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

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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 with 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.

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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; 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; 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; 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; 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-diguanosinemonophospate (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 6 bp 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 *cupA*1 gene, which encodes the fimbrial subunit. In four mutants the transposon was inserted in the *cupA*3 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

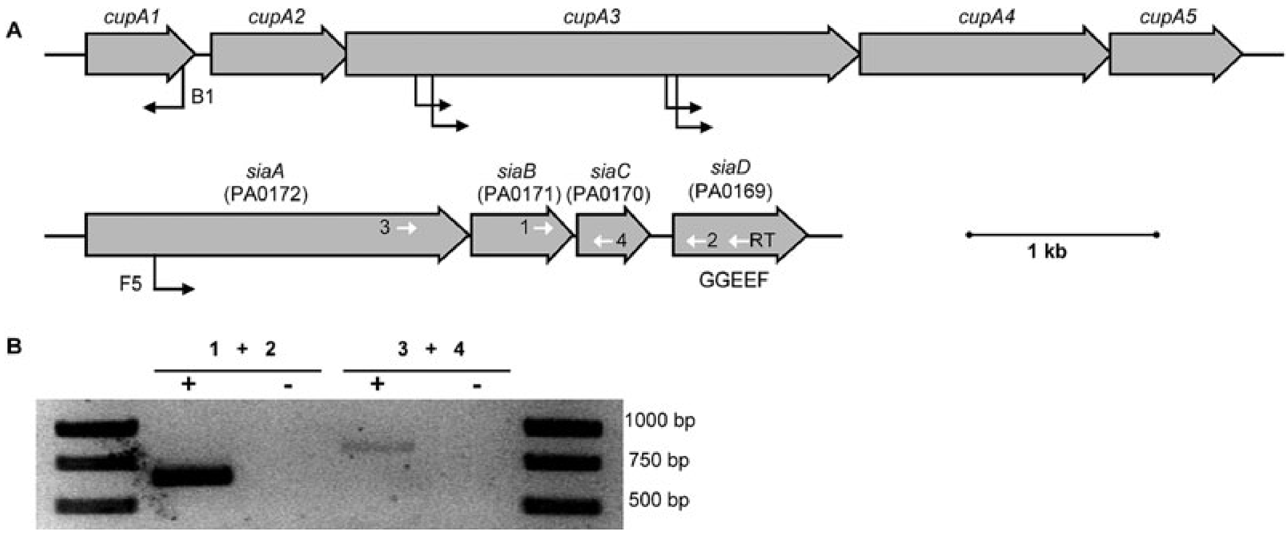

Fig. 1. A. Map of inactivated genes found in transposon mutants of *P. aeruginosa* with a non-aggregative phenotype during growth with SDS. Black arrows indicate the insertion site of the Mariner transposon. The direction of the black arrowhead indicates the orientation of the promoter of the tetracycline resistance gene. Transposon mutants used in this study (B1, F5) and the GGEEF 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 microlitres of the reverse transcriptase reaction (+) or the respective negative control (–) were used in the PCR reactions.

genes PA0172-PA0169 revealed that these genes are co-transcribed (Fig. 1A and B).

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 (data 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 chal-

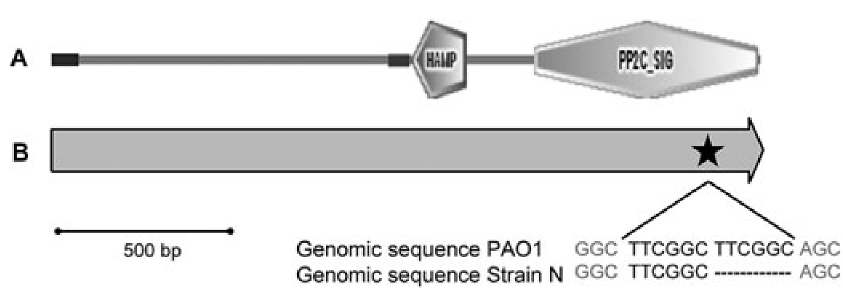

Fig. 2. Predicted domain architecture of the protein encoded by *siaA* (PA0172) in *P. aeruginosa* strain PAO1 and localization of the deletion in strain N.
A. Predicted domain structure of SiaA using the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/).
B. Localization of the 6 bp in-frame deletion (black letters, nucleotides 1834–1845 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.

