounting for the individual strains dropped to 0.5% (KO0169) (Fig. 5b3), or to levels under the detection limit of < 0.5% (B1 and KO0172) (Fig. 5a3, 5d3) compared to the solvent controls (Fig. 5a2, 5b2, 5d3). Similar results were obtained with strains KO0172 pUCP18[0169], KO0172 pUCP18[0172], and B1 pUCP18[4929] (not shown). If strain KO0169 was complemented with pUCP18[0169], its percentage within aggregates formed by the parent strain increased to 45.2% (Fig. 5c1). Complementation with pUCP18[0169] also increased the survival rate of strain KO0169 to 43% after the addition of CCCP (Fig. 4b3).

Northern blot analysis of the cupA1 gene

Complementation of strains KO0169 and KO0172 indicated that PA0172 and PA0169 could represent genes involved in a c-di-GMP dependent signalling pathway that is involved in SDS-induced aggregation. To test whether the *cupA* cluster could represent a target of such a signalling pathway, we performed Northern blot analysis with suspensions of SDS grown- and succinate grown cells with a specific probe for *cupA1*.

By hybridization of RNA samples (10 µg total RNA per lane) with a *cupA1* specific probe, we detected a specific transcript of >700 bases length, which is slightly longer than the *cupA1* gene itself (551Bp). This observation is in agreement with earlier Northern blot analyses of the *cupA1* transcript (Vallet *et al.* 2004). We found that in cells of strain PAO1 grown with SDS, the *cupA1* transcript was increased by about 6-fold compared to cells of strain PAO1 grown with succinate (Fig 6b1, 6b2). In strains KO0169 and KO0172 the level of the *cupA1* transcript was strongly reduced in SDS-grown cells compared to strain PAO1, and even lower than the levels detected in succinate-grown cells of strain PAO1 (Fig. 6b4, 6b7). The levels of the *cupA1* transcript in SDS-grown cells could be decreased in strain PAO1 to levels of succinate-grown cells by expressing the PDE CC3396 from *Caulobacter crescentus* (Fig. 6b3). In this context it should be mentioned that the expression of the potential DGC PA4929 of strains KO0169 and KO0172 during growth with SDS led to increased levels of

the *cupA1* transcript similar to those observed in SDS-grown cells of strain PAO1 (not shown). These results demonstrated an increased detection of *cupA1* transcripts during growth with SDS which could be influenced by the expression of genes manipulating the turnover of c-di-GMP. Furthermore, it strongly indicated the involvement of PA0169 and PA0172 in the regulation of *cupA* transcription.

Expression of pUCP[0169] in strain KO0169 increased the *cupA1* transcript signal in SDS-grown cells to wildtype levels (Fig. 6b5), but had no effect in strain KO0172 (Fig. 6b8). Expression of pUCP18[0172] had no effect on the levels of the *cupA1* transcript in both strains KO0169 and KO0172 (Fig 6b6, 6b9).

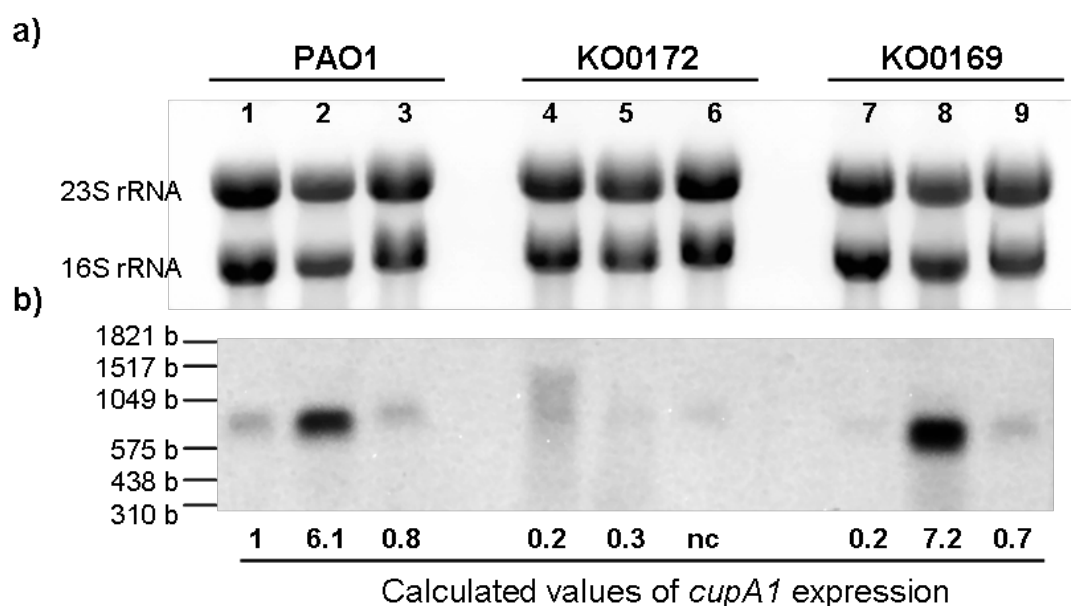


Fig. 6. Northern blot analysis of cell suspension experiments of different *Pseudomonas aeruginosa* strains supplied with 10 mM succinate (1) or 3.5 mM SDS (2-9). a) 23S- and 16S rRNA from the different total RNA samples (10 μ g) after size fractionation (1% agarose gel). b) Signals from the respective RNA samples after transfer to a nitrocellulose membrane and subsequent hybridization with a *cupA1* specific probe. Corresponding length standards of the DIG labeled RNA Molecular Weight Marker I (Roche) are indicated. The values of *cupA1* expression were calculated with the software GelScan5™ software (BioSciTec) as described in material and methods. Numeration indicates plasmids expressed in the individual strains: pUCP18 (1, 2, 4, 7), pBBR[CC3396] (3), pUCP18[0169] (5, 8), pUCP18[0172] (6, 9).

Transcriptional analysis of the cupA operon

To investigate whether the results of the Northern blot analysis were a consequence of increased transcription of the *cupA* operon, we used the previously described transcriptional fusion vector pMP220[*cupA1L::lacZ*], to determine the specific β -galactosidase activity in cell-free extracts of SDS- and succinate-grown cells (Vallet *et al.* 2004).

In cell-free extracts of SDS-grown cells, we found only a slight increase in specific β -galactosidase activity in strain PAO1, compared to strains KO0169 and KO0172 (Fig. 7), which is in contrast to the levels of *cupA1* transcripts detected in these cells (Fig. 6). We also found no significant difference in specific β -galactosidase activity in cell-free extracts of SDS-grown cells compared to succinate-grown cells of strain PAO1 (Fig. 7). In cell-free extracts of succinate-grown cells of strain PAO-P47 (Diggle *et al.* 2002), we found about 10-fold higher β -galactosidase activity compared to cell-free extracts of SDS- or succinate-grown cells of strains PAO1, KO0172, and KO0169. This increased transcription can be explained by the fact that the transcriptional regulator MvaT acts as a repressor of the *cupA* operon (Vallet *et al.* 2004). In addition, we found a 4-fold increase in specific β -galactosidase activity in cell-free extracts of SDS-grown cells of strain PAO-P47 as compared to succinate-grown cells, indicating a transcriptional activation of the *cupA* gene cluster during growth with SDS in the absence of the transcriptional regulator MvaT. In cell-free extracts of succinate-grown cells, the specific β -galactosidase activity was slightly decreased in strain PAO-P47-0169 compared to cell-free extracts of strain PAO-P47 under these conditions. But more importantly, in cell-free extracts of SDS-grown cells the specific β -galactosidase activity was not increased in strain PAO-P47 compared to succinate-grown cells. These results strongly suggested that the transcriptional activation of the *cupA* gene cluster in the absence of the transcriptional regulator MvaT was dependent on the presence of PA0169. However, in strains where the transcriptional regulator MvaT was present, such differential transcription of the *cupA* operon could not be observed, indicating a complex transcriptional control of the *cupA* operon and/or posttranscriptional modifications of the respective mRNA.

