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SiaA and SiaD are essential for inducing autoaggregation as a specific response to detergent stress in *Pseudomonas aeruginosa*

Janosch Klebensberger,¹,² Antoinette Birkenmaier,¹ Robert Geffers,³ Staffan Kjelleberg² and Bodo Philipp¹*

¹Universität Konstanz, Fachbereich Biologie, Mikrobielle Ökologie, Fach M654, 78457 Konstanz, Germany.
²Centre for Marine Bio-Innovation, School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, New South Wales, Australia
³Array Facility / Cell Biology, HCI - Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany

*Corresponding author. E-mail: bodo.philipp@uni-konstanz.de; Tel. (+49) 7531 884541; Fax (+49) 7531 884047.

Running title: Induction of aggregation in *Pseudomonas aeruginosa*
Summary

Cell aggregation is a stress response and serves as a survival strategy for *Pseudomonas aeruginosa* strain PAO1 during growth with the toxic detergent Na-dodecylsulfate (SDS). This process involves the *psl* operon and is linked to c-di-GMP signalling. The induction of cell aggregation in response to SDS was studied. Transposon and site-directed mutagenesis revealed that the *cupA*-operon and the co-transcribed genes *siaA* (PA0172) and *siaD* (PA0169) were essential for SDS-induced aggregation. While *siaA* encodes a putative membrane protein with a HAMP and a PP2C-like phosphatase domain, *siaD* encodes a putative diguanylate cyclase involved in the biosynthesis of c-di-GMP. Complementation studies uncovered that the loss of SDS-induced aggregation in the formerly isolated spontaneous mutant strain N was caused by a non-functional *siaA* allele. DNA-microarray analysis of SDS-grown cells revealed consistent activation of eight genes, including *cupA1*, with known or presumptive important functions in cell aggregation in the parent strain compared to non-aggregating *siaA* and *siaD* mutants. A *siaAD*-dependent increase of *cupA1* mRNA levels in SDS-grown cells was also shown by Northern blots. These results clearly demonstrate that SiaAD are essential for inducing cell aggregation as a specific response to SDS and suggest that they are responsible for perceiving and transducing SDS-related stress.
Introduction

Individual cells within bacterial populations can occur as freely suspended single cells or in cell aggregates, either freely floating or attached to surfaces as biofilms. Formation of aggregates and the dispersal of single cells from aggregates are highly dynamic and coordinated processes, which can be triggered by various environmental cues (Bossier and Verstraete, 1996; Stanley and Lazazzera, 2004; Romeo, 2006). These environmental cues include the availability of carbon and energy sources (Burdman et al., 1998; Sauer et al., 2004; Gjermansen et al., 2005; Thormann et al., 2005; Schleheck et al., 2009) and various stresses. Regarding the latter, dispersal of single cells from cell aggregates can be triggered by oxidative or nitrosative stress (Webb et al., 2003; Barraud et al., 2006), whereas the formation of aggregates can be triggered by toxic compounds such as antibiotics (Hoffman et al., 2005; Gotoh et al., 2008), chlorophenols (Farrell and Quilty, 2002; Fakhruddin and Quilty, 2007) or detergents (Schleheck et al., 2000; Klebensberger et al., 2006; Klebensberger et al., 2007).

Active formation of cell aggregates as a stress response to toxic chemicals is feasible because cells in aggregates are more resistant towards biocides (Lewis, 2001; Gilbert et al., 2002; Drenkard, 2003; Fux et al., 2005). In this respect, aggregation could represent an adaptive strategy for bacteria that use toxic compounds as growth substrates. Such a strategy requires specific molecular modules for sensing and transducing stress signals that indicate cell damage by a toxic substance. These molecular modules subsequently induce aggregation
by affecting the expression or activity of target modules which are responsible for
the production of adhesive surface structures, such as surface proteins or
exopolysaccharides. While knowledge about various target modules and their
regulation is available, information about molecular modules that induce
aggregation is still limited.

Recently, we described cell aggregation as a stress response and survival
strategy in *Pseudomonas aeruginosa* strain PAO1 during growth with the toxic
detergent Na-dodecylsulfate (SDS; Klebensberger *et al.*, 2006; Klebensberger *et
al.*, 2007). We have shown that stress caused by SDS triggers cell aggregation in
an energy-dependent manner. Through genetic studies, we have demonstrated
that the Psl exopolysaccharide is required for SDS-induced cell aggregation.
Furthermore, we have isolated a spontaneous mutant, strain N, which does not
form cell aggregates in response to SDS-stress.

The autoaggregative phenotype of *P. aeruginosa* strain PAO1 during growth with
SDS is reminiscent to previously described constitutively autoaggregative
variants of this organism, such as the small colony variants (SCVs; Häussler,
2004) and the wrinkly spreader (Spiers *et al.*, 2002; Spiers *et al.*, 2003; Hickman
*et al.*, 2005). In contrast, autoaggregation during growth with SDS is a facultative
response, and the isolation of non-aggregative mutants of *P. aeruginosa* strain
PAO1 demonstrates that aggregation is no prerequisite for growth with this toxic
detergent. However, under strong energy limitation by applying the uncoupler
carbonyl cyanide 3-chlorophenylhydrazone (CCCP) as an additional stress, SDS-
induced aggregation was found to confer a strong survival advantage to
aggregated cells in comparison to suspended cells (Klebensberger et al., 2006; Klebensberger et al., 2007). Thus, cell-aggregation can be regarded as a pre-adaptive survival strategy that is inducible by sub lethal stress in order to be prepared for resisting additional stress effects, which might emerge in the near future. Consequently, studies on SDS-induced aggregation offer the chance for identifying the aforementioned molecular modules for inducing autoaggregation in response to a toxic chemical compound.

In SCVs and the wrinkly spreader, autoaggregation is often caused by mutations leading to a constitutive high level of the bacterial second messenger cyclic-diguanosinemonophosphate (c-di-GMP) (Meissner et al., 2007; Starkey et al., 2009). Numerous studies revealed that c-di-GMP is related to a sessile mode of growth and to cell aggregation in Eubacteria (Jenal and Malone, 2006; Hengge, 2009). Diguanylatecyclases (DGCs) and specific phosphodiesterases (PDEs) are responsible for the biosynthesis and the degradation of c-di-GMP, respectively. We obtained strong evidence of c-di-GMP being involved in SDS-induced aggregation because aggregation could be specifically restored in strain N by the overexpression of two genes encoding a known (PA1107; Kulasakara et al., 2006) and a putative (PA4929) DGC. However, both genes were not mutated in strain N, and their insertional inactivation in the wild type strain PAO1 did not cause a loss of SDS-induced aggregation. This indicates that the DGCs encoded by PA1107 and PA4929 are not essential for SDS-induced aggregation.

