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

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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 s*iaD* 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-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 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 *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 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

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

163 *Determination of survival rates in SDS shock experiments*

164 In our previous studies we had shown that aggregated cells had strongly
165 increased survival rates when challenged with SDS in the presence of CCCP
166 (Klebensberger *et al.*, 2006; Klebensberger *et al.*, 2007). In order to test whether
167 this was also true for mutants isolated in this study, we exemplarily evaluated two
168 non-aggregating mutants, one with a defect in *cupA*-encoded adhesive fimbriae
169 (strain B1) and one with a defect in the putative DGC PA0169 (strain KO0169) by
170 comparing their survival rates in SDS-shock experiments in the presence and
171 absence of CCCP. In these experiments, cell suspensions were first supplied
172 with SDS before CCCP was added to allow aggregation of those strains, which
173 were capable of aggregation. For the non-aggregating strains B1 and KO0169,
174 the addition of CCCP caused a dramatic drop of the survival rates by about 4
175 orders of magnitude compared to strain PAO1 (Fig. 4). When strain KO0169 was
176 complemented with pUCP18[0169] (Fig. 4) or pUCP18[4929] (not shown), the

177 survival rate could be restored to the level of the wild type strain PAO1. These
178 results clearly demonstrated that strains with the ability to form aggregates during
179 growth with SDS had an about 1000-fold increased survival rate under these
180 conditions.

181 *Complementation of non-aggregating mutants*

182 To investigate whether the DGCs PA4929 or PA1107, which restored SDS-
183 induced aggregation of strain N, could also complement the mutants deficient in
184 PA0172 and PA0169, we transformed strains F5 and KO0169 with
185 pUCP18[4929] and pUCP18[1107] and evaluated their colony morphology and
186 aggregation during growth with SDS. We found that formation of rough colonies
187 and of cell aggregates during growth with SDS could be restored in strains F5
188 and KO0169 by PA4929 (Fig. 3AB) and by PA1107 (not shown). In addition,
189 complementation of F5 and KO0169 with pUCP18[0172] and pUCP18[PA0169],
190 respectively, restored the SDS-specific rough colony morphology (not shown)
191 and the autoaggregative phenotype in liquid medium (Fig. 5). In contrast,
192 expression of pUCP18[0172] in strain KO0169 or pUCP18[0169] in strain F5 did
193 not restore SDS-induced aggregation (Fig. 6). If succinate was supplied instead
194 of SDS, none of the mutants complemented with pUCP18[0169] or
195 pUCP18[0172] formed aggregates, indicating a specificity of these genes for
196 inducing aggregation as a response to SDS (not shown).
197 In addition, we found that the formation of rough colonies and of cell aggregates
198 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

220 To investigate global differences between cells that do and do not show cell-
221 aggregation during growth with SDS we performed a transcriptome analysis of
222 strains PAO1, N and KO0169 grown with either SDS or succinate. In this
223 analysis, we focussed on the identification of genes that are specifically activated
224 in cells showing in SDS-induced aggregation. For this, we performed statistical
225 analysis of the micoarray data and selected four subsets of data, datasets A, B,
226 C and D, for further analysis (Tables S1-S4 in *Supplementary materials*).
227 Dataset A contains 111 genes that were activated in SDS-grown cells compared
228 to succinate-grown cells of strain PAO1. Dataset B contains 29 genes that were
229 activated in SDS-grown cells of strain PAO1 compared to SDS-grown cells of
230 strain N. Dataset C contains 356 genes that were activated in SDS-grown cells of
231 strain PAO1 compared to SDS-grown cells of strain KO0169.
232 Datasets A, B and C have an overlap of 36 genes (Fig. 6A, Table 1). Eight genes
233 are found in all three datasets, and five of these genes have been related to
234 biofilm formation in earlier studies. For *cupA1* (PA2128) an essential function in
235 biofilm formation has been demonstrated (Vallet *et al.*, 2001). The genes
236 PA4623-4625, which encode hypothetical exported proteins, were found to be
237 activated in a constitutively aggregating *wspF* mutant (Hickman *et al.*, 2005) and
238 in SCVs (Starkey *et al.*, 2009). The gene *mexE* (PA2493) was found to be
239 repressed in the biofilm-defective PpyR (PA2663) mutant compared to biofilm-
240 forming wild type cells (Attila *et al.*, 2008).
241 Further genes with a specific function in biofilm formation, autoaggregation or
242 involved in the regulation of these traits include *ompD* (PA4208) in the overlap of