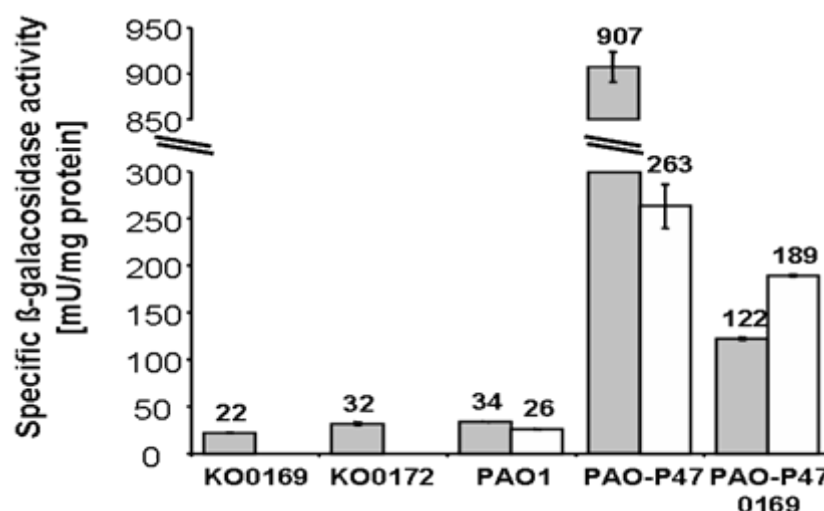


Fig. 7. Specific β -galactosidase activity in cell-free extracts of different *Pseudomonas aeruginosa* strains harbouring a *cupA1-lacZ* transcriptional fusion pMP220[*cupA1L-lacZ*]. Cell-free extracts were obtained from suspensions of succinate-grown (*white bars*) or SDS-grown cells (*grey bars*) incubated with their respective substrate for 90 min at 30°C with shaking at 120 rpm. *Error bars* indicate standard deviation ($n = 3$).

mRNA stability studies

Posttranscriptional modifications of mRNA cannot be detected with transcriptional fusions. Such modifications include mechanisms which influence the translation of the respective mRNA or their stability (Carpousis 2003; Gottesman 2005; Storz *et al.* 2005). As mentioned above, the results of the β -galactosidase assay comparing SDS-grown cells and succinate-grown cells did not reflect the results obtained with the Northern blot analysis. Thus, we speculated whether this could be the result of a different stability of the respective mRNA in cells grown with SDS compared to cells grown with succinate. To test this hypothesis we determined the half-life time of the *cupA1* mRNA from cells of strains PAO-P47 and PAO-P47-0169 grown with SDS or succinate.

In succinate-grown cells of strain PAO-P47, we determined a half-life time of the *cupA1* transcript of about 14.5 min (Fig. 8). When cells of strain PAO-P47 were grown with SDS, the half-life time of the *cupA1* transcript increased almost 2-fold to about 27 min, indicating enhanced stability under these conditions. This stabilising effect was completely lost in SDS-grown cells of strain PAO-P47-0169, and the calculated half-life time of the *cupA1* transcript in this strain was even shorter than in succinate-grown cells of strain PAO-P47 (10 min. vs. 14.5 min).

These results strongly indicated a difference in mRNA stability of the *cupA1* transcript during growth with SDS, and an important role for PA0169 in the regulation of this process.

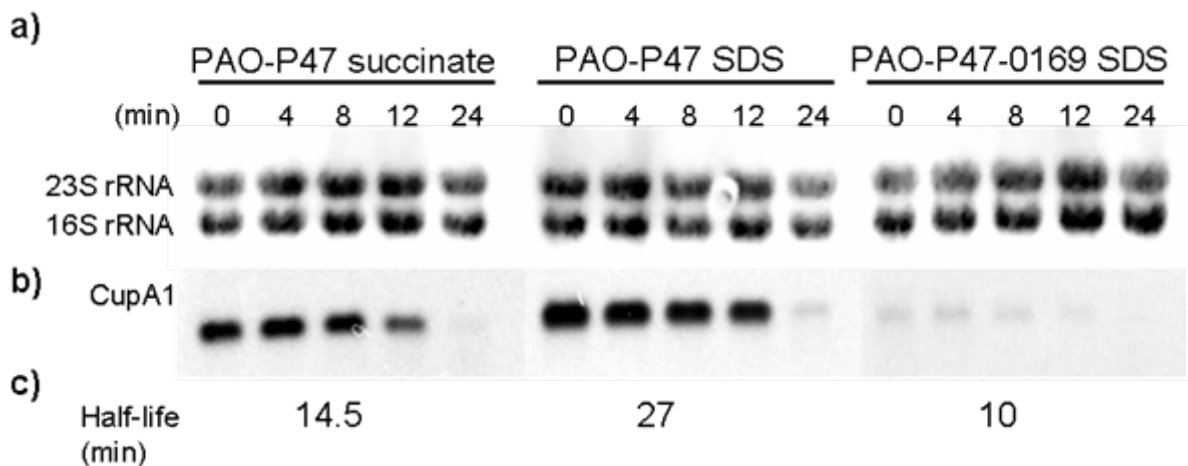


Fig. 8. Northern blot analysis of mRNA stability (*cupA1*) in different *Pseudomonas aeruginosa* strains. a) 23S- and 16S rRNA from the different RNA samples (5 μ g) after size fractionation (1% agarose gel). b) Signal obtained from respective RNA samples (a) after transfer to a nitrocellulose membrane and subsequent hybridization with a *cupA1* specific probe. c) Half-life of the *cupA1* mRNA. The half-life was calculated from the regression line obtained by plotting the calculated values of the *cupA1* transcript with the software GelScan5[™] software (BioSciTec) as described in material and methods.

7.5 Discussion

The goal of our study was to find genes involved in the SDS-induced aggregation in *Pseudomonas aeruginosa* and to investigate the potential c-di-GMP signalling pathway that might be required in this process. By transposon mutagenesis we identified several mutants with a non-aggregative phenotype during growth with SDS. In a previous study, we described two of those mutants defective in the *psl* gene cluster (*pslF*, *pslJ*). We demonstrated that the *psl* cluster is required for aggregate formation during growth with SDS and for the ability of the cells to integrate into aggregates formed by the parent strain. These results are in good agreement with other studies that demonstrated the importance of this gene cluster in adhesion and in maintaining the structure within biofilms (Ma *et al.* 2006; Overhage *et al.* 2005).

In the present study, we describe another set of mutants with a non-aggregative phenotype during growth with SDS. Five of these mutants were mutated in the *cupA* gene cluster. The *cupA* gene cluster represents one of three different clusters (*cupA*, *cupB*, *cupC*) which encode genes responsible for the synthesis and assembly of adhesive fimbria in *Pseudomonas aeruginosa* (Vallet *et al.* 2001). The *cupA* gene cluster has been shown to have essential functions in adhesion to abiotic surfaces and the formation of biofilms. For the *cupB* and *cupC* gene cluster, a role in pellicle formation at the air-liquid interface has been demonstrated (Kulasekara *et al.* 2005).

We found that mutants deficient in *cupA1* or *cupA3* were unable to form aggregates during growth with SDS and had a strongly decreased ability to co-integrate into aggregates formed by strain PAO1 upon SDS exposure. This observation demonstrated an essential role of the *cupA* gene cluster in the formation of aggregates in strain PAO1 during SDS exposure. This conclusion was confirmed by Northern blot analysis evaluating the levels of the *cupA1* transcript. We clearly showed increased levels of the *cupA1* transcript in SDS-grown cells in strain PAO1. Furthermore, we found that these levels could be strongly influenced by the expression of genes manipulating the turnover of the second messenger c-di-GMP. The hypothesis, that a c-di-GMP signalling pathway is involved in regulation of the *cupA* gene cluster is further supported by the recent identification of a two-component system which controls the expression of the *cupB* and *cupC* gene cluster (Kulasekara *et al.* 2005). In this system, the sensor kinase RocS1 interacts with two different response regulators namely, the DNA binding protein RocA1, which acts as a transcriptional activator of the *cupB* and *cupC* genes, and RocR which is an antagonist of RocA1 activity. Interestingly, RocR harbours a conserved EAL (EVL) domain, suggesting that this protein might exhibit enzymatic activity by hydrolysing the second messenger c-di-GMP (Christen *et al.* 2005; Galperin *et al.* 2001).

