Detergent-induced cell aggregation in subpopulations of *Pseudomonas aeruginosa* as a preadaptive survival strategy

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Summary

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

Introduction

The ubiquitous bacterium *Pseudomonas aeruginosa* is characterized by a great metabolic versatility (Clarke, 1982) and a high adaptability to cope with different environmental stress factors (Rodrigue *et al.*, 2000). These traits support its successful colonization of hostile anthropogenic environments where it encounters toxic organic substances, such as detergents and disinfectants, some of which can be utilized as growth substrates. Examples for such environments are industrial wastewaters and healthcare settings where *P. aeruginosa* is a major cause for hospital infections (Emori and Gaynes, 1993). Sinks for disposal of disinfectants and detergents have been repeatedly identified as environmental sources of several *P. aeruginosa* outbreaks in clinical settings (Muscarella, 2004). Understanding survival and growth strategies of *P. aeruginosa* is thus a feasible approach for developing effective measures to prevent the establishment of this opportunistic pathogen in anthropogenic environments.

Recently, we have reported that *P. aeruginosa* strain PAO1 can grow with the anionic detergent SDS (Na-dodecylsulfate) as sole source of carbon and energy (Klebensberger *et al.*, 2006). At the same time the alkyl sulfatase SdsA, which catalyses the first step of SDS degradation in *P. aeruginosa*, was identified and characterized (Hagelueken *et al.*, 2006). SDS is a common ingredient of many household products and very toxic to bacteria because it damages membranes and proteins (Helenius and Simons, 1975). Bacteria require energy-dependent resistance mechanisms such as efflux pumps and Clp-proteases for growing in the presence of detergents (Rajagopal *et al.*, 2002; Poole, 2004). We have shown that SDS was also toxic for strain PAO1 and that surviving of SDS exposure required energy (Klebensberger *et al.*, 2006). If SDS is used for growth, the cells have to find a trade-off for energy investment: to generate energy for growth, they have to uptake SDS, thereby risking to be injured. For protection and repair, they have to invest part of this energy which is consequently not available for growth. This dilemma calls attention to further adaptive strategies that allow growth with a toxic compound as sole source of carbon and energy. We reported that strain PAO1 formed macroscopic cell

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aggregates as an active, energy-requiring stress response to SDS. The formation of cell aggregates or biofilms is an important adaptive strategy of *P. aeruginosa* and other bacteria to colonize adverse environments because residing in such structures confers increased resistance to biocides such as antibiotics, disinfectants and detergents (Lewis, 2001; Gilbert *et al.*, 2002; Drenkard, 2003; Fux *et al.*, 2005).

The formation of cell aggregates or biofilms requires the production of extracellular polymeric substances (EPS). The EPS of *P. aeruginosa* biofilms have been shown to contain DNA (Whitchurch *et al.*, 2002; Steinberger and Holden, 2005; Allesen-Holm *et al.*, 2006) and different polysaccharides (Linker and Jones, 1964; Friedman and Kolter, 2004; Tielen *et al.*, 2005; Ude *et al.*, 2006). The EPS of the macroscopic aggregates formed by strain PAO1 during growth with SDS contained acidic polysaccharides and DNA (Klebensberger *et al.*, 2006). By treatment with DNase, these aggregates were disintegrated and viable cells were released. The formation of cell aggregates and biofilms in *P. aeruginosa* and other bacteria has been shown to involve cyclic-di-guanosine monophosphate (c-di-GMP) signalling (Römling *et al.*, 2005; Jenal and Malone, 2006). This intracellular second messenger molecule was originally found in *Gluconacetobacter xylinus* where it acts as an allosteric regulator of cellulose synthase (Ross *et al.*, 1986). Cyclic-di-guanosine monophosphate biosynthesis from two GTP is catalysed by diguanylate cyclases (DGCs) containing a characteristic GGDEF domain as the active centre (Chan *et al.*, 2004; Ryjenkov *et al.*, 2005). The hydrolysis of c-di-GMP is catalysed by specific phosphodiesterases (PDEs) containing either an EAL or a HD-GYP domain (Christen *et al.*, 2005; Ryan *et al.*, 2006). *Pseudomonas aeruginosa* harbours 17 genes with a GGDEF domain, six genes with an EAL domain and 14 genes containing both domains (Kulesekara *et al.*, 2006). A function in cell aggregation or biofilm formation has been demonstrated only for a few of them so far (D'Argenio *et al.*, 2002; Drenkard and Ausubel, 2002; Hickman *et al.*, 2005; Hoffman *et al.*, 2005).