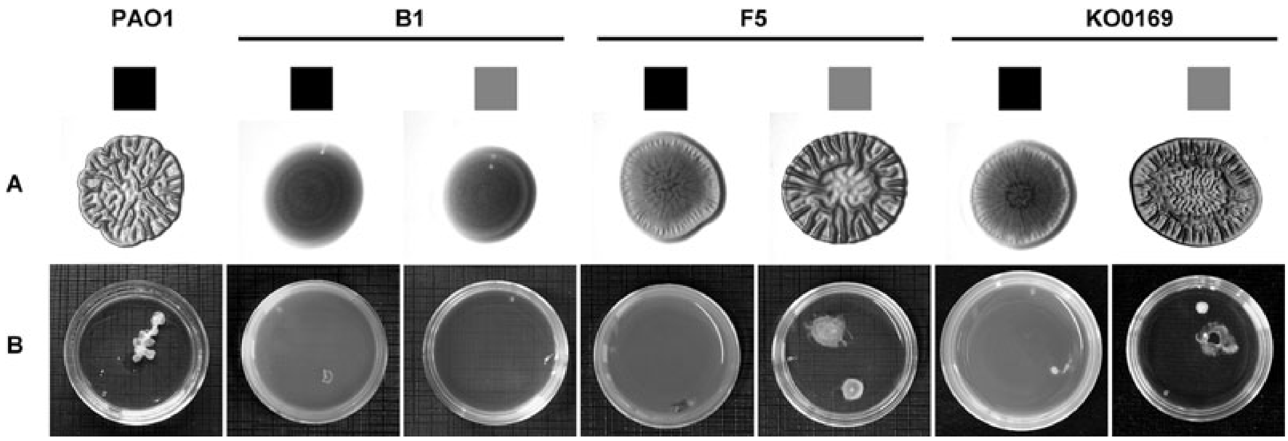

Fig. 3. Phenotypes of the *P. aeruginosa* strains PAO1, the *cupA1* transposon mutant B1, the *siaA* transposon mutant F5 and the *siaD* mutant KO0169 during growth with 3.5 mM SDS after transformation with pUCP18 (■) or pUCP18[4929] (■).
A. Colony morphology on M9 agar containing 0.15% SDS after incubation for 3 days 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 r.p.m.

lenged with SDS in the presence of CCCP (Klebensberger *et al.*, 2006; 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 four orders of magnitude compared with 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.

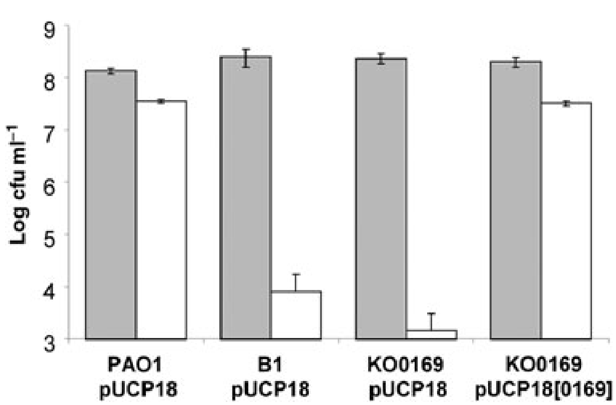

Fig. 4. Colony-forming units (cfu) counts of the *P. aeruginosa* strains PAO1, the *cupA1* transposon mutant B1 and the *siaD* mutant KO0169 after 45 min of exposure to 3.5 mM SDS and a subsequent incubation for an additional 60 min in the presence of 1 mM CCCP (*white bars*) or methanol as a solvent control (*grey bars*). *Error bars* indicate standard deviation ($n = 3$).