In the present study, we identified genes (PA0172 and PA0169) that are most likely components of a c-di-GMP signalling system which is involved in the regulation of the *cupA* expression and SDS-induced aggregation. Mutants in these genes were deficient in SDS-induced aggregation and showed no increase in *cupA1* transcript levels during growth with SDS. Expression of PA0169 in strain KO0169 but not in strain KO0172 restored SDS-induced aggregation and increased the *cupA1* transcript levels to wildtype levels during growth with SDS, suggesting regulation of PA0169 activity by PA0172. However, as strain KO0172 could also not be complemented by the expression of PA0172, we cannot exclude possible polar effects in this mutant. Interestingly, we could not find significant differences in *cupA1* transcription between the two mutants and the parent strain in transcriptional β -galactosidase assays. This raised the question whether the results of the Northern blot analysis were actually due to transcriptional activation of the *cupA* operon or a consequence of posttranscriptional regulation of the respective mRNA. Environmental signals and additional factors which regulate transcription of the *cupA* gene cluster besides MvaT have not been identified so far. We clearly demonstrated that in the absence of MvaT, SDS triggered transcriptional activation of the *cupA* genes by about 4-fold, and that this activation was strictly dependent on the presence of PA0169. Given that PA0169 has DGC activity, it would be very likely that c-di-GMP is such a factor that activates transcription of the *cupA* gene cluster either directly or indirectly. In addition, we also obtained strong evidence of PA0169-dependent increase in stability of the *cupA1* transcript upon SDS exposure. Regulation of degradation and translation of a respective mRNA are key mechanisms of posttranscriptional regulation. In these processes, mRNA is regulated by small noncoding RNAs which either bind directly to the respective mRNA by RNA:RNA pairing, or they bind to proteins that interacts with the respective mRNA (Carpousis 2003; Gottesman 2005; Storz *et al.* 2005). In the context of c-di-GMP signalling pathways, it has also been speculated that posttranscriptional regulation of specific targets could be mediated by c-di-GMP itself by direct interactions with the respective mRNA or small regulatory RNAs (Jenal and Malone 2006). Whether SDS-induced regulation of the *cupA1* is a consequence of transcriptional activation, posttranscriptional modification, or both processes remains to be determined. Nevertheless, the expression of the adhesive fimbria encoded by the *cupA* gene cluster appears to be controlled by a complex regulatory network including the potential c-di-GMP signalling pathway identified in this study. This conclusion is further supported by a recent study which uncovered that the *cupA* genes are subject to phase-variable expression in *Pseudomonas aeruginosa* (Vallet-Gely *et al.* 2005). The authors speculated that this phase-variable expression could contribute to the overall fitness of a population under varying conditions, and postulated specific environmental stimuli that trigger phase-variable expression. As outlined already in the introduction, we found that aggregation is a

dispensable but pre-adaptive response of a subpopulation towards SDS to ensure survival under varying environmental conditions. We therefore speculate that SDS-induced stress represents such an environmental stimulus which triggers aggregation via PA0172 and PA0169 by regulation of phase-variable *cupA* gene expression.

7.6 □□ Acknowledgments

The authors like to thank Urs Jenal, Jacob Malone, and Alexander Boehm from the Biozentrum Basel for the gift of pALMAR3 and pBBR[CC3396] as well as for helpful discussions. Marc Rochas and Jens Steinbrenner from the plant physiology and biochemistry group of Iwona Adamska are acknowledged for helpful discussions about RNA work. Technical assistance from Antje Karst and continuous support from Bernhard Schink is acknowledged. This work was funded by a grant from the *Deutsche Forschungsgemeinschaft* to BP (PH711/2-1).

8 Discussion

Growth with toxic detergents as sole source of carbon and energy is a challenge for bacteria. On the one hand, they have to invest part of their energy into protection and repair mechanisms in order to resist the toxic effects of the detergent. On the other hand, they have to take up the detergent in order to metabolize it, thereby increasing the risk of intracellular damage. To find out how bacteria can cope with such a problematic situation, we investigated the growth of the opportunistic pathogen *Pseudomonas aeruginosa* with the toxic anionic detergent sodium dodecyl sulfate (SDS) as model substrate. From the results of this thesis, we established the model shown in Fig. 1 that provides the basis for the general discussion.

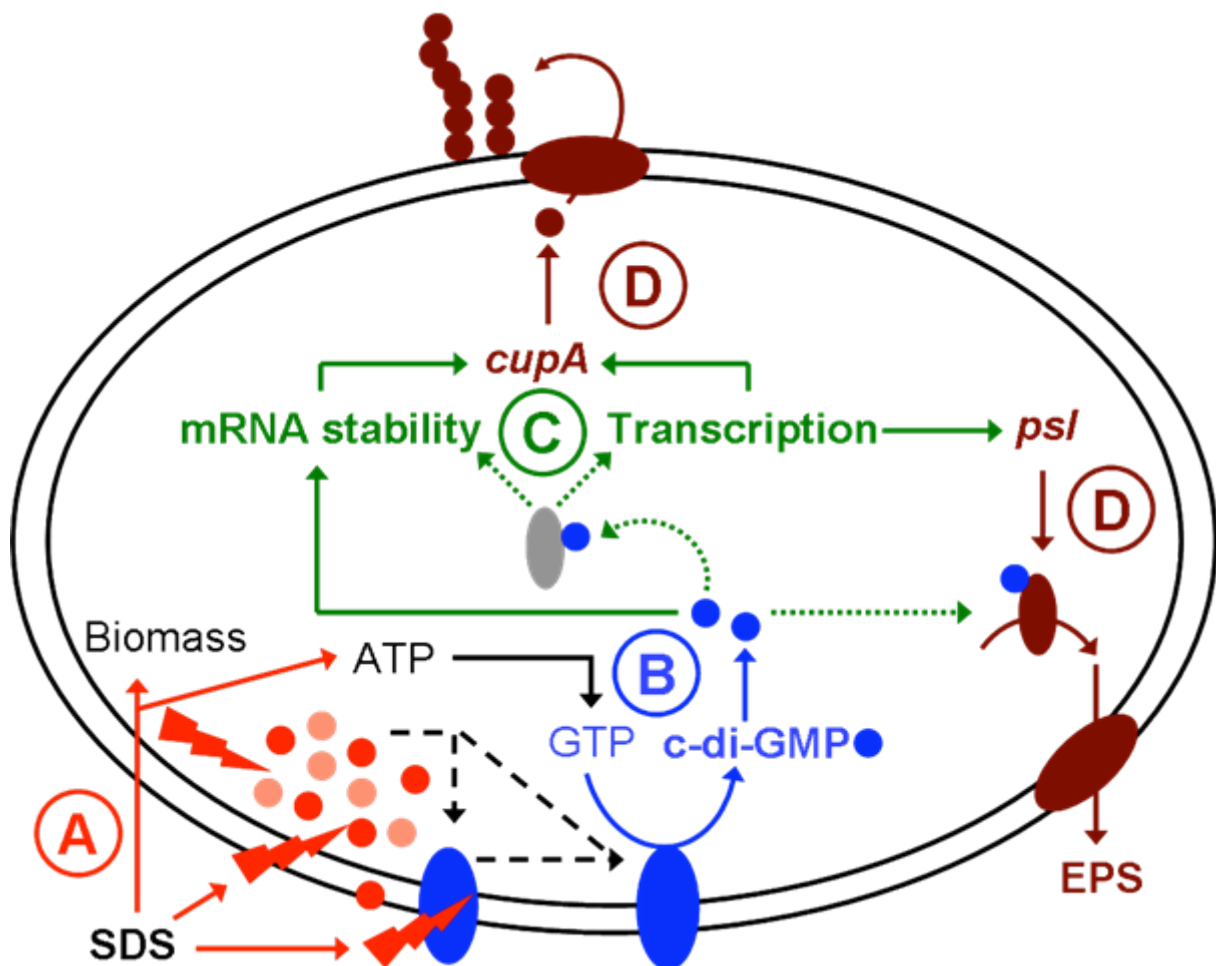


Fig. 1 Proposed model for the SDS-induced regulation of aggregate formation in *Pseudomonas aeruginosa* strain PAO1. A) Putative signals for aggregate formation in a subpopulation of cells triggered by SDS. B) Signal transduction to the second messenger c-di-GMP via the pathway encoded by the genes PA0172-PA0169. C-D) Mechanisms and Targets for c-di-GMP signalling. Further details of this model are discussed in the text.