243 dataset A and B (Southey-Pillig *et al.*, 2005), *pslK* (PA2241) in the overlap of
244 datasets B and C, and finally *cupA3* (PA2130) (Vallet *et al.*, 2004), PA2126
245 (Vallet-Gely *et al.*, 2007), PA2440 (Hickman *et al.*, 2005; Starkey *et al.*, 2009)
246 and *algA* (PA3551) in dataset C. In addition to these genes, dataset C contains
247 PA0172.
248 Dataset D contains 95 genes that were activated in SDS-grown cells compared
249 to succinate-grown cells of strain N. This dataset has a large overlap of 53 genes
250 with genes from dataset A (Fig. 6B; Table S5 in *Supplementary materials*), which
251 contains many genes with potential functions in the proposed pathway of SDS
252 degradation. These genes include *sdsA1* (PA0740), which encodes the
253 alkylsulfatase catalyzing the hydrolysis of SDS to 1-dodecanol (Hagelueken *et*
254 *al.*, 2006), two putative dehydrogenases (PA0364 and PA0366), which might be
255 responsible for oxidation of 1-dodecanol to lauric acid, and several genes
256 encoding putative enzymes for β-oxidation of lauric acid, among them a long-
257 chain-fatty-acid CoA-ligase (PA3299), two acyl-CoA-degydrogenases (PA0506
258 and 0508), a 3-hydroxyl-acyl-CoA dehydrogenase (PA3014) and an acyl-CoA-
259 thiolase (PA3925). Consistent with the formation of acetyl-CoA units as the end
260 products of β-oxidation, the genes encoding the enzymes of the glyoxylate shunt,
261 isocitrate lyase AceA (PA2634) and malate synthase AceB (PA0482), are also
262 found in the overlap of datasets A and D.
263 Induction of these genes is feasible because earlier physiological studies had
264 shown that succinate-grown cells are not induced for SDS-degradation
265 (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).

Dataset D did not overlap with dataset B and had only three overlaps with dataset 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 to 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 (551bp). 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 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 *cupA*1

289 transcript levels compared to strain PAO1 during growth with SDS.
290 Complementation of strain KO0169 with pUCP18[0169] led to increased *cupA1*
291 transcript levels in SDS-grown cells similar to those observed in cells of strain
292 PAO1 under these conditions. In contrast, expression of pUCP18[0172] had no
293 effect on the transcript levels of strain KO0169 in SDS-grown cells. Furthermore,
294 *cupA*1 transcript levels in SDS-grown cells could be decreased in strain PAO1 to
295 levels of succinate-grown cells by the expression of the known PDE CC3396
296 from *Caulobacter crescentus* (Klebensberger *et al.*, 2007).

297 **Discussion**

298 The goal of our study was to identify molecular modules that are specific and
299 essential for inducing autoaggregation in *P. aeruginosa* strain PAO1 in response
300 to SDS. By random- and site-directed mutagenesis, we found two genes with
301 such a function, namely PA0169 and PA0172, which are co-transcribed as an
302 operon together with PA0171 and PA0170. A clear function for this operon has
303 not been shown so far. Transcript levels of PA0169-0172 were elevated in a
304 constitutively aggregating *wspF* mutant of *P. aeruginosa* strain PAO1 (Hickman
305 *et al.*, 2005), and a PA0171 transposon mutant showed a permanently
306 aggregating phenotype (D'Argenio *et al.*, 2002) and decreased twitching motility
307 (Shan *et al.*, 2004), suggesting a general function of this operon in cell
308 aggregation. Here, we clearly demonstrate that PA0172 and PA0169 had an
309 essential function in SDS-induced cell-aggregation because their inactivation
310 caused a loss of this phenotype. Further, we show these two genes are
311 responsible for cell-aggregation as a specific response in the presence of SDS.