Thus, the goal of our study was to identify molecular modules that are both, specific and essential for inducing autoaggregation in response to SDS. For this,
we isolated and characterized transposon mutants lacking SDS-induced aggregation. Based on these transposon mutants, we could identify such a molecular module and demonstrated that a 6bp deletion in one of the corresponding genes was sufficient for the loss of SDS-induced aggregation in the spontaneous mutant strain N. Finally, we compared aggregating and non-aggregating cells on the transcriptome level.

**Results**

*Physiological characterization of transposon mutants*

To identify molecular modules that are both, specific and essential for inducing autoaggregation in response to SDS, we screened a transposon mutant library constructed with a mariner transposon for colonies with a smooth appearance on SDS-containing agar plates as described earlier (Klebensberger et al., 2007). Out of 106 smooth colonies, we isolated 22 clones that did not show SDS-induced aggregation in liquid culture, and in 8 of these clones the transposon insertion sites were identified (Fig. 1A).

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 one mutant, strain B1, the mariner transposon was inserted in the *cupA1* gene, which encodes the fimbrial subunit. In four mutants the transposon was inserted in the *cupA3* gene, which encodes the so-called usher protein.
In a further mutant, strain F5, the transposon was inserted in the gene PA0172, which encodes a putative membrane protein of unknown function (Fig. 1A). Domain and sequence analysis of the protein encoded by this ORF with the SMART software tool (http://smart.embl-heidelberg.de/) predicted the existence of two transmembrane helices and revealed two conserved domains, a sigma factor PP2C-like phosphatase and a HAMP domain, which are both known to be involved in signal transduction (Fig. 2A; Bork et al., 1996; Aravind and Ponting, 1999; Appleman et al., 2003). According to the Pseudomonas Genome Database (Winsor et al., 2009), PA0172 is predicted to be co-transcribed with at least two other genes, PA0171 and PA0170, encoding proteins of unknown function. The gene PA0169 located directly downstream of this cluster encodes a protein with a GGEEF domain, which is characteristic for DGCs involved in the biosynthesis of c-di-GMP. Reverse transcription (RT) with a gene specific primer for PA0169 and a subsequent PCR-based analysis using primers targeting the genes PA0172-PA0169 revealed that these genes are co-transcribed (Fig. 1AB).

All transposon mutants mentioned above showed a similar phenotype during growth with SDS. As shown for the mutant strains B1 and F5, these mutants formed smooth colonies on SDS-containing agar plates in contrast to the rough and structured colonies of strain PAO1 (Fig. 3A). In liquid medium, the mutants did not form macroscopic aggregates during growth with SDS (Fig. 3B), and they had a higher growth rate and reached higher final optical densities than strain PAO1 (not shown).

Physiological characterization of the deletion mutant KO0169
The co-transcription of PA0169 encoding a putative DGC together with the gene PA0172 involved in SDS-induced aggregation suggested that PA0169 has a role in SDS-induced aggregation, too. To test this hypothesis, we constructed the deletion mutant strain KO0169. Physiological characterization of this strain during growth with SDS revealed a similar phenotype as strain F5, namely the formation of smooth and unstructured colonies on SDS-containing agar plates (Fig. 3A) and the lack of aggregation during with SDS in liquid medium (Fig. 3B). In addition, strain KO0169 had a higher growth rate and reached a higher final optical density in liquid medium than strain PAO1 (data not shown).

**Determination of survival rates in SDS shock experiments**

In our previous studies we had shown that aggregated cells had strongly increased survival rates when challenged with SDS in the presence of CCCP (Klebensberger et al., 2006; Klebensberger et al., 2007). In order to test whether this was also true for mutants isolated in this study, we exemplarily evaluated two non-aggregating mutants, one with a defect in cupA-encoded adhesive fimbriae (strain B1) and one with a defect in the putative DGC PA0169 (strain KO0169) by comparing their survival rates in SDS-shock experiments in the presence and absence of CCCP. In these experiments, cell suspensions were first supplied with SDS before CCCP was added to allow aggregation of those strains, which were capable of aggregation. For the non-aggregating strains B1 and KO0169, the addition of CCCP caused a dramatic drop of the survival rates by about 4 orders of magnitude compared to strain PAO1 (Fig. 4). When strain KO0169 was complemented with pUCP18[0169] (Fig. 4) or pUCP18[4929] (not shown), the
survival rate could be restored to the level of the wild type strain PAO1. These results clearly demonstrated that strains with the ability to form aggregates during growth with SDS had an about 1000-fold increased survival rate under these conditions.

Complementation of non-aggregating mutants

To investigate whether the DGCs PA4929 or PA1107, which restored SDS-induced aggregation of strain N, could also complement the mutants deficient in PA0172 and PA0169, we transformed strains F5 and KO0169 with pUCP18[4929] and pUCP18[1107] and evaluated their colony morphology and aggregation during growth with SDS. We found that formation of rough colonies and of cell aggregates during growth with SDS could be restored in strains F5 and KO0169 by PA4929 (Fig. 3AB) and by PA1107 (not shown). In addition, complementation of F5 and KO0169 with pUCP18[0172] and pUCP18[PA0169], respectively, restored the SDS-specific rough colony morphology (not shown) and the autoaggregative phenotype in liquid medium (Fig. 5). In contrast, expression of pUCP18[0172] in strain KO0169 or pUCP18[0169] in strain F5 did not restore SDS-induced aggregation (Fig. 6). If succinate was supplied instead of SDS, none of the mutants complemented with pUCP18[0169] or pUCP18[0172] formed aggregates, indicating a specificity of these genes for inducing aggregation as a response to SDS (not shown).

In addition, we found that the formation of rough colonies and of cell aggregates during growth with SDS could not be restored by pUCP18[4929] in any of the
mutants carrying the transposon in the cupA operon, as shown for the mutant strain B1 (Fig. 3AB).

Identification of a mutation in strain N

As the spontaneous mutant strain N showed a similar phenotype as strains F5 and KO0169, and as all three strains could be similarly complemented by PA4929 and PA1107, we speculated that strain N might be mutated in one of the genes PA0172 or PA0169. To test this hypothesis, we first transformed strain N with the plasmids pUCP18[0172] and pUCP18[0169]. Whereas pUCP18[0169] had no effect, pUCP18[0172] could partially restore the SDS-induced aggregation in strain N (Fig. 5).