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. 3A and B) 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. 3A and B).

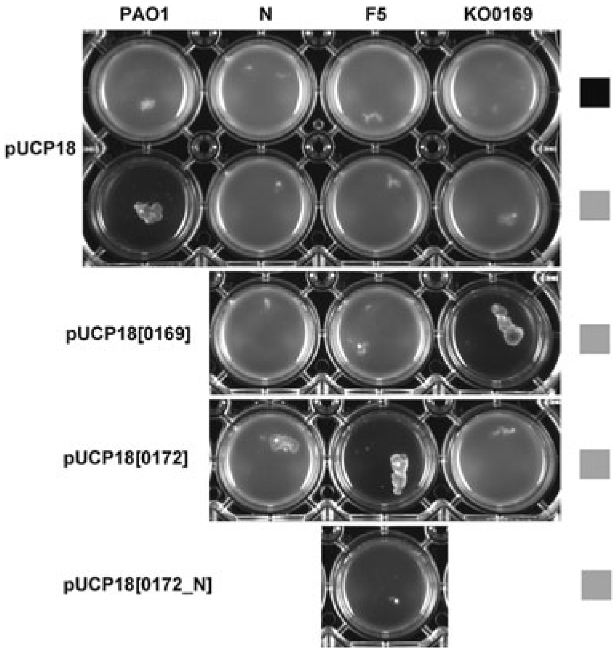

Fig. 5. Phenotypes of the *P. aeruginosa* strains PAO1, the spontaneous *siaA* mutant N, the *siaA* transposon mutant F5 and the *siaD* mutant KO0169 during growth in liquid medium after transformation with pUCP18, pUCP18[0169], pUCP18[0172] and pUCP18[0172_N]. Cells were grown in M9 medium (12-well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

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 six base pairs 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 micoarray data and selected four subsets of data, data sets A, B, C and D, for further analysis (Tables S1–S4).

Data set A contains 111 genes that were activated in SDS-grown cells compared with succinate-grown cells of strain PAO1. Data set B contains 29 genes that were

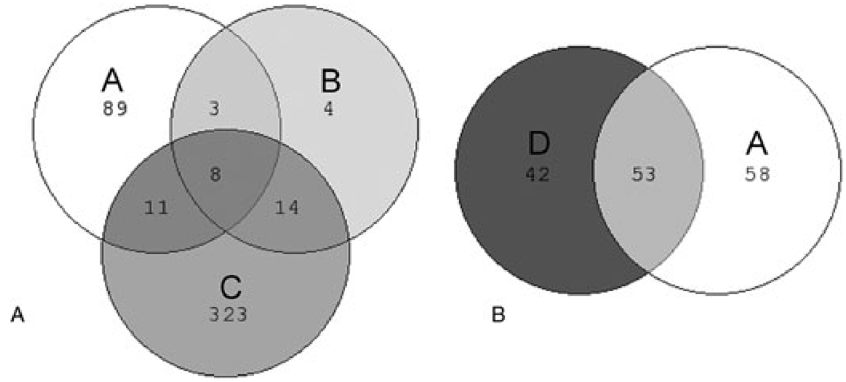

Fig. 6. Venn diagram showing overlaps of data sets A–D that were derived from transcriptome analysis with DNA microarrays of the *P. aeruginosa* strains PAO1, the spontaneous *siaA* mutant N and the *siaD* mutant KO0169. Genes of all data sets are listed in Tables S1–S4.
A. Data set A (*white*): genes activated in SDS-grown cells compared with succinate-grown cells of strain PAO1. Data set B (*light grey*): genes activated in SDS-grown cells of strain PAO1 compared with SDS-grown cells of the spontaneous *siaA* mutant strain N. Data set C (*dark grey*): genes activated in SDS-grown cells of strain PAO1 compared with SDS-grown cells of the *siaD* mutant strain KO0169 (*siaD*).
B. Data set D (*dark grey*): genes activated in SDS-grown cells compared with succinate-grown cells of the spontaneous *siaA* mutant strain N. Data set A (*white*). Genes overlapping between data sets A–C are listed in Table 1; genes overlapping between data sets A and D are listed in Table S5.