312 In respect of these essential and specific functions and the fact that the genes
313 PA0172, PA0171, PA0170 and PA0169 represent a transcriptional unit, we
314 propose to name these genes *siaABCD*, respectively, for *S*DS-*i*nduced-
315 *a*ggregation.
316 The physiological characterization and the complementation analysis suggest
317 that SiaA and SiaD are part of a molecular module involved in signal perception
318 and signal transduction, respectively. This is further supported by the domain
319 structure of both predicted proteins.
320 SiaA harbours a HAMP-domain which is a frequent and essential domain in
321 transmembrane receptors involved in bacterial two-component signal
322 transduction pathways, in particular in chemoreceptors (Hazelbauer *et al.*, 2008)
323 and references therein). The function of HAMP domains in such proteins is to link
324 input and output modules of transmembrane receptors. The PP2C-like
325 phosphatase domain represents such an output domain in bacterial
326 transmembrane receptors, for example in stress signalling in *Bacillus subtilis*,
327 such as RsbP (Vijay *et al.*, 2000) and RsbU (Hardwick *et al.*, 2007). Based on its
328 domain composition, we suggest that SiaA acts as stress sensor in the periplasm
329 or cytoplasm and causes dephosphorylation of downstream signal transduction
330 components after the perception of so far unknown stress signals. The potential
331 sensing domain of SiaA is not known at the present time.
332 Genetic analysis of this gene identified strain N as a natural, non-polar *siaA*
333 mutant. We currently do not know whether strain N harbours more mutations but,
334 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

381 the affiliation of *cupA1* (PA2128), whose essential role we have shown by
382 physiological characterization of the *cupA1* mutant strain B1. Northern blot
383 analysis revealed further that *cupA1* transcript levels are highly elevated in cells
384 exposed to SDS, and that this elevation requires the functional proteins SiaA and
385 SiaD and is linked to intracellular c-di-GMP levels. Recently, it has been shown
386 that anaerobiosis induces a phase-variable *cupA* expression through Anr-
387 mediated activation of the *cgr* genes (PA2127-PA2126), which are located
388 upstream of the *cupA* operon (Vallet-Gely *et al.*, 2007). In our microarray analysis
389 we found that PA2126 is activated in cells showing SDS-induced aggregation
390 compared to a *siaD* mutant (dataset C, Table S3). As the macroscopic
391 aggregates certainly contain zones, in which the cells face microaerophilic
392 conditions, Anr might contribute to the induction of the *cupA* operon.
393 Apart from *cupA1* and *mexE* (PA2493), the other six genes in the overlap of
394 datasets A, B and C encode for hypothetical proteins with putative functions. The
395 consistent activation of the genes PA4623-4625 in different autoaggregative *P.*
396 *aeruginosa* strains indicates that this gene cluster has an important role in cell
397 aggregation under a variety of conditions (Hickman *et al.*, 2005; Starkey *et al.*,
398 2009). Activation of *ompD* (PA4208), which is a part of the *mexGHI*-RND pump,
399 could be linked to increased pyocyanine production accompanying SDS-induced
400 aggregation (Dietrich *et al.*, 2006; Klebensberger *et al.*, 2007).
401 The fact that dataset C contains more genes (356) than datasets A (111) and B
402 (29) suggests that a deletion of *siaD* had impact on further cellular functions
403 apart from SDS-induced aggregation, which are independent of SiaA. In addition,