We proposed that aggregate formation of strain PAO1 is a survival strategy for growth with the toxic detergent SDS. This hypothesis was supported by the fact that aggregated cells of strain PAO1 had a more than 100-fold higher survival rate than freely suspended cells if exposed to SDS in the presence of a second toxic compound, carbonyl cyanide chlorophenylhydrazone (CCCP), which deprives the cells of energy by dissipating the proton motive force (Klebensberger *et al.*, 2006). However, in the cultures growing with SDS, the cells were not homogeneously distributed as they grew in aggregates and as freely suspended cells. This observation forces the question for the exact physiological role of cell aggregation

during growth with SDS. As aggregate formation strictly preceded growth of suspended cells, we hypothesized that cell aggregation is important for initiating growth of strain PAO1 with SDS. If cell aggregation would not be essential for growth with SDS, it should be readily lost because it is carbon- and energy-requiring. In this case non-aggregating mutants would arise and successfully compete against cells that maintain the aggregative phenotype. If cell aggregation is essential such non-aggregating mutants should not arise unless they are more resistant to SDS. Attempts to isolate spontaneous non-aggregating mutants of *P. aeruginosa* strain PAO1 were the starting point of our study to define the physiological role of cell aggregation during growth with the toxic detergent SDS.

Results

Isolation and characterization of strain N

To investigate whether spontaneous non-aggregative mutants of strain PAO1 would enrich in liquid cultures, freely suspended cells from cultures growing with SDS in the early logarithmic phase were repeatedly transferred into fresh medium. After 15 transfers we obtained a culture that contained only few and small aggregates. From this culture, aliquots were spread on solid M9 medium with SDS as carbon and energy source. We observed different colony morphologies, namely rough and highly structured colonies as well as smooth, soft and unstructured colonies. The parental strain PAO1 formed rough colonies on SDS-containing agar plates (Fig. 1, B1) and cell aggregates during growth with SDS in liquid culture (Fig. 1, C1). With succinate as carbon and energy source, strain PAO1 formed smooth colonies on agar plates (Fig. 1, A1) and did not aggregate in liquid cultures (not shown). One strain forming smooth colonies on SDS-containing agar plates, strain N, was characterized further. Strain N formed smooth colonies irrespective of the substrate (Fig. 1, A2 and B2) and did not aggregate in liquid cultures with SDS (Fig. 1, C2). This phenotype was stable without the appearance of revertants after several passages in media without SDS. Therefore, we consider strain N as a spontaneous mutant of strain PAO1 that does not aggregate during growth with SDS.

Strain N reached a higher OD₆₀₀ than the suspended cells of strain PAO1 (Fig. 2A) but their molar growth yields did not differ significantly [$Y = 163 \pm 16$ g dry mass (mol SDS)⁻¹ for strain N and $Y = 196 \pm 30$ g dry mass (mol SDS)⁻¹ for strain PAO1]. The growth rate of strain N with SDS ($\mu = 0.395$ h⁻¹) was higher than the growth rate of those cells of strain PAO1 that grew in suspension ($\mu = 0.3081$ h⁻¹). To test whether the increased growth rate of strain N was caused by a higher specific activity of

Fig. 1. Characterization of different *P. aeruginosa* strains.