activated in SDS-grown cells of strain PAO1 compared with SDS-grown cells of strain N. Data set C contains 356 genes that were activated in SDS-grown cells of strain PAO1 compared with SDS-grown cells of strain KO0169.

Data sets A, B and C have an overlap of 36 genes (Fig. 6A, Table 1). Eight genes are found in all three data sets, 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 with 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 data set A and B (Southey-Pillig *et al*., 2005), *pslK* (PA2241) in the overlap of data sets 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 data set C. In addition to these genes, data set C contains PA0172.

Data set D contains 95 genes that were activated in SDS-grown cells compared with succinate-grown cells of strain N. This data set has a large overlap of 53 genes with genes from data set A (Fig. 6B; Table S5), which contains many genes with potential functions in the proposed pathway of SDS degradation. These genes include *sdsA1* (PA0740), which encodes the alkylsulfatase catalysing 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-degydrogenases (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 data sets 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 *sdsA1* and *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).

Data set D did not overlap with data set B and had only three overlaps with data set C (not shown).

Northern blot analysis of cupA1 transcription

The microarray analysis comparison of succinate-grown cells and SDS-grown cells suggested an important role for the *cupA* operon in SDS-induced aggregation. Furthermore, the lack of increased *cupA* expression in SDS-grown cells of strains N and KO0169 compared with strain PAO1 strongly indicated the involvement of the operon PA0172-PA0169 in the expression of the *cupA* operon under these conditions. In order to test this hypothesis and to confirm these microarray data, we investigated the transcript levels of *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 *cupA*1-specific probe, we detected a specific transcript of > 700 bases length, which is slightly longer than the *cupA*1 gene itself (551 bp). This observation is in agreement with earlier Northern blot analyses of the *cupA1* transcript (Vallet *et al*., 2004). We found that the *cupA*1 transcript was increased by about sixfold in SDS-grown compared with succinate-grown cells of strain PAO1. In contrast, SDS-grown cells of strains N, F5 and KO0169 did not show an increase of *cupA*1 transcript levels compared with 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, *cupA*1 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

Table 1. Transcriptional analysis of different *P. aeruginosa* strains with DNA microarrays.