In the next step, we amplified the gene PA0172 of strain N and determined its DNA sequence. By comparing this sequence with the sequence of the parent strain from the Pseudomonas Genome Database (Winsor et al., 2009) we found an in-frame 6 bp deletion within the predicted PP2C-like phosphatase domain in the C-terminal region of PA0172 (Fig. 2B) causing a deletion of a phenylalanine and a glycine residue. These 6 bp were part of a 12 bp direct repeat encoding the amino acid sequence FGFG. To investigate whether the PA0172 allele of strain N was functional we transformed strain F5 with pUCP18[0172_N] and cultivated it with SDS. While the allele from strain PAO1 restored SDS-induced aggregation in strain F5, the allele of strain N did not (Fig. 5).

Transcriptional analysis of SDS-induced aggregation
To investigate global differences between cells that do and do not show cell-aggregation during growth with SDS we performed a transcriptome analysis of strains PAO1, N and KO0169 grown with either SDS or succinate. In this analysis, we focussed on the identification of genes that are specifically activated in cells showing in SDS-induced aggregation. For this, we performed statistical analysis of the microarray data and selected four subsets of data, datasets A, B, C and D, for further analysis (Tables S1-S4 in Supplementary materials).

Dataset A contains 111 genes that were activated in SDS-grown cells compared to succinate-grown cells of strain PAO1. Dataset B contains 29 genes that were activated in SDS-grown cells of strain PAO1 compared to SDS-grown cells of strain N. Dataset C contains 356 genes that were activated in SDS-grown cells of strain PAO1 compared to SDS-grown cells of strain KO0169. Datasets A, B and C have an overlap of 36 genes (Fig. 6A, Table 1). Eight genes are found in all three datasets, and five of these genes have been related to biofilm formation in earlier studies. For cupA1 (PA2128) an essential function in biofilm formation has been demonstrated (Vallet et al., 2001). The genes PA4623-4625, which encode hypothetical exported proteins, were found to be activated in a constitutively aggregating wspF mutant (Hickman et al., 2005) and in SCVs (Starkey et al., 2009). The gene mexE (PA2493) was found to be repressed in the biofilm-defective PpyR (PA2663) mutant compared to biofilm-forming wild type cells (Attila et al., 2008).

Further genes with a specific function in biofilm formation, autoaggregation or involved in the regulation of these traits include ompD (PA4208) in the overlap of
dataset A and B (Southey-Pillig et al., 2005), pslK (PA2241) in the overlap of datasets B and C, and finally cupA3 (PA2130) (Vallet et al., 2004), PA2126 (Vallet-Gely et al., 2007), PA2440 (Hickman et al., 2005; Starkey et al., 2009) and algA (PA3551) in dataset C. In addition to these genes, dataset C contains PA0172.

Dataset D contains 95 genes that were activated in SDS-grown cells compared to succinate-grown cells of strain N. This dataset has a large overlap of 53 genes with genes from dataset A (Fig. 6B; Table S5 in Supplementary materials), which contains many genes with potential functions in the proposed pathway of SDS degradation. These genes include sdsA1 (PA0740), which encodes the alkylsulfatase catalyzing the hydrolysis of SDS to 1-dodecanol (Hagelueken et al., 2006), two putative dehydrogenases (PA0364 and PA0366), which might be responsible for oxidation of 1-dodecanol to lauric acid, and several genes encoding putative enzymes for β-oxidation of lauric acid, among them a long-chain-fatty-acid CoA-ligase (PA3299), two acyl-CoA-dehydrogenases (PA0506 and 0508), a 3-hydroxyl-acyl-CoA dehydrogenase (PA3014) and an acyl-CoA-thiolase (PA3925). Consistent with the formation of acetyl-CoA units as the end products of β-oxidation, the genes encoding the enzymes of the glyoxylate shunt, isocitrate lyase AceA (PA2634) and malate synthase AceB (PA0482), are also found in the overlap of datasets A and D.

Induction of these genes is feasible because earlier physiological studies had shown that succinate-grown cells are not induced for SDS-degradation (Klebensberger et al., 2006). To confirm these microarray data, we tested four
different transposon mutants defective in two activated genes with essential functions for the utilization of SDS as a growth substrate (Table 2), namely \textit{sdsA1} and \textit{aceA}, for growth with SDS. None of these four mutants did grow with SDS as a sole source of carbon and energy while they could grow with succinate in the presence of SDS (not shown).

Dataset D did not overlap with dataset B and had only three overlaps with dataset C (not shown).

\textit{Northern blot analysis of \textit{cupA1} transcription}

The microarray analysis comparison of succinate-grown cells and SDS-grown cells suggested an important role for the \textit{cupA} operon in SDS induced aggregation. Furthermore, the lack of increased \textit{cupA} expression in SDS-grown cells of strains N and KO0169 compared to strain PAO1 strongly indicated the involvement of the operon PA0172-PA0169 in the expression of the \textit{cupA} operon under these conditions. In order to test this hypothesis and to confirm these microarray data, we investigated the transcript levels of \textit{cupA1} by Northern blot analysis in strains PAO1, KO0169, F5 and N under various conditions (Fig. 7).

By hybridization of RNA samples obtained from strain PAO1 with a \textit{cupA1} specific probe, we detected a specific transcript of >700 bases length, which is slightly longer than the \textit{cupA1} gene itself (551bp). This observation is in agreement with earlier Northern blot analyses of the \textit{cupA1} transcript (Vallet \textit{et al.}, 2004). We found that the \textit{cupA1} transcript was increased by about 6-fold in SDS-grown compared to succinate-grown cells of strain PAO1. In contrast, SDS-grown cells of strains N, F5 and KO0169 did not show an increase of \textit{cupA1}
transcript levels compared to strain PAO1 during growth with SDS. Complementation of strain KO0169 with pUCP18[0169] led to increased cupA1 transcript levels in SDS-grown cells similar to those observed in cells of strain PAO1 under these conditions. In contrast, expression of pUCP18[0172] had no effect on the transcript levels of strain KO0169 in SDS-grown cells. Furthermore, cupA1 transcript levels in SDS-grown cells could be decreased in strain PAO1 to levels of succinate-grown cells by the expression of the known PDE CC3396 from Caulobacter crescentus (Klebensberger et al., 2007).