A. Colony morphology on M9 containing 10 mM succinate after incubation for 1 day at 37°C.

B. Colony morphology on M9 containing 0.15% SDS after incubation for 3 day at 37°C.

C. Growth in liquid M9 medium containing 0.1% SDS after incubation for 18 h at 30°C with shaking at 120 r.p.m.

D. Swarming motility on semi-solid agar after incubation for 24 h at 37°C.

The different strains are indicated by the following numeration: (1) PAO1 pUCP18, (2) N pUCP18, (3) N pUCP18[4929], (4) PAO1-D4 pUCP18, (5) PAO1 pBBR, (6) PAO1 pBBR[CC3396].

enzymes involved in SDS degradation we determined the activity of SDS-alkyl sulfatase. The specific activities of SDS alkyl sulfatase in cell-free extracts of both strains were not significantly different (25 ± 3 mU mg⁻¹ protein for strain N and 31 ± 3 mU mg⁻¹ protein for strain PAO1).

During growth with SDS, strain PAO1 produced about 50% more pyocyanine than strain N (Fig. 2B). As a further difference, strain N had a higher swarming motility than strain PAO1 (Fig. 1, D1 and D2) whereas no differences were observed in swimming motility (not shown).

Fig. 2. Growth and pyocyanine production of different *P. aeruginosa* strains in M9 medium containing 3.5 mM SDS at 30°C with shaking at 200 r.p.m.

A. OD₆₀₀ of strains PAO1 pUCP18 (■), N pUCP18 (▲), N pUCP18[4929] (△), PAO1-D4 pUCP18 (□), PAO1 pBBR (●) and PAO1 pBBR[CC3396] (○).

B. Percentages of pyocyanine produced by the individual strains normalized to pyocyanine production of strain PAO1 pUCP18 (100%). The different strains are indicated by the following numeration: (1) PAO1 pUCP18, (2) N pUCP18, (3) N pUCP18[4929], (4) PAO1-D4 pUCP18, (5) PAO1 pBBR, (6) PAO1 pBBR[CC3396]. Error bars indicate standard deviation ($n = 3$).

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

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

Restoration of SDS-induced aggregation in strain N by complementation

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

that included genes with a GGDEF motif. In particular, these complementing fragments contained genes for the putative DGCs PA1107 (neighbouring by PA1106 and *fljJ*), PA1727 (neighbouring by PA1728 and PA1729) and PA4929 (neighbouring by *alr*). Further subcloning of these three putative DGC genes as single genes (Table 1) revealed that they were responsible for complementation in strain N.

To investigate whether the formation of macroscopic aggregates by these complemented clones of strain N was SDS-dependent or a constitutive phenotype caused by overexpression of DGCs, we compared their phenotypes during growth with SDS and with succinate. Strains N pUCP18[1107] (not shown) and N pUCP18[4929] formed rough colonies on agar plates and macroscopic aggregates in liquid medium only with SDS but not with succinate as carbon and energy source (Fig. 1, A3–C3). In contrast, strain N pUCP18[1727] formed rough colonies and macroscopic aggregates also during growth with succinate (not shown). Swarming motility, growth curves and pyocyanine production of strain N pUCP18[1107] (not shown) and of strain N pUCP18[4929] resembled those of the parental strain PAO1 (Fig. 1, D3; Fig. 2). The macroscopic aggregates formed by strain N complemented with pUCP18[1107] or pUCP18[4929] were indistinguishable from aggregates of strain PAO1.

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

Two of the four remaining complementing plasmids contained fragments of about 1200 bp with the single gene PA1869 which encodes a putative acyl carrier protein (KN2C and NNG1, Table 1). The other two complementing plasmids carried a fragment of about 3900 bp containing the genes *pvcBCD* (KN6B and KN7C, Table 1). Sequence analysis of these genes in strain N revealed no mutations compared with the corresponding sequences in *Pseudomonas* Genome Project database.

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

The restoration of SDS-dependent cell aggregation in strain N by complementation with DGCs strongly suggested that this process involved the second messenger c-di-GMP. To test this hypothesis we transformed strain PAO1 with the plasmid pBBR[CC3396] containing the

gene for the known PDE CC3396 from *Caulobacter crescentus* (Christen *et al.*, 2005). In strain PAO1 pBBR[CC3396] the roughness of colonies on SDS-containing agar plates (Fig. 1, B6) and the formation of macroscopic aggregates in SDS-containing liquid culture (Fig. 1, C6) were strongly reduced compared with the vector control strain PAO1 pBBR. Furthermore, strain PAO1 pBBR[CC3396] showed increased swarming motility (Fig. 1, D6) and a higher growth rate with SDS (Fig. 2A) than the suspended cells of the vector control (Fig. 1, D5; Fig. 2A). Pyocyanine production was only slightly decreased (Fig. 2B).