Gene No.[a]	Gene name and protein description	Fold change in data set A[b]	Fold change in data set B[b]	Fold change in data set C[b]
PA2128	*cupA1*; fimbrial subunit CupA1	18.899	9.954	12.422
PA2493	*mexE*; RND multidrug efflux membrane fusion protein MexE precursor	2.831	2.314	2.377
PA3691	Hypothetical protein; exported protein	4.789	2.428	4.039
PA4498	Probable metallopeptidase	3.881	3.116	2.485
PA4623	Hypothetical protein; exported lipoprotein	3.847	3.598	5.515
PA4625	Hypothetical protein; exported protein	3.111	4.634	7.505
PA4624	Hypothetical protein; outer membrane protein	2.538	3.363	4.505
PA5061	Conserved hypothetical protein; exported lipoprotein	4.461	2.673	2.138
PA0263	*hcpC*; secreted protein Hcp	2.005	2.373	
PA4739	Conserved hypothetical protein; exported lipoprotein	7.987	2.195	
PA5446	Conserved hypothetical protein; lipid metabolism	4.457	6.87	
PA1338	*ggt*; gamma-glutamyltranspeptidase precursor	2.075		2.081
PA1787	*acnB*; aconitate hydratase	3.677		2.169
PA1903	*phzE*; phenazine biosynthesis protein PhzE	3.615		4.039
PA3519	Hypothetical protein	2.755		6.694
PA4208	Probable outer membrane protein precursor	2.726		6.038
PA4258	*rplV*; 50S ribosomal protein L22	2.540		2.379
PA4260	*rplB*; 50S ribosomal protein L2	2.505		2.230
PA4267	*rpsG*; 30S ribosomal protein S7	2.403		2.164
PA4501	*opdP*; glycine-glutamate dipeptide porin OpdP	2.147		2.752
PA4502	Probable binding protein component of ABC transporter	2.122		2.208
PA5348	Probable DNA-binding protein	4.326		2.400
PA0200	Hypothetical protein		9.954	2.328
PA0745	Probable enoyl-CoA hydratase/isomerase		4.634	2.344
PA0812	Hypothetical protein		4.049	3.592
PA0999	*fabH1*; 3-oxoacyl-[acyl-carrier-protein] synthase III		3.598	5.523
PA1183	*dctA*; C4-dicarboxylate transport protein		3.363	2.277
PA1894	Hypothetical protein		2.850	2.232
PA2241	*pslK;* exopolysaccharide biosynthesis		2.464	2.542
PA3194	*edd*; phosphogluconate dehydratase		2.404	6.992
PA3384	*phnC*; ATP-binding component of ABC phosphonate transporter		2.175	3.238
PA3972	Probable acyl-CoA dehydrogenase		2.270	2.157
PA4504	Probable permease of ABC transporter		2.194	2.583
PA4505	Probable ATP-binding component of ABC transporter		2.186	3.848
PA5170	*arcD*; arginine/ornithine antiporter		2.040	2.411
PA5171	*arcA*; arginine deiminase		2.025	2.244

Overlaps of data sets A, B and C in Fig. 6A containing genes activated in SDS-grown cells of strain PAO1 compared with succinate-grown cells of strain PAO1 (data set A), to SDS-grown cells of the spontaneous *siaA* mutant strain N (data set B) and to SDS-grown cells of the *siaD* mutant strain KO0169 (data set C).

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

b. Fold change of mRNA-levels in SDS-grown cells of strain PAO1 was ≥ 2.0 ($P \leq 0.05$) in data sets A and B.