Discussion

The goal of our study was to identify molecular modules that are specific and essential for inducing autoaggregation in P. aeruginosa strain PAO1 in response to SDS. By random- and site-directed mutagenesis, we found two genes with such a function, namely PA0169 and PA0172, which are co-transcribed as an operon together with PA0171 and PA0170. A clear function for this operon has not been shown so far. Transcript levels of PA0169-0172 were elevated in a constitutively aggregating wspF mutant of P. aeruginosa strain PAO1 (Hickman et al., 2005), and a PA0171 transposon mutant showed a permanently aggregating phenotype (D’Argenio et al., 2002) and decreased twitching motility (Shan et al., 2004), suggesting a general function of this operon in cell aggregation. Here, we clearly demonstrate that PA0172 and PA0169 had an essential function in SDS-induced cell-aggregation because their inactivation caused a loss of this phenotype. Further, we show these two genes are responsible for cell-aggregation as a specific response in the presence of SDS.
In respect of these essential and specific functions and the fact that the genes PA0172, PA0171, PA0170 and PA0169 represent a transcriptional unit, we propose to name these genes $siaABCD$, respectively, for SDS-induced-aggregation.

The physiological characterization and the complementation analysis suggest that SiaA and SiaD are part of a molecular module involved in signal perception and signal transduction, respectively. This is further supported by the domain structure of both predicted proteins.

SiaA harbours a HAMP-domain which is a frequent and essential domain in transmembrane receptors involved in bacterial two-component signal transduction pathways, in particular in chemoreceptors (Hazelbauer et al., 2008) and references therein). The function of HAMP domains in such proteins is to link input and output modules of transmembrane receptors. The PP2C-like phosphatase domain represents such an output domain in bacterial transmembrane receptors, for example in stress signalling in Bacillus subtilis, such as RsbP (Vijay et al., 2000) and RsbU (Hardwick et al., 2007). Based on its domain composition, we suggest that SiaA acts as stress sensor in the periplasm or cytoplasm and causes dephosphorylation of downstream signal transduction components after the perception of so far unknown stress signals. The potential sensing domain of SiaA is not known at the present time.

Genetic analysis of this gene identified strain N as a natural, non-polar $siaA$ mutant. We currently do not know whether strain N harbours more mutations but, in any case, the deletion of a phenylalanine and a glycine residue within the
predicted PP2C-domain was sufficient to render the corresponding protein non-functional with respect to the SDS-induced cell aggregation as shown by its inability to complement strain F5. SDS-induced aggregation could not be fully restored in strain N by complementation with the wild-type siaA allele. A plausible explanation for this effect might be that the functionality of many chemoreceptors is essentially related to the formation of dimers of two monomers of the respective sensorprotein (Hazelbauer et al., 2008). In this respect, a mixture of functional and non-functional SiaA monomers may lead to a mixed population of homodimers in strain N, resulting in functional and non-functional chemoreceptor complexes.

The essential function of siaD (PA0169), which encodes a putative DGC with a predicted cytoplasmic localization, strongly supports that SDS-induced aggregation is regulated through a c-di-GMP-dependent signal transduction pathway. SiaD is the smallest of two known (PA2870, PA5487) and two putative (PA0169, PA3177) DGCs that do not contain any further known domains (Kulasakara et al., 2006) and it is, to our knowledge, the first of these four genes, for which a physiological function has been shown.

The mutation of siaA in strains F5 and N and the corresponding loss of SDS-induced aggregation in these strains could not be complemented by siaD and, in turn, the mutation in siaD in strain KO0169 could not be complemented by expressing siaA from a plasmid. This complementation pattern suggests an interdependency of the SiaA and SiaD proteins, and we propose that in SDS-induced aggregation, the SiaD protein requires an activating input from a
functional SiaA protein. As SiaA and SiaD are essential in the SDS-induced aggregation, how can the DGCs PA4929 and PA1107 restore aggregation in two different siaA mutants, strains N and F5, and the siaD mutant KO0169 in an SDS-dependent manner? To explain this specific but non-essential role, we assume that overexpression of PA4929 and PA1107, and most likely increased c-di-GMP synthesis as a consequence of this, bypasses the otherwise essential SiaAD-dependent induction of cell aggregation in response to SDS by a so far unknown mechanism.

In combination with our previous study (Klebensberger et al., 2007) we have now identified three operons with an essential function in SDS-induced aggregation, namely siaABCD, psl, and cupA. The psl and cupA operons are known to be important for biofilm formation (Vallet et al., 2001; Jackson et al., 2004; Overhage et al., 2005; Ma et al., 2006). As all three operons have been shown to be activated by high c-di-GMP levels (Hickman et al., 2005; Meissner et al., 2007; Starkey et al., 2009), the essential function of these operons further supports the involvement of a c-di-GMP signalling for SDS-induced aggregation.

The transcriptional analysis by DNA-microarrays revealed eight genes that are presumably very important for SDS-induced aggregation because they were consistently activated in aggregating cells of strain PAO1 compared to three types of non-aggregating cells, namely to succinate-grown cells of strain PAO1, to SDS-grown cells of the siaD mutant strain KO0169 and to SDS-grown cells of the natural siaA mutant strain N (overlap of datasets A, B and C, Table 1). The importance of these genes for SDS-induced aggregation is strongly supported by
the affiliation of cupA1 (PA2128), whose essential role we have shown by physiological characterization of the cupA1 mutant strain B1. Northern blot analysis revealed further that cupA1 transcript levels are highly elevated in cells exposed to SDS, and that this elevation requires the functional proteins SiaA and SiaD and is linked to intracellular c-di-GMP levels. Recently, it has been shown that anaerobiosis induces a phase-variable cupA expression through Anr-mediated activation of the cgr genes (PA2127-PA2126), which are located upstream of the cupA operon (Vallet-Gely et al., 2007). In our microarray analysis we found that PA2126 is activated in cells showing SDS-induced aggregation compared to a siaD mutant (dataset C, Table S3). As the macroscopic aggregates certainly contain zones, in which the cells face microaerophilic conditions, Anr might contribute to the induction of the cupA operon.

Apart from cupA1 and mexE (PA2493), the other six genes in the overlap of datasets A, B and C encode for hypothetical proteins with putative functions. The consistent activation of the genes PA4623-4625 in different autoaggregative P. aeruginosa strains indicates that this gene cluster has an important role in cell aggregation under a variety of conditions (Hickman et al., 2005; Starkey et al., 2009). Activation of ompD (PA4208), which is a part of the mexGHI-RND pump, could be linked to increased pyocyanine production accompanying SDS-induced aggregation (Dietrich et al., 2006; Klebensberger et al., 2007).