404 the downregulation of *siaA* in strain KO0169 is indicative of a positive feedback
405 regulation of SiaD on *siaA* expression.
406 The consistent activation of genes for SDS degradation in SDS-grown cells of
407 two different strains, strain PAO1 and strain N, supports the reliability of our
408 transcriptional analysis. Furthermore, it shows that degradation and cell
409 aggregation are induced by SDS as independent processes. SiaAD induce
410 aggregation as a response to an environmental stimulus, presumably cell
411 damage caused by SDS, thereby increasing the fitness of cells under conditions
412 that are detrimental for suspended cells. Under unstable environmental
413 conditions, this induction is certainly an advantageous trait for growth with SDS
414 because cells of *P. aeruginosa* will recurrently encounter various stresses in their
415 natural habitats. Under stable laboratory conditions, however, this aggregation is
416 not required for growth with SDS and its induction is readily lost by applying
417 appropriate selection pressure, as shown for the *siaA*-defective strain N. Such a
418 loss of non-essential physiological traits, which imply the formation of
419 multicellular structures, is a common event in the evolution of domesticated
420 laboratory strains (Aguilar *et al.*, 2007). Thus, by identifying genes for the
421 induction of autoaggregation, we could spot *siaA* as a target for the evolution of a
422 domesticated *P. aeruginosa* strain.

423 **Experimental procedures**

424 *Bacterial strains, growth media, growth experiments and cell suspension*
425 *experiments*

426 Bacterial strains and plasmids used in this study are listed in Table 2. Bacteria
427 were cultivated in Luria Bertani (LB) medium or in a modified M9 mineral medium
428 supplied with 3.5 mM SDS or 10 mM Na_2-succinate as carbon and energy
429 sources as described previously (Klebensberger *et al.*, 2006). Plasmid-
430 harbouring *Escherichia coli* strains were selected and maintained on LB agar
431 plates (1.5%, w/v) containing 100 µg/ml ampicillin (Fluka), 15 µg/ml gentamycin
432 (Sigma) or 50 µg/ml tetracycline (Fluka). Plasmid-harbouring strains and
433 insertional mutants of *P. aeruginosa* were selected on Pseudomonas isolation
434 agar (Difco) containing 200 µg/ml carbenicillin, 120 µg/ml gentamycin or 160
435 µg/ml tetracycline. For experiments in liquid M9 medium, the concentrations of
436 carbenicillin, gentamycin and tetracycline were decreased to 50 µg/ml, 10 µg/ml
437 and 20 µg/ml, respectively.
438 Growth experiments with *P. aeruginosa* were performed as described previously
439 (Klebensberger *et al.*, 2006). Colony morphology was evaluated on solid M9
440 medium containing 0.15% SDS or 10 mM Na_2-succinate after incubation for 3
441 days at 30°C. SDS-induced aggregation was tested in 3 ml M9 medium
442 containing 3.5 mM (0.1%) SDS in small Petri dishes (3.5 cm in diameter; Nunc)
443 or in 1.5 ml M9 medium in 12-Well plates (IWAKI Microplate; IWAKI Glass Co) on
444 a rotary shaker at 120 rpm or 150 rpm for 18 h at 30°C.
445 SDS shock experiments with cell suspensions of different *P. aeruginosa* strains
446 were performed as described previously (Klebensberger *et al.*, 2007).

447 *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 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 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

494 construct pUCP18[0172_N] a 2667 bp fragment containing the gene PA0172
495 was amplified from genomic DNA of strain N by PCR using the primer
496 1205_fp2_BamHI (5'- GGATCCGCGGGCCGGGCGAGAAAC-3') and
497 1205_rp_HindIII (5'- AAGCTTCGGGCGGCGTAGCTGCTCCTTGTA-3') and
498 cloned into pALLi10 (Trenzyme GmbH). PA0172_N was then excised as a
499 BamHI-HindIII fragment and subcloned into the respective restriction site of the
500 plasmid pUCP18 to obtain the plasmid pUCP[0172_N]. Correct orientation for
501 expressing of PA0169, PA0172 and PA0172_N from the *lac*-promoter of pUCP18
502 was confirmed by sequencing.