SDS as a trigger for aggregation in a subpopulation of P. aeruginosa (Fig. 1 A)

The response of freely suspended cells towards the presence of the toxic detergent SDS was strictly energy-dependent (Chapter 5). We demonstrated complete lysis of cells under strong energy limitation which confirmed the requirement of energy-dependent resistance mechanisms described by others (Nikaido 2003; Poole 2004; Rajagopal *et al.* 2002). During energy limitation of the cells by addition of potassium cyanide, cyanide-insensitive terminal oxidases generated sufficient energy to survive SDS-exposure, but not enough for the formation of macroscopic aggregates. Only under high-energy supply, and therefore growth permitting conditions, a subpopulation responded with aggregate formation. The accumulation of damaged cells within the aggregates, compared to the cells that remained freely suspended, strongly implicated a direct link between growth, cell damage, and aggregate formation (Chapter 5). This might be a consequence of re-arrangements in surface structures of dividing cells which may increase their susceptibility to the toxic effects of SDS. Thus, these effects could represent putative signals for cells to respond with aggregate formation in order to protect themselves. The formation of aggregates or biofilms as a protective response towards toxic compounds appears feasible, and has been described also for other substances such as organic solvents, hydrogen peroxide, or antibiotics (Bossier and Verstraete 1996a; Farrell and Quilty 2002; Hoffman *et al.* 2005). Another possibility is that undamaged cells might respond to compounds that descend from cell lysis, such as phospholipids and fatty acids. An indication of the involvement of fatty acids in aggregate formation was found by the isolation of a plasmid which restored the formation of aggregates in the non-aggregative strain N (Chapter 6). The plasmid encoded an acyl carrier protein (PA1896), most likely involved in the biosynthesis of fatty acids. However, further investigations with a deletion mutant in PA1896 showed no difference in aggregate formation during growth with SDS compared to strain PAO1 (unpublished). Finally, the signal could also be generated during the metabolism of SDS inside the cell. Several short-chain fatty acids which might accumulate to a certain extent during the degradation of SDS are known to exhibit toxic effects for microorganisms (Nieman 1954; Teh 1974).

The existence of non-aggregative mutants indicated that aggregation is not a prerequisite for growth with SDS (Chapter 6, Chapter 7). This raised the question whether toxicity is indeed the trigger for aggregate formation. However, a direct linkage of toxicity and aggregate formation was found in SDS shock experiments under strong energy limitation. This experiments revealed increased survival rates of aggregated cells compared to freely suspended cells (Chapter 5) and of strains that responded with aggregate formation compared to non-aggregative strains (Chapter 6, Chapter 7). From these results, we concluded that SDS triggered an aggregative phenotype in a subpopulation which represents

an adaptive strategy to ensure survival under varying environmental conditions. Nevertheless, the nature of the signal that is responsible for SDS-induced aggregation remains to be elucidated. One possible approach for signal identification would represent a screen for additional environmental conditions or compounds which activate a similar response in cells of *P. aeruginosa* like the anionic detergent SDS.

To our knowledge, SDS-induced aggregation represents the first identified environmental stimulus which triggers the development of an aggregative phenotype in the opportunistic pathogen *P. aeruginosa*. Phenotypic variants with an autoaggregative phenotype have previously been shown to appear regularly during growth, especially during long-term incubations or in biofilms (Drenkard and Ausubel 2002; Kirisits *et al.* 2005; Webb *et al.* 2004). Furthermore, such phenotypes are often isolated from sputum of patients with cystic fibrosis infected by *Pseudomonas aeruginosa* (Costerton *et al.* 1999; Häußler *et al.* 2003). Cystic fibrosis is a genetic defect that arises by the mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, leading to defective ion transport (Na^+ , Cl^- , HCO_3^-) of epithelial cells. A consequence of this defect is an altered composition of the lung fluid, finally resulting in chronic infections by *Pseudomonas aeruginosa* and other bacterial species (*Staphylococcus aureus*, *Burkholderia cepacia*) (Lyczak *et al.* 2002). Interestingly, most defense mechanisms against bacterial infections within the lung are based on toxic compounds such as cationic antimicrobial peptides, reactive oxygen species (ROS) released by macrophages and neutrophils, and surface active compounds such as phospholipids. Thus, identification of the detergent SDS as a stimulus for aggregation could provide further insights into the pathogenicity of this organism because this response might represent one of the early mechanisms in the establishment in *Pseudomonas aeruginosa* infections. Whether strains able to respond with SDS-induced aggregation are indeed more virulent than non-aggregative strains remains to be determined. However, the significant decrease in production of the virulence factor pyocyanine of the non-aggregative strains N and PAO-D4 during growth with SDS (Chapter 6), might be an indication for this (Lau *et al.* 2004).

Signal transduction (Fig. 1B)

The development of the aggregative phenotypes described above have been repeatedly linked to regulatory systems involved in the metabolism of the second messenger cyclic di-guanosine monophosphate (c-di-GMP) (D'Argenio *et al.* 2002; Drenkard and Ausubel 2002; Goymer *et al.* 2006; Hickman *et al.* 2005). However, information about environmental triggers of such c-di-GMP-dependent signalling pathways are limited. By complementation studies with genes responsible for the turnover of c-di-GMP, we obtained first indications for the involvement of this second messenger in the SDS-induced

aggregation (Chapter 6). By transposon mutagenesis, we finally identified a novel putative c-di-GMP-dependent signalling pathway required for this process (Chapter 7). The pathway is encoded by a gene cluster consisting of at least 4 genes (PA0172-PA0169) with unknown function. Whether the genes upstream of this cluster (PA0173-PA0181), which are likely to be involved in a chemotaxis-like two-component system, are also part of the SDS-induced signalling pathway is unknown so far. However, the conserved domains encoded by PA0172, namely a sigma factor PP2C-like phosphatase and a HAMP domain (Appleman *et al.* 2003; Aravind and Ponting 1999), implicated that this protein represents most likely a sensor for the signal triggered by SDS. The conserved GGDEF domain of PA0169 implicates that this gene encodes for a DGC, responsible for downstream signal propagation via the synthesis of c-di-GMP. Deletion of one of these genes resulted in the loss of the SDS-induced aggregative phenotype. Interestingly, expression of the putative DGC PA0169 restored this loss-of-function in the PA0169 mutant, but not in the PA0172 mutant, suggesting regulation of PA0169 activity by PA0172. However, the putative DGC activity of PA0169 and the possible regulation of activity via PA0172 have to be confirmed experimentally by purification and biochemical characterization of the respective proteins as an example. The role of the genes PA0170 and PA0171 within this pathway is unclear, but a recent study demonstrated that transposon insertion in gene PA0171 results in the development of an autoaggregative phenotype (D'Argenio *et al.* 2002). This strongly indicated activation of the signalling pathway uncoupled from the signal triggered by SDS. Whether this permanent activation is a result of increased expression of genes downstream of the transposon or due to loss of gene PA0171 remains to be determined. Further investigation by site-directed mutagenesis in gene PA0171 in strain PAO1 and the strains KO0172 and KO0169 are currently in progress.