SDS shock experiments with single and mixed cultures

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

In the next step, we investigated whether the non-aggregating strains could be integrated into aggregates of strain PAO1 and, if so, whether they would benefit from this integration during exposure to SDS in the presence of CCCP. For this, we set up SDS shock experiments in which we mixed strain PAO1 with strains N, N pUCP18[4929] and PAO1 pBBR[CC3396] at equal cell numbers prior to addition of SDS, as described under *Experimental procedures*. In all combinations, macroscopic cell aggregates were formed. Strain N accounted for 35.7% of the cfus released from aggregates formed with strain PAO1 (Fig. 4, A1). Thus, although strain N was incapable of SDS-induced aggregation it could be integrated into cell aggregates of the parental strain. This integration was confirmed by confocal laser scanning microscopy (CLSM) analysis of aggregates containing strains PAO1-Tn7-cfp and N-Tn7-yfp (Fig. 5A). Aggregates consisting mainly of strain PAO1 cells were interspersed with single cells and cell clusters of strain N. When mixed cell suspensions with strains PAO1 and N were exposed to SDS in the presence of CCCP after strain N had been integrated into the aggregates, the percentage of strain N decreased to 14% (Fig. 4, A3). Considering the

Fig. 4. SDS shock experiments with mixed cell suspensions of different *P. aeruginosa* strains.

Grey bars indicate percentages of total cfu counts of strains PAO1 pUCP18 (A, B, D) or PAO1 pBBR (C). White bars indicate percentages of total cfu counts of strains N pUCP18 (A), N pUCP18[4929] (B), PAO1 pBBR[CC3396] (C) and PAO1-D4 pUCP18 (D). Percentages were calculated after differentiating single colonies either by their colony morphology on SDS containing agar plates (A, C, D) or by the fluorescence of YFP- and CFP-tagged strains (B).

1. Percentages of individual strains in macroscopic cell aggregates formed after incubation of mixed cell suspensions with SDS for 45 min.

2 and 3. Percentages of individual strains in whole cell suspensions (aggregated plus suspended cells) after incubation with SDS for 45 min and additional incubation with methanol as solvent control (2) or CCCP (3) for 60 min.

Cell aggregates and whole cell suspensions were treated with DNase prior to cfu determination as described in *Experimental procedures*. Error bars indicate standard deviation ($n = 3$).

total cfu counts in these experiments (mean value of 4.33×10^7 cfu ml⁻¹), this percentage equals 6×10^6 cfu ml⁻¹. The cfus of strain N in the absence of strain PAO1 were 7.5×10^3 after exposure to SDS in the presence of CCCP (Fig. 3). Taking into account that twice as much cells of strain N were used in the single strain experiment, the survival rate of strain N was more than 1000-fold higher in the presence of strain PAO1 than in cell suspensions of strain N alone. If strain N was complemented with pUCP18[4929], its percentage of cfus released from aggregates increased to about 50% (Fig. 4,

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

B1). Complementation with pUCP18[4929] also increased the survival rate of strain N after addition of CCCP more than twofold (Fig. 4, B3). If strain PAO1 harboured pBBR[CC3396] its percentage of cfus released from aggregates was slightly reduced to 44.3% (Fig. 4, C1). This value was further decreased to 32.6% if the cells were exposed to SDS in the presence of CCCP (Fig. 4, C3).