The fact that dataset C contains more genes (356) than datasets A (111) and B (29) suggests that a deletion of siaD had impact on further cellular functions apart from SDS-induced aggregation, which are independent of SiaA. In addition,
the downregulation of siaA in strain KO0169 is indicative of a positive feedback regulation of SiaD on siaA expression.

The consistent activation of genes for SDS degradation in SDS-grown cells of two different strains, strain PAO1 and strain N, supports the reliability of our transcriptional analysis. Furthermore, it shows that degradation and cell aggregation are induced by SDS as independent processes. SiaAD induce aggregation as a response to an environmental stimulus, presumably cell damage caused by SDS, thereby increasing the fitness of cells under conditions that are detrimental for suspended cells. Under unstable environmental conditions, this induction is certainly an advantageous trait for growth with SDS because cells of *P. aeruginosa* will recurrently encounter various stresses in their natural habitats. Under stable laboratory conditions, however, this aggregation is not required for growth with SDS and its induction is readily lost by applying appropriate selection pressure, as shown for the siaA-defective strain N. Such a loss of non-essential physiological traits, which imply the formation of multicellular structures, is a common event in the evolution of domesticated laboratory strains (Aguilar *et al.*, 2007). Thus, by identifying genes for the induction of autoaggregation, we could spot siaA as a target for the evolution of a domesticated *P. aeruginosa* strain.

**Experimental procedures**

*Bacterial strains, growth media, growth experiments and cell suspension experiments*
Bacterial strains and plasmids used in this study are listed in Table 2. 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 sources as described previously (Klebensberger et al., 2006). Plasmid-harbouring *Escherichia coli* strains were selected and maintained on LB agar plates (1.5%, w/v) containing 100 µg/ml ampicillin (Fluka), 15 µg/ml gentamycin (Sigma) or 50 µg/ml tetracycline (Fluka). Plasmid-harbouring strains and insertional mutants of *P. aeruginosa* were selected on Pseudomonas isolation agar (Difco) containing 200 µg/ml carbenicillin, 120 µg/ml gentamycin or 160 µg/ml tetracycline. For experiments 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.

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 3.5 mM (0.1%) SDS in small Petri dishes (3.5 cm in diameter; Nunc) or in 1.5 ml M9 medium in 12-Well plates (IWAKI Microplate; IWAKI Glass Co) on a rotary shaker at 120 rpm or 150 rpm for 18 h at 30°C.

SDS shock experiments with cell suspensions of different *P. aeruginosa* strains were performed as described previously (Klebensberger et al., 2007).

*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). A pool of ~20,000 transposon mutants were screened for non-aggregating strains by searching for smooth colonies on M9 agar plates containing 0.15% SDS and 80 µg/ml tetracycline. The exact position of the transposon insertion in mutants showing the respective phenotype was identified by inverse PCR as described previously (Klebensberger *et al.*, 2007).

**Construction of the PA0169 deletion mutant and of complementing plasmids**

For construction of a PA0169 deletion mutant, a 1326 bp fragment containing the gene PA0169 was amplified by PCR (TripleMaster PCR System, Eppendorf) from purified genomic DNA (Puregene DNA Isolation Kit, Gentra) using the primers KO-PA0169-F (5’-GGACCTGCGCCTGCTGTACCTGAA-3’) and KO-PA0169-R (5’-GCCTCGCCCGCGCCTATGG-3’). The amplicon was 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 TopoKO0169 was linearized with Smal, cutting at position 368 within the ORF of PA0169. After purification (PCR Purification Kit, Peqlab) the linearized plasmid was blunt-ended with T4 DNA polymerase (NEB), purified and dephosphorylated using Shrimp alkaline phosphatase (Promega). A blunt-ended res-cat-res cassette obtained from plasmid pKO2a (kindly provided by Theo Smits) was ligated with the linearized plasmid TopoKO0169, resulting in the plasmid TopoKO0169[Cm]. Finally, the fragment containing PA0169[Cm] was excised with XbaI-HindIII, treated with T4
DNA polymerase and subsequently subcloned in the blunt-ended suicide vector pEX18Ap (Hoang et al., 1998) digested with EcoRI-HindIII. The resulting plasmid pEXKO0169 was 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 excise 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, a clone sensitive towards chloramphenicol and gentamycin was obtained and designated KO0169.

To construct plasmid pUCP18[0169], 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 KO-0172-F (5’-CAACCTGCTCGCCGGCCTGCTCAC-3’) and pKO171-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]. To
construct pUCP18[0172_N] a 2667 bp fragment containing the gene PA0172 was amplified from genomic DNA of strain N by PCR using the primer 1205_fp2_BamHI (5’- GGATCCGCGGGCCGGGAGAAAC-3’) and 1205_rp_HindIII (5’- AAGCTTCGGGCGGCGTAGCTGCTCCTTGTA-3’) and cloned into pALLi10 (Trenzyme GmbH). PA0172_N was then excised as a BamHI-HindIII fragment and subcloned into the respective restriction site of the plasmid pUCP18 to obtain the plasmid pUCP[0172_N]. Correct orientation for expressing of PA0169, PA0172 and PA0172_N from the lac-promoter of pUCP18 was confirmed by sequencing.

RNA isolation

For Microarray- and Northern blot analysis, suspensions (OD600 = 1.5) of succinate-grown cells or of SDS-grown cells were supplied with their respective substrate (10 mM succinate or 3.5 mM SDS) in triplicates 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 manufacturer’s instructions. After repurification with an RNeasy column (QIAGEN), the samples were quantified spectrophotometrically and stored at −60°C until further analysis.
For reverse transcriptase reactions, cells of *P. aeruginosa* were grown in 10 ml LB medium in a plastic tube (Greiner) with shaking at 200 rpm at 37°C. Cells were harvested during exponential phase (OD = 0.8) by centrifugation at 5,000 × g at 4°C for 3 min, and RNA was extracted from the cells with the PureLink Micro-to-Midi total RNA purification system (Invitrogen) according to the manufacturer’s instructions. Contaminating DNA was removed with an off-column RNase-free DNase I treatment (QIAGEN) according to the manufacturer’s instructions. After repurification with an PureLink Micro-to-Midi column (Invitrogen), the samples stored at −80°C until further analysis.