From our results, we speculate that the signal triggered by SDS-exposure is perceived by the protein encoded by gene PA0172. Signal perception leads to altered activity of the phosphatase domain, resulting in activation of the putative DGC encoded by gene PA0169 which is responsible for increased biosynthesis of c-di-GMP.

Regulation of target genes (Fig. 1C-D)

Beside the identification of the signalling transduction system described above, we also uncovered specific target genes of this pathway which are organized in two gene clusters and responsible for the expression of adhesive surface structures (Chapter 6, Chapter 7). The *psl* gene cluster was required for aggregate formation and co-integration of cells into aggregates formed by the parent strain, as shown with the isolation of two non-aggregative transposon mutants deficient in the genes *pslF* and *pslJ* (Chapter 6). This finding is in agreement with recent studies which demonstrated essential

functions of the *psl* genes in biofilm formation and development (Campisano *et al.* 2006; Matsukawa and Greenberg 2004; Overhage *et al.* 2005). Staining with fluorescently labeled ConA lectins indicated that the mannose- and glucose-rich *psl*-dependent polysaccharide could be a part of the EPS in SDS-induced aggregates (Friedman and Kolter 2004).

The *cupA* gene cluster responsible for the expression of adhesive fimbria was also found to be essential for aggregate formation during growth with SDS (Chapter 7). We demonstrated transcriptional activation of the *cupA* gene cluster during growth with SDS by Northern Blot analysis (Chapter 7) and confirmed this result by microarray analysis comparing SDS-grown cells and succinate-grown cells (unpublished). Fimbria expression of cells was supported by the high amount of curli or fiber-like structures detected within SDS-induced aggregates by Scanning Electron Microscopy (Fritz 2004). These results represent so far the first defined environmental trigger that activates transcription of the *cupA* genes. The existence of such specific triggers have been proposed in a recent study which demonstrated a phase variable expression of the *cupA* genes (Vallet-Gely *et al.* 2005). The dependency of the transcriptional activation from the signalling pathway described above strongly implicated that the second messenger c-di-GMP is responsible for the regulation of *cupA* gene transcription either directly or indirectly. This hypothesis was further supported by decreased levels of the *cupA1* transcript in strain PAO1 expressing the known phosphodiesterase CC3396 and the identification of c-di-GMP-dependent regulatory system of adhesive fimbria encoded by the gene clusters *cupB* and *cupC* in a recent study (Kulasekara *et al.* 2005). In addition, we obtained strong evidence for another regulatory mechanism of the *cupA* gene expression via posttranscriptional stabilization of the respective mRNA during growth with SDS (Chapter 7). Like the transcriptional activation of the *cupA* gene cluster, also the posttranscriptional regulation was shown to be strictly dependent on the presence of the putative DGC encoded by gene PA0169, indicative of a c-di-GMP-dependent process.

Our knowledge about specific target molecules and the mechanisms involved in c-di-GMP-dependent signalling is limited. However, it has been suggested that c-di-GMP could regulate cellular functions at different levels such as transcription, posttranscription, translation, and enzyme activity. We currently do not know how the signal transduction via PA0172-PA0169 regulates the expression of *psl* genes. Transcriptional control of the *psl* gene cluster appears very likely, because such a mechanism has already been suggested in a recent study (Hickman *et al.*). For the *cupA* genes we demonstrated transcriptional activation in response to SDS, which could be mediated by binding of c-di-GMP to a so far unidentified regulator, resulting in altered transcription of the *cupA* genes. The existence of a regulatory sequence (*cis*-controlling element) within the intergenic region upstream of the *cupA* gene cluster which could bind such a transcription factor has been suggested recently

(Vallet *et al.* 2004). A similar mechanism could also be responsible for the posttranscriptional control of the stability of the respective mRNA. In such a scenario, c-di-GMP might interact with mRNA binding proteins, such as Hfq, thereby influencing the stability of the transcript within the cell (Gottesman 2004). Interestingly, c-di-GMP is itself a RNA molecule. Therefore, a direct binding of c-di-GMP to specific mRNA sequences by RNA::RNA pairing could be responsible for changes in secondary structure of the target mRNA, finally resulting in altered turnover of the mRNA. Such a mechanism has been postulated recently, but experimental evidence is currently missing (Jenal and Malone 2006).

With our data, we demonstrated the complexity of cellular responses in *Pseudomonas aeruginosa* towards SDS which has been shown to be mediated by a novel signalling system controlling aggregation via expression of adhesive surface structures. To explore the molecular mechanisms of these cellular responses, we currently try to identify the transcriptome of the PA0169 knockout mutant during growth with SDS.

9 Appendix

Record of achievement

Unless mentioned otherwise, the results described in this thesis were exclusively performed by myself or under my direct supervision.

Research done in cooperation

Chapter 5. Scanning Electron Microscopy was done by Christiane Dittrich from the chair of neurobiology at the University of Konstanz. Determination of sulfatase activities in cell-free extracts and the establishment of a photometrical assay for the quantification of sodium dodecyl sulfate were done by Oliver Rui during his diploma thesis at the chair of microbial ecology at the University of Konstanz (Rui 2003).

Chapter 6. The position of transposon insertion sites was determined in cooperation with Trenzyme GmbH, Konstanz, Germany. Confocal Laser Scanning Microscopy was done in cooperation with Daniel Bressler and Jost Wingender from the Biofilm Centre at the University of Duisburg-Essen.

Chapter 7. The identification of the insertion sites within the mutants obtained by transposon mutagenesis was done in cooperation with Trenzyme GmbH, Konstanz.

10 References

- Aldridge, P., and Jenal, U.** (1999) Cell cycle-dependent degradation of a flagellar motor component requires a novel-type response regulator. *Mol Microbiol* **32**:379-391.
- Aldridge, P., Paul, R., Goymer, P., Rainey, P., and Jenal, U.** (2003) Role of the GGDEF regulator PleD in polar development of *Caulobacter crescentus*. *Mol Microbiol* **47**:1695-1708.
- Allesen-Holm, M., Barken, K. B., Yang, L., Klausen, M., Webb, J. S., Kjelleberg, S., Molin, S., Givskov, M., and Tolker-Nielsen, T.** (2006) A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol* **59**:1114-1128.
- Alonso, A., Rojo, F., and Martinez, J. L.** (1999) Environmental and clinical isolates of *Pseudomonas aeruginosa* show pathogenic and biodegradative properties irrespective of their origin. *Environ Microbiol* **1**:421-430.
- Appleman, J. A., Chen, L. L., and Stewart, V.** (2003) Probing conservation of HAMP linker structure and signal transduction mechanism through analysis of hybrid sensor kinases. *J Bacteriol* **185**:4872-4882.
- Aravind, L., and Ponting, C. P.** (1999) The cytoplasmic helical linker domain of receptor histidine kinase and methyl-accepting proteins is common to many prokaryotic signalling proteins. *FEMS Microbiol Lett* **176**:111-116.
- Bao, Y., Lies, D. P., Fu, H., and Roberts, G. P.** (1991) An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. *Gene* **109**:167-168.
- Barraud, N., Hassett, D. J., Hwang, S. H., Rice, S. A., Kjelleberg, S., and Webb, J. S.** (2006) Involvement of Nitric Oxide in Biofilm Dispersal of *Pseudomonas aeruginosa*. *J Bacteriol* **188**:7344-7353.
- Beveridge, T. J.** (1999) Structures of gram-negative cell walls and their derived membrane vesicles. *J Bacteriol* **181**:4725-4733.
- Bjarnsholt, T., and Givskov, M.** (2006) The role of quorum sensing in the pathogenicity of the cunning aggressor *Pseudomonas aeruginosa*. *Anal Bioanal Chem.* **387**:409-414.
- Bossier, P., and Verstraete, W.** (1996a) *Comamonas testosteroni* colony phenotype influences exopolysaccharide production and coaggregation with yeast cells. *Appl Environ Microbiol* **62**:2687-2691.
- Bossier, P., and Verstraete, W.** (1996b) Triggers for microbial aggregation in activated sludge? *Appl Microbiol Biotechnol* **45**:1-6.