Co-cultivation of strains PAO1 and N

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

Characterization of the transposon mutant strain PAO1-D4

The discovery that smooth colony morphology on SDS-containing agar plates correlated with a non-aggregating

phenotype during growth with SDS in liquid culture enabled us to conveniently screen for non-aggregating transposon mutants of strain PAO1. We created a mariner transposon mutant library of strain PAO1 and searched for smooth colonies on SDS-containing agar plates. From a first screening we isolated the transposon mutants PAO1-D1 and PAO1-D4. Sequence analysis revealed that the mariner transposons were inserted in the genes *pslF* and *pslJ* respectively. These genes encode hypothetical proteins with homology to a putative glycosyltransferase (*pslF*) and polysaccharide polymerase (*pslJ*). They are part of an operon (*pslA-O*) which is responsible for exopolysaccharide biosynthesis and biofilm formation in *P. aeruginosa* (Friedman and Kolter, 2004; Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004; Ma *et al.*, 2006). Both strains did not aggregate in liquid cultures with SDS. A role for *pslF* in biofilm formation had already been described (Friedman and Kolter, 2004). We characterized the *pslJ* mutant strain PAO1-D4 further. Its physiological properties resembled those of strain N, namely smooth colony morphology on SDS-containing agar plates (Fig. 1, A4), no aggregation and a higher growth rate with SDS in liquid culture (Fig. 1, B4; Fig. 2A), increased swarming motility (Fig. 1, D4), and strongly decreased pyocyanine production (Fig. 2B). As an important difference to strain N, SDS-induced aggregation could not be restored in strain PAO1-D4 by transformation with pUCP18[4929].

In SDS shock experiments strain PAO1-D4 resembled strains N and PAO1 pBBR[CC3396] as treatment with

SDS and CCCP caused a drop of cfu counts by more than four orders of magnitude compared with the solvent control (Fig. 3). In cell aggregates formed with strain PAO1 we found that strain PAO1-D4 accounted only for 1.3% of the cfus released from aggregates (Fig. 4, D1). If these cell suspensions were supplied with CCCP the cfus of strain PAO1-D4 were below the detection limit of 0.85% (Fig. 4, D3).

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

Discussion

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

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

growing with SDS were divided into aggregating and non-aggregating subpopulations calls attention to the molecular basis of this phenotypic variation.

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

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

P. aeruginosa could be detected by HPLC analysis only upon overexpression of certain DGCs during growth in rich medium indicates that the levels of this second messenger are generally low in this organism (Kulesekara *et al.*, 2006).

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

The complementing effect of PA1869 and *pvcBCD* cannot be explained at the present time. All four genes were not mutated in strain N and a non-polar deletion of PA1869 in strain PAO1 had no phenotype (J. Klebensberger and B. Philipp, unpublished).

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

upstream of c-di-GMP signalling. The respective gene product could be involved in transducing SDS-induced stress to the postulated c-di-GMP signalling pathway.

We have shown that integration into aggregates of strain PAO1 conferred a substantial survival advantage for strain N upon exposure to SDS in the presence of CCCP. In this respect, cell aggregation could ensure genotypic and phenotypic diversity of *P. aeruginosa* populations which supports survival and growth in environments with fluctuating loads of multiple toxic chemicals, such as the aforementioned industrial wastewaters or clinical settings. Aggregating variants could increase population's survival chances under strongly adverse conditions while non-aggregating and fast-growing variants could increase the population's chances for rapid colonization of a new habitat when conditions improve. Pyocyanine production which accompanied cell aggregation in our experiments would be a means to inhibit competing microorganisms. To understand this survival strategy of *P. aeruginosa* in more detail, we are currently investigating the presumptive stress signals, the signal transduction pathways and the molecular targets involved in SDS-induced aggregation.

Experimental procedures

Bacterial strains and growth media

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

Growth experiments and swarming assay

Growth experiments with *P. aeruginosa* were performed as described previously (Klebensberger *et al.*, 2006). For determination of molar growth yields (Y) with SDS, cultures were incubated until onset of the stationary phase. Cells were harvested immediately by centrifugation at 15 000 g for 10 min at 7°C. To obtain the complete biomass, biofilms attached to the glass surface were scraped off and added to the fraction of suspended cells. The cells were washed once with 30 ml of distilled water, resuspended in a small volume of