**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 the 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 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 pre-hybridized 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)-labelled 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’- CCCAGCGGCCGAGGTCGTATT-3’). Hybridization was performed overnight at 50°C with 15 ng DIG-labelled 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 (Bio-Rad) for further analysis. Signal intensities obtained from the cupA1 hybridisation as well as the ethidium bromide fluorescence intensities of the 23S- and 16S RNA from the respective agarose gel were quantified using GelScan5™ software (BioSciTec). All signal intensities obtained from the cupA1 hybridisation were normalized to the total RNA of the respective sample (combined ethidium bromide fluorescence intensities of the 23S- and 16S RNA).

DNA microarray hybridization and data analysis

Quality and integrity of the total RNA isolated from strains PAO1, KO0169 (siaD) and N grown with either SDS or succinate was controlled by running all samples
on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies). For biotin-
labelled target synthesis starting from 10 µg of total RNA, reactions were
performed using standard protocols supplied by the manufacturer (Affymetrix).
Briefly, 10 µg total RNA was converted to cDNA using random hexamers. The
cDNA was then fragmented by DNaseI and labelled with terminal transferase in
the presence of biotin-ddUTP to biotinylate cDNA at the 3’ termini. Samples were
hybridized to an identical lot of Affymetrix GeneChip Pae_G1a for 16 hours.
After hybridisation the GeneChips were washed, stained with SA-PE and read
using an Affymetrix GeneChip fluidic station and scanner. DNA microarray
hybridization was performed in duplicates.
Analysis of microarray data was performed using the Affymetrix GCOS 1.2 using
the MAS5 algorithm. For normalization all array experiments were scaled to a
target intensity of 150, otherwise using the default values of GCOS 1.2. For
further downstream analysis Array Assist 4.0 software (Stratagene) were applied.
The entire dataset was cleaned for genes with no reliable signal measurements
indicated by the detection call of MAS5.0 algorithm. Therefore, genes showing
more than 50% “Present” calls across the dataset were selected for further
calculations. Comparisons of groups consisting of two biological replicates were
performed as indicated. Each signal intensity value was compared to the mean
intensity of the corresponding control group. Relative gene expressions were
determined by log2 ratios. A Student’s T-test was used to identify significant
expression changes. From these data, selected subsets (datasets A-D) were
chosen for further comparison (Tables S1-S4 in Supplementary materials) with the software GeneVenn (Pirooznia et al., 2007)

Reverse transcription and subsequent PCR reactions

For each reverse transcriptase reaction, 2 µg purified total RNA, 2 pmol *siaD*/PA0169 specific primer PA0169RT (5´-TTGACGGTCTGGAATAGGTTT-3´) and 10 nmol dNTPs were mixed on ice in a sterile 0.2 µl PCR tube and incubated at 65°C for 5 min. After cooling the tubes on ice for 5 min, first-strand cDNA synthesis was carried out by using SuperScriptIII Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions at 55°C for 50 min. Controls consisted of reactions without the addition of the SuperScriptIII enzyme. After heat inactivation at 70°C for 10 min and subsequent incubation with 2 units RNase H (Invitrogen) at 37°C for 20 min, the first-strand reaction mixtures were used as a template for subsequent PCR reactions. PCR was carried out by using PWO DNA Polymerase (Roche Applied Science) with 2 µl of the first-strand reaction mixtures and 15 pmol of each primer. Primer pairs were designed to obtain one 840 bp PCR product (PA0172F_End_RT (5´-CTGGCGCCGGCTTGAGCCGTACC-3´), 0170R_RT (5´-GTGGACTCGGTTGATGTCGTG-3´)) and one 651 bp PCR product (PA0171F_RT (5´-GCGCCGTGATCTGACCGTTTT-3´), PA0169R (5´-AGGGCCGCAGTCTGGTGTTT-3´)), which included the intergenic sequences between PA0172-PA0170 and PA0171-PA0169, respectively. Controls consisted of PCR reactions containing 2 µl of the control first-strand reaction mixtures described above. All PCR reactions were performed in an
Mastercycler personal thermocycler (Eppendorf) using a program with an initial
denaturing step at 98°C for 2 min and 30 cycles of 96°C for 20 sec, 60°C for 15
sec and 72°C for 1 min. For analysis, 10 µl of each PCR reaction was size
fractionated by using a 1% (w/v) agarose gel, stained with ethidium bromide and
finally visualized by using a Gel Doc XR gel documentation system (Bio-Rad).

Photography and image processing

Macroscopic images of colonies and liquid cultures were taken with a Canon
Powershot G6 camera. Images were processed with Paint Shop Pro 4.

Supplementary material

The following supplementary material is available with four subsets of DNA-
microarray data with selected comparisons of P. aeruginosa strains PAO1, the
spontaneous siaA mutant N and the siaD mutant KO0169 grown with either SDS
or succinate:

Table S1: Dataset A: Genes activated in SDS-grown cells compared to
succinate-grown cells of strain PAO1.

Table S2: Dataset B: Genes activated in SDS-grown cells of strain PAO1
compared to SDS-grown cells of the spontaneous siaA mutant strain N.

Table S3: Dataset C: Genes activated in SDS-grown cells of strain PAO1
compared to SDS-grown cells of the siaD mutant strain KO0169.

Table S4: Dataset D: Genes activated in SDS-grown cells compared to
succinate-grown cells of the spontaneous siaA mutant strain N.
Table S5: Overlaps of datasets A and D in Figure 6B. Genes activated in SDS-grown cells compared to succinate-grown cells of strain PAO1 (dataset A) and in SDS-grown compared to succinate-grown cells of the spontaneous siaA mutant strain N (dataset D).

Acknowledgements

The authors like to thank Ilona Kindinger for excellent technical assistance and Bernhard Schink for continuous support. This work was funded by grants from the Deutsche Forschungsgemeinschaft (projects PH71/2-1 and B9 in SFB 454) and from the University of Konstanz (project 58/03) to BP.

References


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.


are controlled by the transcriptional regulator MvaT. *J Bacteriol* **186**: 2880-2890.


**Table 1.** Transcriptional analysis of different *P. aeruginosa* strains with DNA microarrays. Overlaps of datasets A, B and C in Figure 6A containing genes activated in SDS-grown cells of strain PAO1 compared to succinate-grown cells of strain PAO1 (dataset A), to SDS-grown cells of the spontaneous siaA mutant strain N (dataset B) and to SDS-grown cells of the siaD mutant strain KO0169 (dataset C).