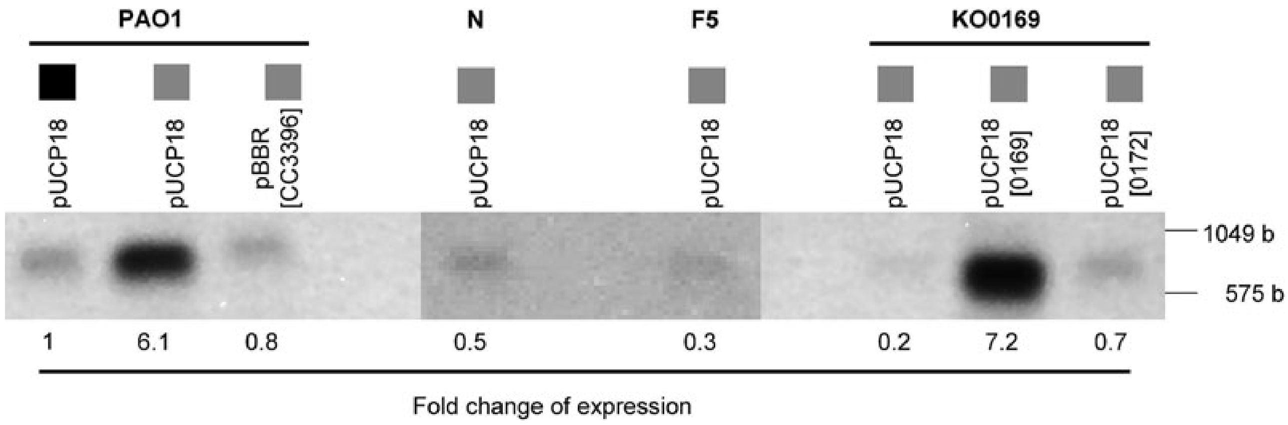

Fig. 7. Northern blot analysis with a *cupA1*-specific probe for determination of *cupA1* transcript levels in RNA preparations derived from cell suspensions ($OD_{600} = 1$) of the *P. aeruginosa* strains PAO1, the spontaneous *siaA* mutant N, the *siaA* transposon mutant F5 and the *siaD* mutant KO0169. 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 labelled 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 (■).

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. Furthermore, 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 an 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 sensor-protein (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 in 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 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 with three types of non-aggregating cells, namely with succinate-grown cells of strain PAO1, with SDS-grown cells of the *siaD* mutant strain KO0169 and with SDS-grown cells of the natural *siaA* mutant strain N (overlap of data sets 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 with a *siaD* mutant (data set 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 data sets 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 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 data set C contains more genes (356) than data sets 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 Na_2-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^{-1} ampicillin (Fluka), 15 μg ml^{-1} gentamycin (Sigma) or 50 μg ml^{-1} tetracycline (Fluka). Plasmid-harbouring strains and insertional mutants of *P. aeruginosa* were selected on *Pseudomonas* isolation agar (Difco) containing 200 μg ml^{-1} carbenicillin, 120 μg ml^{-1} gentamycin or 160 μg ml^{-1} tetracycline. For experiments in liquid M9 medium, the concentrations of carbenicillin, gentamycin and tetracycline were decreased to 50, 10 and 20 μg ml^{-1} 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 Na_2-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 or 150 r.p.m. 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^{-1} 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′-GGACCTGCGCCTGCTGTACC TGAA-3′) and KO-PA0169-R (5′-GCCTCGCCCGCGCCTA TGG-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 SmaI, 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^{-1} 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′-CAACCTGCTCGCCGGCC TGCTCAC-3′) and pKO171-R (5′-CGGGCGGCGTAGCTGC TCCTTGTA-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′- GGATCCGCGG GCCGGGCGAGAAAC-3′) and 1205_rp_HindIII (5′- AAGC TTCGGGCGGCGTAGCTGCTCCTTGTA-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 (OD_{600} = 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 r.p.m. 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 three 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 (Quiagen), the samples were quantified spectrophotometrically and stored at −60°C until further analysis.