Table 1. Strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics	Source or reference
<i>P. aeruginosa</i>		
PAO1	Wild type of strain PAO1 (RH)	Holloway collection
N	Spontaneous mutant of strain PAO1 (SH)	This study
PAO1-Tn7-yfp	PAO1 with Tn7 chromosomal insertion of <i>yfp</i> (RH)	This study
N-Tn7-cfp	N with Tn7 chromosomal insertion of <i>cfp</i> (SH)	This study
PAO1-D1	Mariner transposon mutant of strain PAO1, insertion at position 596 of gene <i>pslF</i> (1178 bp) (SH)	This study
PAO1-D4	Mariner transposon mutant of strain PAO1, insertion at position 1118 of gene <i>pslJ</i> (1437 bp) (SH)	This study
PAO1-KO[1107]	PAO1, PA1107::pKO[1107] (RH)	This study
PAO1-KO[1727]	PAO1, PA1727::pKO[1727] (RH)	This study
PAO1-KO[4929]	PAO1, PA4929::pKO[4929] (RH)	This study
<i>E. coli</i>		
JM109	<i>endA1 recA1 gyrA96 thi hsd R17 (r_K⁻, m_K⁺), relA1 supE44 Δ(lac-proAB) [F⁺ traD36 proAB⁺ lacI^q lacZΔM15]</i>	Promega
SM10::λpir	<i>thi1 thr1 leuB6 supE44 tonA21 lacY1 recA::RP4-2-Tc::Mu Km^r λpir</i>	Miller and Mekalanos (1988)
HB101	<i>thi-1 hsd S20 (r_B⁻, m_B⁻) supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20 (str^r) xyl-5 mtl-1 galK2</i>	Promega
S17-1	<i>thi pro hsdR hsdM⁺ recA RP4-2-Tc::Mu-Km::Tn7</i>	Simon <i>et al.</i> (1983)
Plasmids		
pUCP18	<i>Escherichia</i> – <i>Pseudomonas</i> shuttle vector (Ap ^r)	West <i>et al.</i> (1994)
pKnockout-G	Suicide vector used for gene inactivation (Ap ^r , Gm ^r)	Windgassen <i>et al.</i> (2000)
pALMAR3	Plasmid harbouring a mariner transposon used for transposon mutagenesis (Tet ^r)	Jenal lab
pBK-miniTn7(gm)	Plasmid for chromosomal integration of <i>yfp</i> (Ap ^r , Gm ^r , Cm ^r)	Klausen <i>et al.</i> (2003)
PA1 _{104/03} - <i>eyfp-a</i>		
pBK-miniTn7(gm)	Plasmid for chromosomal integration of <i>cfp</i> (Ap ^r , Gm ^r , Cm ^r)	Klausen <i>et al.</i> (2003)
PA1 _{104/03} - <i>ecfp-a</i>		
pBBR1MSC-5	Broad-host-range cloning vector (Gm ^r)	Kovach <i>et al.</i> (1995)
pBBR[CC3396]	pBBR1MSC-5 containing gene CC3396 from <i>C. crescentus</i>	Jenal lab
KN6B and KN7C	Complementing plasmids harbouring the genes <i>pvcBCD</i> (c. 3900 bp)	This study
KN2C and NNG1	Complementing plasmids harbouring the single gene PA1869 (c. 1200 bp)	This study
pUCP18[1107]	pUCP18 harbouring a SmaI fragment (2008 bp) encoding the single gene PA1107	This study
pUCP18[1727]	pUCP18 harbouring a EcoRI-HindIII fragment (2476 bp) encoding the single gene PA1727	This study
pUCP18[4929]	pUCP18 harbouring a Sall fragment (2426 bp) encoding the single gene PA4929	This study
pKO[1107]	pKnockout-G harbouring an internal EcoRI fragment (883 bp) of PA1107	This study
pKO[1727]	pKnockout-G harbouring an internal HincII fragment (702 bp) of PA1727	This study
pKO[4929]	pKnockout-G harbouring an internal BamHI-PstI fragment (908 bp) of PA4929	This study
pUX-BF13	Plasmid providing the Tn7 transposase genes (Ap ^r)	Bao <i>et al.</i> (1991)
pRK 600	<i>ori ColE1 RK2-Mob⁺ RK2-Tra⁺ (Cm^r)</i>	Kessler <i>et al.</i> (1992)

(RH) indicates rough colony morphology on SDS-containing agar. (SH) indicates smooth colony morphology on SDS-containing agar.

distilled water, transferred to weight-constant test tubes, and centrifuged at 2000 *g* for 10 min at 7°C. Supernatants were decanted and cell pellets were completely dried at 70°C. After cooling the tubes in a desiccator, the dry weights were determined. SDS concentrations at the beginning and at the end of growth were determined as described previously (Klebensberger *et al.*, 2006).