503 *RNA isolation*

504 For Microarray- and Northern blot analysis, suspensions (OD600 = 1.5) of
505 succinate-grown cells or of SDS-grown cells were supplied with their respective
506 substrate (10 mM succinate or 3.5 mM SDS) in triplicates in small Petri dishes
507 (3.5 cm in diameter, Nunc) in a final volume of 3 ml. After incubation with shaking
508 at 120 rpm at 30°C for 60 min, these triplicates were combined in a plastic tube
509 (Greiner) filled with 30 ml ice-cold DNase buffer. Cells were harvested by
510 centrifugation at 15.000 × g at 4°C for 1 min, and RNA was extracted from the
511 cells with the Purescript RNA Isolation Kit (Gentra Systems) according to the
512 manufacturer's instructions. RNA from 3 independent experiments was
513 combined, and contaminating DNA was removed with an off-column RNase-free
514 DNase I treatment (QIAGEN) according to the manufacturer's instructions. After
515 repurification with an RNeasy column (Quiagen), the samples were quantified
516 spectrophotometrically and stored at –60°C until further analysis

517 For reverse transcriptase reactions, cells of *P. aeruginosa* were grown in 10 ml
518 LB medium in a plastic tube (Greiner) with shaking at 200 rpm at 37°C. Cells
519 were harvested during exponential phase (OD = 0.8) by centrifugation at 5.000 ×
520 g at 4°C for 3 min, and RNA was extracted from the cells with the PureLink
521 Micro-to-Midi total RNA purification system (Invitrogen) according to the
522 manufacturer's instructions. Contaminating DNA was removed with an off-column
523 RNase-free DNase I treatment (QIAGEN) according to the manufacturer's
524 instructions. After repurification with an PureLink Micro-to-Midi column
525 (Invitrogen), the samples stored at –80°C until further analysis.

526 *Northern blot analysis*

527 For Northern blot hybridization, 1% agarose gels containing 3.5% formaldehyde
528 (w/v) were cast and run in 1 × MOPS buffer (20 mM morpholinopropansulfonic
529 acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) for size fractionation of RNA
530 samples. The loading dye for denaturation of the RNA samples contained 50%
531 formamide, 6% formaldehyde, 1 × MOPS buffer, 0.01% bromophenol blue, and
532 0.2% ethidium bromide.
533 For Northern blot analysis, 10 µg of total RNA were used. Total RNA was
534 transferred to positively charged nylon membranes (Roche) overnight with a
535 Turboblotter (Schleicher & Schuell) using 20 × SSC solution (3 M sodium
536 chloride, 0.3 M sodium citrate, pH 7). After UV-cross linking and washing with 2 ×
537 SSC solution for 1 h, the membranes were pre-hybridized with high-SDS-
538 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* 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

561 on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies). For biotin-
562 labelled target synthesis starting from 10 µg of total RNA, reactions were
563 performed using standard protocols supplied by the manufacturer (Affymetrix).
564 Briefly, 10 µg total RNA was converted to cDNA using random hexamers. The
565 cDNA was then fragmented by DNaseI and labelled with terminal transferase in
566 the presence of biotin-ddUTP to biotinylate cDNA at the 3´termini. Samples were
567 hybridized to an identical lot of Affymetrix GeneChip Pae_G1a for 16 hours.
568 After hybridisation the GeneChips were washed, stained with SA-PE and read
569 using an Affymetrix GeneChip fluidic station and scanner. DNA microarray
570 hybridization was performed in duplicates.
571 Analysis of microarray data was performed using the Affymetrix GCOS 1.2 using
572 the MAS5 algorithm. For normalization all array experiments were scaled to a
573 target intensity of 150, otherwise using the default values of GCOS 1.2. For
574 further downstream analysis Array Assist 4.0 software (Stratagene) were applied.
575 The entire dataset was cleaned for genes with no reliable signal measurements
576 indicated by the detection call of MAS5.0 algorithm. Therefore, genes showing
577 more than 50% “Present” calls across the dataset were selected for further
578 calculations. Comparisons of groups consisting of two biological replicates were
579 performed as indicated. Each signal intensity value was compared to the mean
580 intensity of the corresponding control group. Relative gene expressions were
581 determined by log2 ratios. A Student’s T-test was used to identify significant
582 expression changes. From these data, selected subsets (datasets A-D) were