-
- Caiazza, N. C., and O'Toole, G. A.** (2004) SadB is required for the transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas aeruginosa* PA14. *J Bacteriol* **186**:4476-4485.
- Campisano, A., Schröder, C., Schemionek, M., Overhage, J., and Rehm, B. H.** (2006) PsID is a secreted protein required for biofilm formation by *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **72**:3066-3068.
- Carpousis, A. J.** (2003) Degradation of targeted mRNAs in *Escherichia coli*: regulation by a small antisense RNA. *Genes Dev* **17**:2351-2355.
- Chan, C., Paul, R., Samoray, D., Amiot, N. C., Giese, B., Jenal, U., and Schirmer, T.** (2004) Structural basis of activity and allosteric control of diguanylate cyclase. *Proc Natl Acad Sci USA* **101**:17084-17089.
- Chang, A. L., Tuckerman, J. R., Gonzalez, G., Mayer, R., Weinhouse, H., Volman, G., Amikam, D., Benziman, M., and Gilles-Gonzalez, M. A.** (2001) Phosphodiesterase A1, a regulator of cellulose synthesis in *Acetobacter xylinum*, is a heme-based sensor. *Biochemistry* **40**:3420-3426.
- Christen, M., Christen, B., Folcher, M., Schauerte, A., and Jenal, U.** (2005) Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. *J Biol Chem* **280**:30829-30837.
- Clarke, P. H.** (1982) The metabolic versatility of pseudomonads. *Antonie Van Leeuwenhoek* **48**:105-130.
- Cooper, M., Tavankar, G. R., and Williams, H. D.** (2003) Regulation of expression of the cyanide-insensitive terminal oxidase in *Pseudomonas aeruginosa*. *Microbiology* **149**:1275-1284.
- Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., and Lappin-Scott, H. M.** (1995) Microbial biofilms. *Annu Rev Microbiol* **49**:711-745.
- Costerton, J. W., Stewart, P. S., and Greenberg, E. P.** (1999) Bacterial biofilms: a common cause of persistent infections. *Science* **284**:1318-1322.
- Cunningham, L., Pitt, M., and Williams, H. D.** (1997) The *cioAB* genes from *Pseudomonas aeruginosa* code for a novel cyanide-insensitive terminal oxidase related to the cytochrome bd quinol oxidases. *Mol Microbiol* **24**:579-591.
- D'Argenio, D. A., Calfee, M. W., Rainey, P. B., and Pesci, E. C.** (2002) Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. *J Bacteriol* **184**:6481-6489.
- Déziel, E., Comeau, Y., and Villemur, R.** (2001) Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpilated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *J Bacteriol* **183**:1195-1204.

- Diggle, S. P., Winzer, K., Lazdunski, A., Williams, P., and Cámara, M.** (2002) Advancing the quorum in *Pseudomonas aeruginosa*: MvaT and the regulation of N-acylhomoserine lactone production and virulence gene expression. *J Bacteriol* **184**:2576-2586.
- Drenkard, E., and Ausubel, F. M.** (2002) *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**:740-743.
- Ellis, A. J., Hales, S. G., Ur-Rehman, N. G., and White, G. F.** (2002) Novel alkylsulfatases required for biodegradation of the branched primary alkyl sulfate surfactant 2-butyloctyl sulfate. *Appl Environ Microbiol* **68**:31-36.
- Farrell, A., and Quilty, B.** (2002) Substrate-dependent autoaggregation of *Pseudomonas putida* CP1 during the degradation of mono-chlorophenols and phenol. *J Ind Microbiol Biotechnol* **28**:316-324.
- Friedman, L., and Kolter, R.** (2004) Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. *J Bacteriol* **186**:4457-4465.
- Fritz, E.** (2004) Untersuchungen zur Tensid-induzierten Zell-Zell-Aggregation bei *Pseudomonas aeruginosa*. Diplomarbeit, Department of Biology, University of Konstanz.
- Fux, C. A., Costerton, J. W., Stewart, P. S., and Stoodley, P.** (2005) Survival strategies of infectious biofilms. *Trends Microbiol* **13**:34-40.
- Galperin, M. Y., Nikolskaya, A. N., and Koonin, E. V.** (2001) Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* **203**:11-21.
- Gilbert, P., Maira-Litran, T., McBain, A. J., Rickard, A. H., and Whyte, F. W.** (2002) The physiology and collective recalcitrance of microbial biofilm communities. *Adv Microb Physiol* **46**:202-256.
- Gjermansen, M., Ragas, P., Sternberg, C., Molin, S., and Tolker-Nielsen, T.** (2005) Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. *Environ Microbiol* **7**:894-906.
- Gottesman, S.** (2004) The small RNA regulators of *Escherichia coli*: roles and mechanisms*. *Annu Rev Microbiol* **58**:303-328.
- Gottesman, S.** (2005) Micros for microbes: non-coding regulatory RNAs in bacteria. *Trends Genet* **21**:399-404.
- Goymer, P., Kahn, S. G., Malone, J. G., Gehrig, S. M., Spiers, A. J., and Rainey, P. B.** (2006) Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. II. Role of the GGDEF regulator WspR in evolution and development of the wrinkly spreader phenotype. *Genetics* **173**:515-526.

-
- Hagelueken, G., Adams, T. M., Wiehlmann, L., Widow, U., Kolmar, H., Tümmler, B., Heinz, D. W., and Schubert, W. D.** (2006) The crystal structure of SdsA1, an alkylsulfatase from *Pseudomonas aeruginosa*, defines a third class of sulfatases. *Proc Natl Acad Sci USA* **103**:7631-7636.
- Häußler, S.** (2004) Biofilm formation by the small colony variant phenotype of *Pseudomonas aeruginosa*. *Environ Microbiol* **6**:546-551.
- Häußler, S., Ziegler, I., Lottel, A., von Gotz, F., Rohde, M., Wehmhohner, D., Saravanamuthu, S., Tümmler, B., and Steinmetz, I.** (2003) Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *J Med Microbiol* **52**:295-301.
- Hecht, G. B., and Newton, A.** (1995) Identification of a novel response regulator required for the swarmer-to-stalked-cell transition in *Caulobacter crescentus*. *J Bacteriol* **177**:6223-6229.
- Helenius, A., and Simons, K.** (1975) Solubilization of membranes by detergents. *Biochim Biophys Acta* **415**:29-79.
- Hickman, J. W., Tifrea, D. F., and Harwood, C. S.** (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc Natl Acad Sci USA* **102**:14422-14427.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J., and Schweizer, H. P.** (1998) 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**:77-86.
- Hoben, H. J., and Somasegaran, P.** (1982) Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium spp.* in inoculants made from presterilized peat. *Appl Environ Microbiol* **44**:1246-1247.
- Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z., Jones, R. A., and Miller, S. I.** (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* **436**:1171-1175.
- Hummerjohann, J., Laudénbach, S., Retey, J., Leisinger, T., and Kertesz, M. A.** (2000) The sulfur-regulated arylsulfatase gene cluster of *Pseudomonas aeruginosa*, a new member of the *cys* regulon. *J Bacteriol* **182**:2055-2058.
- Irani, V. R., and Rowe, J. J.** (1997) Enhancement of transformation in *Pseudomonas aeruginosa* PAO1 by Mg²⁺ and heat. *Biotechniques* **22**:54-6.
- Jackson, K. D., Starkey, M., Kremer, S., Parsek, M. R., and Wozniak, D. J.** (2004) Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J Bacteriol* **186**:4466-4475.