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

Gene library construction and complementation of mutants

Genomic DNA of strain PAO1 was purified (Puregene DNA Isolation Kit, Gentra) and partially digested with Sau3AI. DNA

fragments between 1 kb and 8 kb were extracted and purified (E.Z.N.A Gel Extraction Kit, Peqlab) from agarose gels and ligated into the BamHI restriction site of pUCP18 (West *et al.*, 1994). Competent *E. coli* JM109 cells (Promega) were transformed with these plasmids and submitted to ampicillin selection and blue-white screening. Six thousand positive clones were transferred into 96-well plates by pooling five clones per well in 200 µl of LB with ampicillin and grown with shaking at 200 r.p.m. for 24 h at 37°C. All pools of transformants were combined at equal volumes, washed twice in LB medium and frozen as glycerol stocks (40% v/v) in liquid nitrogen. The plasmids from this gene library were extracted (peqGold Plasmid Miniprep Kit I, Peqlab) and transformed into *P. aeruginosa* strain N (Irani and Rowe, 1997). Transformants were screened on M9 agar plates containing 0.15% SDS and 125 µg ml⁻¹ carbenicillin. Clones with rough colony morphol-

Table 2. Primers used in this study.

Primers	Sequence
Chromosomal insertion analysis	
pKO-G	5'-GCGCGTTGGCCGATTCATTA-3'
PA1107-Check-R	5'-CTGGTCGGCGGCGCTGTAGAG-3'
PA1727-Check-R	5'-CCGGCAGGCGTTTGAGATACAG-3'
PA4929-Check-R	5'-CTGGTCGGCGGCCCTTGAGAGTTT-3'
Tn7-GlmS	5'-AATCTGGCCCAAGTCGGTGAC-3'
Tn7R109	5'-CAGCATAACTGGACTGATTTTCAG-3'
Transposon insertion analysis	
Marseq-F	5'-TGAATGCGCAAACCAACCCTTGGC-3'
Marseq-R	5'-GGAAACAGCTATGACCATGATTACGCC-3'
Sequence analysis	
PA1107-F	5'-GACGCGCCGGAGCCCTGTTCG-3'
PA1107-R	5'-GAGCGCCACGGACCATCTTA-3'
PA 1727-F	5'-GGGAATTCCATGAGACGCACCTCCTGT-3'
PA 1727-R	5'-GCGATGGGCGAATCTGAAGCTTCTGAC-3'
PA4929-F	5'-GAAGGGCGCCGGACCGAAACACTC-3'
PA4929-R	5'-TGCGGCCGGGAATCATGCTCTACG-3'
PA 1869-F	5'-CGGGCCCTGGCGGAAGT-3'
PA 1869-R	5'-GCGGGCGGCGGCAAAAA-3'
pvc-F	5'-CTCGCCGCGGTGAGCCAGTG-3'
pvc-R	5'-CGTTTCCGCGGCGTTCATCC-3'

ogy were further tested for SDS-induced aggregation in 3 ml of M9 medium containing 0.1% SDS in small Petridishes (3.5 cm in diameter; Nunc) on a rotary shaker at 120 r.p.m. (Orbital Incubator S150, Stuart Scientific) for 18 h at 30°C. Plasmids that restored aggregation of strain N in liquid media were isolated, the chromosomal fragments were sequenced and identified by comparison with the *Pseudomonas* Genome Project database (<http://v2.pseudomonas.com/>).