<table>
<thead>
<tr>
<th>Gene no*</th>
<th>Gene name and protein description</th>
<th>Fold change in dataset A&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fold change in dataset B&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fold change in dataset C&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA2128</td>
<td><em>cupA1</em>; fimbrial subunit CupA1</td>
<td>18.899</td>
<td>9.954</td>
<td>12.422</td>
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<td>PA2493</td>
<td><em>mexE</em>; RND multidrug efflux membrane fusion protein MexE precursor</td>
<td>2.831</td>
<td>2.314</td>
<td>2.377</td>
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<td>PA3691</td>
<td>hypothetical protein; exported protein</td>
<td>4.789</td>
<td>2.428</td>
<td>4.039</td>
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<tr>
<td>PA4498</td>
<td>probable metallopeptidase</td>
<td>3.881</td>
<td>3.116</td>
<td>2.485</td>
</tr>
<tr>
<td>PA4623</td>
<td>hypothetical protein; exported lipoprotein</td>
<td>3.847</td>
<td>3.598</td>
<td>5.515</td>
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<td>PA4625</td>
<td>hypothetical protein; exported protein</td>
<td>3.111</td>
<td>4.634</td>
<td>7.505</td>
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<tr>
<td>PA4624</td>
<td>hypothetical protein; outer membrane protein</td>
<td>2.538</td>
<td>3.363</td>
<td>4.505</td>
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<td>PA5061</td>
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<td>4.461</td>
<td>2.673</td>
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<td>PA0263</td>
<td><em>hcpC</em>; secreted protein Hcp</td>
<td>2.005</td>
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<td>PA4739</td>
<td>conserved hypothetical protein; exported lipoprotein</td>
<td>7.987</td>
<td>2.195</td>
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<td>PA5446</td>
<td>conserved hypothetical protein; lipid metabolism</td>
<td>4.457</td>
<td>6.87</td>
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<td>PA1338</td>
<td><em>ggt</em>; gamma-glutamyltranspeptidase precursor</td>
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<td>PA1787</td>
<td><em>acnB</em>; aconitate hydratase</td>
<td>3.677</td>
<td>2.169</td>
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<tr>
<td>PA1903</td>
<td><em>phzE</em>; phenazine biosynthesis protein PhzE</td>
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<td>4.039</td>
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<td>PA3519</td>
<td>hypothetical protein</td>
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<tr>
<td>PA4208</td>
<td>probable outer membrane protein precursor</td>
<td>2.726</td>
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<td>PA4258</td>
<td><em>rplV</em>; 50S ribosomal protein L22</td>
<td>2.540</td>
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<td>PA4260</td>
<td><em>rplB</em>; 50S ribosomal protein L2</td>
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<td><em>rpsG</em>; 30S ribosomal protein S7</td>
<td>2.403</td>
<td>2.164</td>
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<td>PA4501</td>
<td><em>opdP</em>; glycine-glutamate dipeptide porin OpdP</td>
<td>2.147</td>
<td>2.752</td>
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<td>PA4502</td>
<td>probable binding protein component of ABC transporter</td>
<td>2.122</td>
<td>2.208</td>
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<tr>
<td>PA5348</td>
<td>probable DNA-binding protein</td>
<td>4.326</td>
<td>2.400</td>
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<td>PA0200</td>
<td>hypothetical protein</td>
<td>9.954</td>
<td>2.328</td>
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<td>PA0745</td>
<td>probable enoyl-CoA hydratase/isomerase</td>
<td>4.634</td>
<td>2.344</td>
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<td>PA0812</td>
<td>hypothetical protein</td>
<td>4.049</td>
<td>3.592</td>
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<td>PA0999</td>
<td><em>fabH1</em>; 3-oxoacyl-[acyl-carrier-protein] synthase III</td>
<td>3.598</td>
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<td>PA1183</td>
<td><em>dctA</em>; C4-dicarboxylate transport protein</td>
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<td>PA1894</td>
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<td>PA number</td>
<td>Gene Product</td>
<td>Fold Change</td>
<td>P-value</td>
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<td>------------------------------------------------------------------------------</td>
<td>-------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>PA2241</td>
<td>pslK; exopolysaccharide biosynthesis</td>
<td>2.464</td>
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<td>PA3194</td>
<td>edd; phosphogluconate dehydratase</td>
<td>2.404</td>
<td>6.992</td>
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<td>PA3384</td>
<td>phnC; ATP-binding component of ABC phosphonate transporter</td>
<td>2.175</td>
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<td>PA3972</td>
<td>probable acyl-CoA dehydrogenase</td>
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<td>probable permease of ABC transporter</td>
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<td>PA4505</td>
<td>probable ATP-binding component of ABC transporter</td>
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<td>3.848</td>
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<td>PA5170</td>
<td>arcD; arginine/ornithine antiporter</td>
<td>2.040</td>
<td>2.411</td>
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<td>PA5171</td>
<td>arcA; arginine deiminase</td>
<td>2.025</td>
<td>2.244</td>
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</table>

PA numbers according to the Pseudomonas Genome Database (Winsor et al., 2009).