- Jacobs, M. A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Chun-Rong, L., Guenther, D., Bovee, D., Olson, M. V., and Manoil, C. (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **100**:14339-14344.
- Jenal, U., and Malone, J. (2006) Mechanisms of Cyclic-di-GMP Signaling in Bacteria. *Annu Rev Genet* **40**:385-407.
- Jørgensen, F., Bally, M., Chapon-Herve, V., Michel, G., Lazdunski, A., Williams, P., and Stewart, G. S. (1999) RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*. *Microbiology* **145**:835-844.
- Kadurugamuwa, J. L., and Beveridge, T. J. (1995) Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J Bacteriol* **177**:3998-4008.
- Kessler, B., de Lorenzo, V., and Timmis, K. N. (1992) A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. *Mol Gen Genet* **233**:293-301.
- Kirisits, M. J., Prost, L., Starkey, M., and Parsek, M. R. (2005) Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **71**:4809-4821.
- Klausen, M., Aes-Jørgensen, A., Molin, S., and Tolker-Nielsen, T. (2003a) Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol Microbiol* **50**:61-68.
- Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aes-Jørgensen, A., Molin, S., and Tolker-Nielsen, T. (2003b) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* **48**:1511-1524.
- Klebensberger, J., Rui, O., Fritz, E., Schink, B., and Philipp, B. (2006) Cell aggregation of *Pseudomonas aeruginosa* strain PAO1 as an energy-dependent stress response during growth with sodium dodecyl sulfate. *Arch Microbiol* **185**:417-427.
- Klebensberger, J., Lautenschlager, K., Bressler, D., Wingender, J., and Philipp, B. (2007) Detergent-induced cell aggregation in subpopulations of *Pseudomonas aeruginosa* as a pre-adaptive survival strategy. *Environ Microbiol* **Accepted**.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop II, R. M., and Peterson, K. M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**:175-176.

-
- Kulasekara, H. D., Ventre, I., Kulasekara, B. R., Lazdunski, A., Filloux, A., and Lory, S.** (2005) A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial *cup* genes. *Mol Microbiol* **55**:368-380.
- Kulasekara, H. D., Lee, V., Brencic, A., Liberati, N., Urbach, J., Miyata, S., Lee, D. G., Neely, A. N., Hyodo, M., Hayakawa, Y., Ausubel, F. M., and Lory, S.** (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. *Proc Natl Acad Sci USA* **103**:2839-2844.
- Lampe, D. J., Akerley, B. J., Rubin, E. J., Mekalanos, J. J., and Robertson, H. M.** (1999) Hyperactive transposase mutants of the Himar1 mariner transposon. *Proc Natl Acad Sci USA* **96**:11428-11433.
- Lau, G. W., Ran, H., Kong, F., Hassett, D. J., and Mavrodi, D.** (2004) *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. *Infect Immun* **72**:4275-4278.
- Lewis, K.** (2001) Riddle of biofilm resistance. *Antimicrob Agents Chemother* **45**:999-1007.
- Linker, A., and Jones, R. S.** (1964) A Polysaccharide Resembling Alginic Acid From A *Pseudomonas* Micro-Organism. *Nature* **204**:187-188.
- Lyczak, J. B., Cannon, C. L., and Pier, G. B.** (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect* **2**:1051-1060.
- Lyczak, J. B., Cannon, C. L., and Pier, G. B.** (2002) Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* **15**:194-222.
- Ma, L., Jackson, K. D., Landry, R. M., Parsek, M. R., and Wozniak, D. J.** (2006) Analysis of *Pseudomonas aeruginosa* conditional Psl variants reveals roles for the Psl polysaccharide in adhesion and maintaining biofilm structure post-attachment. *J Bacteriol.* **188**:8213-8221.
- Manoil, C., and Beckwith, J.** (1985) TnpA: a transposon probe for protein export signals. *Proc Natl Acad Sci USA* **82**:8129-8133.
- Marchesi, J. R., Owen, S. A., White, G. F., House, W. A., and Russell, N. J.** (1994) SDS-degrading bacteria attach to riverine sediment in response to the surfactant or its primary biodegradation product dodecan-1-ol. *Microbiology* **140**:2999-3006.
- Matsukawa, M., and Greenberg, E. P.** (2004) Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **186**:4449-4456.
- Miller, V. L., and Mekalanos, J. J.** (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170**:2575-2583.

- Morgan, R., Kohn, S., Hwang, S. H., Hassett, D. J., and Sauer, K.** (2006) BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. *J Bacteriol* **188**:7335-7343.
- Muscarella, L. F.** (2004) Contribution of tap water and environmental surfaces to nosocomial transmission of antibiotic-resistant *Pseudomonas aeruginosa*. *Infect Control Hosp Epidemiol* **25**:342-345.
- Nickerson, K. W., and Aspedon, A.** (1992) Detergent-shock response in enteric bacteria. *Mol Microbiol* **6**:957-961.
- Nieman, C.** (1954) Influence of trace amounts of fatty acids on the growth of microorganisms. *Bacteriol Rev* **18**:147-163.
- Nikaido, H.** (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* **67**:593-656.
- Nikaido, H., and Vaara, M.** (1985) Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49**:1-32.
- Nyström, T.** (2005) Role of oxidative carbonylation in protein quality control and senescence. *Embo J* **24**:1311-1317.
- O'Toole, G. A., and Kolter, R.** (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30**:295-304.
- Overhage, J., Schemionek, M., Webb, J. S., and Rehm, B. H.** (2005) Expression of the *psl* operon in *Pseudomonas aeruginosa* PAO1 biofilms: PslA performs an essential function in biofilm formation. *Appl Environ Microbiol* **71**:4407-4413.
- Paul, R., Weiser, S., Amiot, N. C., Chan, C., Schirmer, T., Giese, B., and Jenal, U.** (2004) Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. *Genes Dev* **18**:715-727.
- Payne, W. J., and Feisal, V. E.** (1963) Bacterial utilization of dodecyl sulfate and dodecyl benzene sulfonate. *Appl Microbiol* **11**:339-344.
- Poole, K.** (2004) Efflux-mediated multiresistance in Gram-negative bacteria. *Clin Microbiol Infect* **10**:12-26.
- Rainey, P. B., and Travisano, M.** (1998) Adaptive radiation in a heterogeneous environment. *Nature* **394**:69-72.
- Rainey, P. B., and Rainey, K.** (2003) Evolution of cooperation and conflict in experimental bacterial populations. *Nature* **425**:72-74.
- Rajagopal, S., Eis, N., and Nickerson, K. W.** (2003) Eight gram-negative bacteria are 10,000 times more sensitive to cationic detergents than to anionic detergents. *Can J Microbiol* **49**:775-779.

-
- Rajagopal, S., Sudarsan, N., and Nickerson, K. W.** (2002) Sodium dodecyl sulfate hypersensitivity of *clpP* and *clpB* mutants of *Escherichia coli*. *Appl Environ Microbiol* **68**:4117-4121.
- Rodrigue, A., Quentin, Y., Lazdunski, A., Méjean, V., and Foglino, M.** (2000) Two-component systems in *Pseudomonas aeruginosa*: why so many? *Trends Microbiol* **8**:498-504.
- Römling, U.** (2005) Characterization of the *rdar* morphotype, a multicellular behaviour in Enterobacteriaceae. *Cell Mol Life Sci* **62**:1234-1246.
- Römling, U., Gomelsky, M., and Galperin, M. Y.** (2005) C-di-GMP: the dawning of a novel bacterial signalling system. *Mol Microbiol* **57**:629-639.
- Ross, P., Aloni, Y., Weinhouse, H., Michaeli, D., Weinberger-Ohana, P., Mayer, R., and Benziman, M.** (1986) Control of cellulose synthesis *Acetobacter xylinum*. A unique guanyl oligonucleotide is the immediate activator of the cellulose synthase. *Carbohydrate Research* **149**:101-117.
- Rui, O.** (2003) Untersuchungen zum Abbau von und zur Resistenz gegenüber SDS bei *Pseudomonas aeruginosa* Stamm PAO1. Diplomarbeit, Department of Biology, University of Freiburg.
- Rusconi, F., Valton, E., Nguyen, R., and Dufourc, E.** (2001) Quantification of sodium dodecyl sulfate in microliter-volume biochemical samples by visible light spectroscopy. *Anal Biochem* **295**:31-37.
- Ryan, R. P., Fouhy, Y., Lucey, J. F., Crossman, L. C., Spiro, S., He, Y. W., Zhang, L. H., Heeb, S., Cámara, M., Williams, P., and Dow, J. M.** (2006) Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. *Proc Natl Acad Sci USA* **103**:6712-6717.
- Ryjenkov, D. A., Tarutina, M., Moskvina, O. V., and Gomelsky, M.** (2005) Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J Bacteriol* **187**:1792-1798.
- Sambrook, J., Fritsch, E. F., and Maniatis, T.** (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, CSH, NY.
- Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W., and Davies, D. G.** (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* **184**:1140-1154.
- Sauer, K., Cullen, M. C., Rickard, A. H., Zeef, L. A., Davies, D. G., and Gilbert, P.** (2004) Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J Bacteriol* **186**:7312-26.