583 chosen for further comparison (Tables S1-S4 in *Supplementary materials*) with
584 the software GeneVenn (Pirooznia *et al.*, 2007)

585 *Reverse transcription and subsequent PCR reactions*

586 For each reverse transcriptase reaction, 2 µg purified total RNA, 2 pmol
587 *siaD*/PA0169 specific primer PA0169RT (5´-TTGACGGTCTGCGAATAGGTTT-
588 3´) and 10 nmol dNTPs were mixed on ice in a sterile 0.2 µl PCR tube and
589 incubated at 65˚C for 5 min. After cooling the tubes on ice for 5 min, first-strand
590 cDNA synthesis was carried out by using SuperScriptIII Reverse Transcriptase
591 (Invitrogen) according to the manufacturer's instructions at 55˚C for 50 min.
592 Controls consisted of reactions without the addition of the SuperScriptIII enzyme.
593 After heat inactivation at 70˚C for 10 min and subsequent incubation wit 2 units
594 RNase H (Invitrogen) at 37˚C for 20 min, the first-strand reaction mixtures were
595 used as a template for subsequent PCR reactions. PCR was carried out by using
596 PWO DNA Polymerase (Roche Applied Science) with 2 µl of the first-strand
597 reaction mixtures and 15 pmol of each primer. Primer pairs were designed to
598 obtain one 840 bp PCR product (PA0172F_End_RT (5´-
599 CTGGCGCCGGGCTGGACCTTCTACC-3´), 0170R_RT (5´-
600 GTGGACTGGGTGCCGGGTATGTGC-3´)) and one 651 bp PCR product
601 (PA0171F_RT (5´-GCGCCGTGATCTGACCCCGTGTTT-3´), PA0169R (5´-
602 AGGCGCCGCAGCTGCTTGTGGTAG-3´)), which included the intergenic
603 sequences between PA0172-PA0170 and PA0171-PA0169, respectively.
604 Controls consisted of PCR reactions containing 2 µl of the control first-strand
605 reaction mixtures described above. All PCR reactions were performed in an

606 Mastercycler personal thermocycler (Eppendorf) using a program with an initial
607 denaturing step at 98˚C for 2 min and 30 cycles of 96˚C for 20 sec, 60˚C for 15
608 sec and 72˚C for 1 min. For analysis, 10 µl of each PCR reaction was size
609 fractionated by using a 1% (w/v) agarose gel, stained with ethidium bromide and
610 finally visualized by using a Gel Doc XR gel documentation system (Bio-Rad).

611 *Photography and image processing*

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

614 **Supplementary material**

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

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

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

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

625 **Table S4:** Dataset D: Genes activated in SDS-grown cells compared to
626 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).

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827

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

Gene no[a].	Gene name and protein description	Fold change in dataset A[b]	Fold change in dataset B[b]	Fold change in dataset 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

[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 $\geq$ 2.0 ($P \leq 0.05$) in datasets A and B.