Table 2. Strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics	Source or reference
Pseudomonas aeruginosa		
PAO1	Wild-type of strain PAO1	Holloway collection
N	Spontaneous mutant of strain PAO1	Klebensberger *et al.* (2007)
B1	*cupA1*::mariner mutant (nucleotide position 480) in strain PAO1, Tet^r	This study
F5	*siaA*/PA0172::mariner mutant (nucleotide position 732) of strain PAO1, Tet^r	This study
KO0169	Insertional knockout mutant of *siaD*/PA0169 (resolvase site at position 368) in strain PAO1	This study
MPAO1 [11402] and [42553]	*sdsA1* (PA0740) insertional mutants derived from strain MPAO1	Jacobs *et al.* (2003); Washington Genome Center
MPAO1 [11153] and [20796]	*aceA* (PA2634) insertional mutants derived from strain MPAO1	Jacobs *et al.* (2003); Washington Genome Center
Escherichia coli		
JM109	*endA1 recA1 gyrA96 thi hsd R17 (r_k^-, m_k^+), relA1 supE44* Δ*(lac-proAB)* [F′ *traD36 pro*AB$^+$ *lac*I^q *lacZ*ΔM15]	Promega
CC118	*araD139* Δ*(ara leu)*7697 Δ*lac*X74 *phoA*Δ20 *galE thi rpsB argE$_{am}$ recA1*	Manoil and Beckwith (1985)
Plasmids		
pALMAR3	Plasmid harbouring a mariner transposon used for transposon mutagenesis, Tet^r	Jenal lab
pUCP18	*Escherichia-Pseudomonas* shuttle vector, Ap^r	West *et al.* (1994)
pUCP18[0169]	Plasmid pUCP18 harbouring a XbaI-HindIII fragment (1439 bp) encoding *siaD*/PA0169	This study
pUCP18[0172]	Plasmid pUCP18 harbouring a BamHI fragment (2708 bp) encoding *siaA*/PA0172 from the parent strain	This study
pUCP18[0172_N]	Plasmid pUCP18 harbouring a BamHI-HindIII fragment (2661 bp) encoding *siaA*/PA0172 from strain N	This study
pUCP18[4929]	pUCP18 harbouring a SalI fragment (2426 bp) encoding PA4929	Klebensberger *et al.* (2007)
pBBR1MSC-5	Broad-host-range cloning vector (Gm^r)	Kovach *et al.* (1995)
pBBR[CC3396]	pBBR1MSC-5 containing the gene CC3396 from *C. crescentus*	Jenal lab
pEX18AP	Gene replacement vector, Ap^r, *sacB*	Hoang *et al.* (1998)
pKO2b	pUC18Sfi containing a *res-cat-res* casette, Ap^r, Cm^r	Smits, unpublished
pUCPParA	*parA* as EcoRI-HindIII fragment in pUCP24, Gm^r	Smits *et al.* (2002)
pRK 600	*ori* ColE1 RK2-Mob$^+$ RK2-Tra$^+$ (Cm^r), helper strain in tri-parental matings	Kessler *et al.* (1992)

For reverse transcriptase reactions, cells of *P. aeruginosa* were grown in 10 ml LB medium in a plastic tube (Greiner) with shaking at 200 r.p.m. at 37°C. Cells were harvested during exponential phase ($OD_{600} = 0.8$) 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 morpholinopropansulfonic 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 was used. Total RNA was transferred to positively charged nylon membranes (Roche) overnight with a Turboblotter (Schleicher and 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)-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′-CCCCAGCGGCCGCAGAGGTCGTATT-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* hybridization 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* hybridization 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 h.

After hybridization 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) was applied. The entire data set 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 data set were selected for further calculations. Comparisons of groups consisting of two biological replicates were performed as indicated. Each signal intensity value was compared with the mean intensity of the corresponding control group. Relative gene expressions were determined by log2 ratios. Student's *t*-test was used to identify significant expression changes. From these data, selected subsets (data sets A–D) were chosen for further comparison (Tables S1–S4) 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′-TTGACGGTCTGCGAATAGGTTT-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 wit 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′-CTGGCGCC GGGCTGGACCTTCTACC-3′; 0170R_RT 5′-GTGGACTGG GTGCCGGGTATGTGC-3′) and one 651 bp PCR product (PA0171F_RT 5′-GCGCCGTGATCTGACCCCGTGTTT-3′; PA0169R (5′-AGGCGCCGCAGCTGCTTGTGGTAG-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 s, 60°C for 15 s 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.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

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. Data set A: genes activated in SDS-grown cells compared with succinate-grown cells of strain PAO1.

Table S2. Data set B: genes activated in SDS-grown cells of strain PAO1 compared with SDS-grown cells of the spontaneous *siaA* mutant strain N.

Table S3. Data set C: genes activated in SDS-grown cells of strain PAO1 compared with SDS-grown cells of the *siaD* mutant strain KO0169.

Table S4. Data set D: genes activated in SDS-grown cells compared with succinate-grown cells of the spontaneous *siaA* mutant strain N.

Table S5. Overlaps of data sets A and D in Fig. 6B. Genes activated in SDS-grown compared with succinate-grown cells of strain PAO1 (data set A) and in SDS-grown compared with succinate-grown cells of the spontaneous *siaA* mutant strain N (data set D).