- Schleheck, D., Dong, W., Denger, K., Heinzle, E., and Cook, A. M.** (2000) An alpha-proteobacterium converts linear alkylbenzenesulfonate surfactants into sulfophenylcarboxylates and linear alkyldiphenyletherdisulfonate surfactants into sulfodiphenylethercarboxylates. *Appl Environ Microbiol* **66**:1911-1916.
- Schleheck, D., Lechner, M., Schonenberger, R., Suter, M. J., and Cook, A. M.** (2003) Desulfonation and degradation of the disulfodiphenylethercarboxylates from linear alkyldiphenyletherdisulfonate surfactants. *Appl Environ Microbiol* **69**:938-944.
- Schleheck, D., Knepper, T. P., Fischer, K., and Cook, A. M.** (2004) Mineralization of individual congeners of linear alkylbenzenesulfonate by defined pairs of heterotrophic bacteria. *Appl Environ Microbiol* **70**:4053-4063.
- Scott, M. J., and Jones, M. N.** (2000) The biodegradation of surfactants in the environment. *Biochim Biophys Acta* **1508**:235-251.
- Simon, R., Priefer, U., Pühler, A.** (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Biotechnology* **1**:784-791.
- Smits, T. H., Balada, S. B., Witholt, B., and van Beilen, J. B.** (2002) Functional analysis of alkane hydroxylases from gram-negative and gram-positive bacteria. *J Bacteriol* **184**:1733-1742.
- Spiers, A. J., Kahn, S. G., Bohannon, J., Travisano, M., and Rainey, P. B.** (2002) Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. I. Genetic and phenotypic bases of wrinkly spreader fitness. *Genetics* **161**:33-46.
- Spiers, A. J., Bohannon, J., Gehrig, S. M., and Rainey, P. B.** (2003) Biofilm formation at the air-liquid interface by the *Pseudomonas fluorescens* SBW25 wrinkly spreader requires an acetylated form of cellulose. *Mol Microbiol* **50**:15-27.
- Spiers, A. J., and Rainey, P. B.** (2005) The *Pseudomonas fluorescens* SBW25 wrinkly spreader biofilm requires attachment factor, cellulose fibre and LPS interactions to maintain strength and integrity. *Microbiology* **151**:2829-2839.
- Spoering, A. L., and Lewis, K.** (2001) Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol* **183**:6746-6751.
- Stavskaia, S. S., Nikovskaia, G. N., Shamolina, I. I., Samoilenko, L. S., Grigor'eva, T. I., and Lusta, K. A.** (1989) [Degradation of alkylsulfates by a *Pseudomonas aeruginosa* culture immobilized on a polyvinyl alcohol fiber]. *Mikrobiologiya* **58**:607-610.
- Steinberger, R. E., and Holden, P. A.** (2005) Extracellular DNA in single- and multiple-species unsaturated biofilms. *Appl Environ Microbiol* **71**:5404-5410.
- Stoodley, P., Sauer, K., Davies, D. G., and Costerton, J. W.** (2002) Biofilms as complex differentiated communities. *Annu Rev Microbiol* **56**:187-209.

-
- Storz, G., Altuvia, S., and Wassarman, K. M.** (2005) An abundance of RNA regulators. *Annu Rev Biochem* **74**:199-217.
- Suh, S. J., Silo-Suh, L., Woods, D. E., Hassett, D. J., West, S. E., and Ohman, D. E.** (1999) Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* **181**:3890-3897.
- Sutherland, I.** (2001) Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* **147**:3-9.
- Tal, R., Wong, H. C., Calhoon, R., Gelfand, D., Fear, A. L., Volman, G., Mayer, R., Ross, P., Amikam, D., Weinhouse, H., Cohen, A., Sapir, S., Ohana, P., and Benziman, M.** (1998) Three *cdg* operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: genetic organization and occurrence of conserved domains in isoenzymes. *J Bacteriol* **180**:4416-4425.
- Teh, J. S.** (1974) Toxicity of short-chain fatty acids and alcohols towards *Cladosporium resinae*. *Appl Microbiol* **28**:840-844.
- Thomas, O. R., and White, G. F.** (1989) Metabolic pathway for the biodegradation of sodium dodecyl sulfate by *Pseudomonas sp.* C12B. *Biotechnol Appl Biochem* **11**:318-327.
- Tielen, P., Strathmann, M., Jaeger, K. E., Flemming, H. C., and Wingender, J.** (2005) Alginate acetylation influences initial surface colonization by mucoid *Pseudomonas aeruginosa*. *Microbiol Res* **160**:165-176.
- Tredget, E. E., Shankowsky, H. A., Rennie, R., Burrell, R. E., and Logsetty, S.** (2004) *Pseudomonas* infections in the thermally injured patient. *Burns* **30**:3-26.
- Ude, S., Arnold, D. L., Moon, C. D., Timms-Wilson, T., and Spiers, A. J.** (2006) Biofilm formation and cellulose expression among diverse environmental *Pseudomonas* isolates. *Environ Microbiol* **8**:1997-2011.
- Vallet, I., Olson, J. W., Lory, S., Lazdunski, A., and Filloux, A.** (2001) The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation. *Proc Natl Acad Sci USA* **98**:6911-6916.
- Vallet, I., Diggle, S. P., Stacey, R. E., Cámara, M., Ventre, I., Lory, S., Lazdunski, A., Williams, P., and Filloux, A.** (2004) Biofilm formation in *Pseudomonas aeruginosa*: fimbrial *cup* gene clusters are controlled by the transcriptional regulator MvaT. *J Bacteriol* **186**:2880-2890.
- Vallet-Gely, I., Donovan, K. E., Fang, R., Joung, J. K., and Dove, S. L.** (2005) Repression of phase-variable *cup* gene expression by H-NS-like proteins in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **102**:11082-11087.

- Webb, J. S., Thompson, L. S., James, S., Charlton, T., Tolker-Nielsen, T., Koch, B., Givskov, M., and Kjelleberg, S.** (2003) Cell death in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **185**:4585-4592.
- Webb, J. S., Lau, M., and Kjelleberg, S.** (2004) Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **186**:8066-8073.
- Weber, H., Pesavento, C., Possling, A., Tischendorf, G., and Hengge, R.** (2006) Cyclic-di-GMP-mediated signalling within the sigma network of *Escherichia coli*. *Mol Microbiol* **62**:1014-1034.
- West, S. E., Schweizer, H. P., Dall, C., Sample, A. K., and Runyen-Janecky, L. J.** (1994) Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **148**:81-86.
- Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C., and Mattick, J. S.** (2002) Extracellular DNA required for bacterial biofilm formation. *Science* **295**:1487.
- Whiteley, M., Bangera, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S., and Greenberg, E. P.** (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413**:860-864.
- Widdel, F., and Pfennig, N.** (1981) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. *Arch Microbiol* **129**:395-400.
- Windgassen, M., Urban, A., and Jäger, K. E.** (2000) Rapid gene inactivation in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **193**:201-205.
- Wozniak, D. J., Wyckoff, T. J., Starkey, M., Keyser, R., Azadi, P., O'Toole, G. A., and Parsek, M. R.** (2003) Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci USA* **100**:7907-7912.