Fold change of mRNA-levels in SDS-grown cells of strain PAO1 was >2.0 (P ≤ 0.05) in datasets A and B.
Table 2. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td><strong>P. aeruginosa</strong></td>
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<tr>
<td>PAO1</td>
<td>Wild-type of strain PAO1</td>
<td>Holloway collection</td>
</tr>
<tr>
<td>N</td>
<td>Spontaneous mutant of strain PAO1</td>
<td>Klebensberger <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>B1</td>
<td><em>cupA1::mariner</em> mutant (nucleotide position 480) in strain PAO1, Tet'</td>
<td>This study</td>
</tr>
<tr>
<td>F5</td>
<td><em>siaA/PA0172::mariner</em> mutant (nucleotide position 732) of strain PAO1, Tet'</td>
<td>This study</td>
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<tr>
<td>KO0169</td>
<td>Insertional knockout mutant of <em>siaD/PA0169</em> (resolvase site at position 368) in strain PAO1</td>
<td>This study</td>
</tr>
<tr>
<td>MPAO1 [11402] and [42553]</td>
<td><em>sdsA1</em> (PA0740) insertional mutants derived from strain MPAO1</td>
<td>Jacobs <em>et al.</em> (2003); Washington Genome Center</td>
</tr>
<tr>
<td>MPAO1 [11153] and [20796]</td>
<td><em>aceA</em> (PA2634) insertional mutants derived from strain MPAO1</td>
<td>Jacobs <em>et al.</em> (2003); Washington Genome Center</td>
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<td><strong>E. coli</strong></td>
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<td>JM109</td>
<td>*endA1 recA1 gyrA96 thi hsd R17 (rK', mK'), relA1 supE44 Δ(lac-proAB) [F' traD36 proAB' lacIq lacZΔM15]</td>
<td>Promega</td>
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<tr>
<td>CC118</td>
<td>*araD139 Δ(ara leu)7697 ΔlacX74 phoAΔ20 galE thi rpsB argE'Δ recA1</td>
<td>Manoil and Beckwith (1985)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td>pALMAR3</td>
<td>Plasmid harbouring a mariner transposon used for transposon mutagenesis, Tet'</td>
<td>Jenal lab</td>
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<tr>
<td>pUCP18[0169]</td>
<td>Plasmid pUCP18 harboring a XbaI-HindIII fragment (1439 bp) encoding <em>siaD/PA0169</em></td>
<td>This study</td>
</tr>
<tr>
<td>pUCP18[0172]</td>
<td>Plasmid pUCP18 harboring a BamHI fragment (2708 bp) encoding <em>siaA/PA0172</em> from the parent strain</td>
<td>This study</td>
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<tr>
<td>pUCP18[0172_N]</td>
<td>Plasmid pUCP18 harboring a BamHI-HindIII fragment (2661 bp) encoding <em>siaA/PA0172</em> from strain N</td>
<td>This study</td>
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<td>pUCP18[4929]</td>
<td>pUCP18 harboring a Sall fragment (2426 bp) encoding PA4929</td>
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<td>pBBR1MSC-5</td>
<td>Broad-host-range cloning vector, (Gm')</td>
<td>Kovach <em>et al.</em> (1995)</td>
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<td>pBBR1MSC-5 containing the gene CC3396 from <em>C. crescentus</em></td>
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<td>Vector</td>
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<td>Reference</td>
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<tr>
<td>pUCPParA</td>
<td>parA as EcoRI-HindIII fragment in pUCP24, Gm'</td>
<td>Smits et al. (2002)</td>
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<td>pRK 600</td>
<td>ori ColE1 RK2-Mob+ RK2-Tra+, (Cm'), helper strain in tri-parental matings</td>
<td>Kessler et al. (1992)</td>
</tr>
</tbody>
</table>
Legends to the Figures

Fig. 1. A. Map of inactivated genes found in transposon mutants of *P. aeruginosa* with a non-aggregative phenotype during growth with SDS. Black arrows indicate the insertion site of the Mariner transposon. The direction of the black arrowhead indicates the orientation of the promoter of the tetracycline resistance gene. Transposon mutants used in this study (B1, F5) and the GEEF-motif of the putative DGC encoded by the gene *siaD* (PA0169) are indicated. Binding sites and orientation of oligonucleotides used for the reverse transcriptase reactions (RT) from total RNA extractions of *P. aeruginosa* cells and subsequent PCR amplification (1, 2, 3, 4) are indicated by white arrows. B. Size fractionation of 10 µl of the PCR reactions performed with primer pairs 1 + 2 or 3 + 4 by using a 1%, agarose gel (w/v). Two µl of the reverse transcriptase reaction (+) or the respective negative control (−) were used in the PCR reactions.

Fig. 2. Predicted domain architecture of the protein encoded by *siaA* (PA0172) in *P. aeruginosa* strain PAO1 and localization of the deletion in strain N. A. Predicted domain structure of SiaA using the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). B. Localization of the 6bp in-frame deletion (black letters, nucleotides 1840-1852 of the ORF) leading to a loss of a phenylalanine and a glycine residue within the predicted PP2C_SIG-like domain in the C-terminal region of the *siaA* allele in strain N.

Fig. 3. Phenotypes of the *P. aeruginosa* strains PAO1, the *cupA1* transposon mutant B1, the *siaA* transposon mutant F5 and the *siaD* mutant KO0169 during growth with 3.5 mM SDS after transformation with pUCP18 (◼) or pUCP18[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 in small Petri dishes (3 cm diameter, Nunc) after incubation for 18 h at 30°C with shaking at 120 rpm.
Fig. 4. CFU counts of the *P. aeruginosa* strains PAO1, the *cupA1* transposon mutant B1 and the *siaD* mutant KO0169 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*). Error bars indicate standard deviation (n = 3).

Fig. 5. Phenotypes of the *P. aeruginosa* strains PAO1, the spontaneous *siaA* mutant N, the *siaA* transposon mutant F5 and the *siaD* mutant KO0169 during growth in liquid medium after transformation with pUCP18, pUCP18[0169], pUCP18[0172], and pUCP18[0172_N]. Cells were grown in M9 medium (12 well plates) containing 10 mM succinate (■) or 3.5 mM SDS (■) for 18 h at 30°C with shaking at 150 rpm.

Fig. 6. Venn diagram showing overlaps of datasets A, B, C and D that were derived from transcriptome analysis with DNA microarrays of the *P. aeruginosa* strains PAO1, the spontaneous *siaA* mutant N and the *siaD* mutant KO0169. Genes of all datasets are listed in Tables S1-S4 in Supplementary materials. A. Dataset A (*white*): genes activated in SDS-grown cells compared to succinate-grown cells of strain PAO1. Dataset B (*light grey*): genes activated in SDS-grown cells of strain PAO1 compared to SDS-grown cells of the spontaneous *siaA* mutant strain N. Dataset C (*dark grey*): genes activated in SDS-grown cells of strain PAO1 compared to SDS-grown cells of the *siaD* mutant strain KO0169 (*siaD*). B. Dataset D (*dark grey*): genes activated in SDS-grown cells compared to succinate-grown cells of the spontaneous *siaA* mutant strain N. Dataset A (*white*). Genes overlapping between datasets A-C are listed in Table 1; genes overlapping between datasets A and D are listed in Table S5 in Supplementary materials.
**Fig. 7.** Northern blot analysis with a cupA1 specific probe for determination of cupA1 transcript levels in RNA-preparations derived from cell suspensions (OD600 = 1) of the *P. aeruginosa* strains PAO1, the spontaneous siaA mutant N, the siaA transposon mutant F5 and the siaD mutant KO0169. Suspensions were prepared from cultures grown in M9 medium containing 10 mM succinate (■) or 3.5 mM SDS (▲); 10 µg total RNA was used for size fractionation and blotting. Corresponding length standards of the DIG labeled RNA Molecular Weight Marker I (Roche) are indicated. Calculated expression values of the cupA1 transcript from the Northern Blot analysis using the GelScan5™ software (BioSciTec) are indicated below the blot. The expression values represent changes of the signal intensity from the cupA1 specific probe of RNA-preparations in comparison to strain PAO1 grown with succinate (■).