837
838 **Table 2.** Strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics	Source or reference
P. 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
E. 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 harboring a XbaI-HindIII fragment (1439 bp) encoding *siaD*/PA0169	This study
pUCP18[0172]	Plasmid pUCP18 harboring a BamHI fragment (2708 bp) encoding *siaA*/PA0172 from the parent strain	This study
pUCP18[0172_N]	Plasmid pUCP18 harboring a BamHI-HindIII fragment (2661 bp) encoding *siaA*/PA0172 from strain N	This study
pUCP18[4929]	pUCP18 harboring 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)

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840 **Legends to the Figures**
841

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

854

855 **Fig. 2.** Predicted domain architecture of the protein encoded by *siaA* (PA0172) in
856 *P. aeruginosa* strain PAO1 and localization of the deletion in strain N. **A.**
857 Predicted domain structure of SiaA using the Simple Modular Architecture
858 Research Tool (SMART; http://smart.embl-heidelberg.de/). **B.** Localization of the
859 6bp in-frame deletion (black letters, nucleotides 1840-1852 of the ORF) leading
860 to a loss of a phenylalanine and a glycine residue within the predicted
861 PP2C_SIG-like domain in the C-terminal region of the *siaA* allele in strain N.

862
863
864 **Fig. 3.** Phenotypes of the *P. aeruginosa* strains PAO1, the *cupA1* transposon
865 mutant B1, the *siaA* transposon mutant F5 and the *siaD* mutant KO0169 during
866 growth with 3.5 mM SDS after transformation with pUCP18 (■) or pUCP18[4929]
867 (■). **A.** Colony morphology on M9 agar containing 0.15% SDS after incubation for
868 3 d at 37°C. **B.** Growth in liquid M9 medium containing 0.1% SDS in small Petri
869 dishes (3 cm diameter, Nunc) after incubation for 18 h at 30°C with shaking at
870 120 rpm.

871 **Fig. 4.** CFU counts of the *P. aeruginosa* strains PAO1, the *cupA1* transposon
872 mutant B1 and the *siaD* mutant KO0169 after 45 min of exposure to 3.5 mM SDS
873 and a subsequent incubation for an additional 60 min in the presence of 1 mM
874 CCCP (*white bars*) or methanol as a solvent control (*grey bars*). *Error bars*
875 indicate standard deviation (n = 3).
876
877 **Fig. 5.** Phenotypes of the *P. aeruginosa* strains PAO1, the spontaneous *siaA*
878 mutant N, the *siaA* transposon mutant F5 and the *siaD* mutant KO0169 during
879 growth in liquid medium after transformation with pUCP18, pUCP18[0169],
880 pUCP18[0172], and pUCP18[0172_N]. Cells were grown in M9 medium (12 well
881 plates) containing 10 mM succinate (■) or 3.5 mM SDS (■) for 18 h at 30°C with
882 shaking at 150 rpm.
883
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885 **Fig. 6.** Venn diagram showing overlaps of datasets A, B, C and D that were
886 derived from transcriptome analysis with DNA microarrays of the *P. aeruginosa*
887 strains PAO1, the spontaneous *siaA* mutant N and the *siaD* mutant KO0169.
888 Genes of all datasets are listed in Tables S1-S4 in *Supplementary materials*. **A**.
889 Dataset A (*white*): genes activated in SDS-grown cells compared to succinate-
890 grown cells of strain PAO1. Dataset B (*light grey*): genes activated in SDS-grown
891 cells of strain PAO1 compared to SDS-grown cells of the spontaneous *siaA*
892 mutant strain N. Dataset C (*dark grey*): genes activated in SDS-grown cells of
893 strain PAO1 compared to SDS-grown cells of the *siaD* mutant strain KO0169
894 (*siaD*). **B**. Dataset D (*dark grey*): genes activated in SDS-grown cells compared
895 to succinate-grown cells of the spontaneous *siaA* mutant strain N. Dataset A
896 (*white*). Genes overlapping between datasets A-C are listed in Table 1; genes
897 overlapping between datasets A and D are listed in Table S5 in *Supplementary*
898 *materials*.
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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 (■).