Construction of plasmids and insertional mutants

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

Transposon mutagenesis

For random transposon mutagenesis of strain PAO1, the vector pALMAR3 carrying a mariner transposon (Lampe *et al.*, 1999) with a tetracycline resistance gene was used (kindly provided by Urs Jenal). For bi-parental matings, *E. coli* S17-1 harbouring pALMAR3 (donor) was grown in LB medium with shaking at 150 r.p.m. at 37°C, while strain PAO1 (recipient) was grown in LB medium with shaking at 50 r.p.m. at 42°C. After incubation overnight, 5×10^8 cells of the donor and 1×10^9 cells of the recipient were harvested by

centrifugation at 10 000 r.p.m. for 1.5 min (5415 D; Eppendorf), washed twice in 2 ml of prewarmed LB medium, and finally resuspended in 50 µl of LB medium. Donor and recipient were carefully mixed by pipetting and spread onto sterile membrane filters (OE66 0.2 µm 25 mm diameter; Schleicher and Schuell) that were placed on prewarmed LB agar plates. After incubation for 6 h at 37°C, the filters were transferred to a 50 ml plastic tube (Greiner) containing 2 ml of NaCl (0.9%). After vortexing, aliquots of the cell suspensions were spread on PIA agar plates containing 160 µg ml⁻¹ tetracycline to select for transposon mutants of strain PAO1. After incubation for 48 h at 37°C, all colonies (~20 000) were scraped off, transferred into a plastic tube containing 25 ml of NaCl (0.9%, w/v), washed twice and finally resuspended in 4 ml of LB medium.

Transposon mutants with smooth colony morphology on M9 agar plates containing 0.15% SDS and 80 µg ml⁻¹ tetracycline were further screened for SDS-induced aggregation in liquid culture. In non-aggregating mutants, the exact position of the transposon insertion was identified by inverse PCR with primers indicated in Table 2 in cooperation with Trenzyme GmbH (Konstanz). Transposon insertion sites were identified by comparison with the *Pseudomonas* Genome Project database. Strains PAO1-Tn7-yfp and N-Tn7-cfp were constructed through site-directed transposon insertion into the chromosome via four-parental mating as described previously (Klausen *et al.*, 2003).

SDS shock experiments

SDS shock experiments with cell suspensions of single cultures of different *P. aeruginosa* strains were performed as described previously (Klebensberger *et al.*, 2006). In SDS shock experiments with mixed cell suspensions of different *P. aeruginosa* strains, 500 µl from cell suspensions (OD₆₀₀ = 1.5) of two different strains were combined in one well of a 24-well microtitre plate (Nunc Surface, Nunc),

supplied with SDS (3.5 mM) and incubated on a rotary shaker at 200 r.p.m. and 30°C for 45 min. After macroscopic aggregates had formed, they were separated from the suspended cells. For this, aggregates were transferred into 30 ml of DNase buffer (50 mM Tris-HCl with 10 mM MgCl₂ at pH 7.2) with a pipette, harvested by centrifugation at 80 g for 5 min, washed twice and finally resuspended in 3 ml of DNase buffer. Aggregates were disintegrated by treatment with DNase I (Type II, stock solution in water, Sigma) for 30 min at 37°C. Cells released from aggregates and cells that had remained in suspension during SDS shock were quantified by cfu counts as described previously (Klebensberger *et al.*, 2006). As controls, single cultures of the individual strains were submitted to the same procedure in parallel.

To determine the survival rates of strains in single or mixed culture after exposure to SDS plus CCCP (Sigma), SDS shock experiments were set up as described above. After 45 min, cell suspensions were supplied with CCCP (1 mM) and incubated for additional 60 min with shaking at 75 r.p.m. Then, the complete cell suspensions (suspended cells plus aggregates) were transferred into 30 ml of DNase buffer and harvested by centrifugation at 10 000 g for 8 min at room temperature. After washing, the cells were resuspended in DNase buffer in a final volume of 3 ml and treated with DNase I for 30 min at 37°C. Colony-forming unit counts were determined as described above. Cell suspensions with methanol were used as controls.

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

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

Other analytical methods

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

For pyocyanine determination, aliquots (5 ml) of supernatants from SDS-grown cultures from early stationary phase were extracted with chloroform (3 ml) in a plastic tube (Sarstaedt). After centrifugation at 2000 g for 2 min, 2 ml of

the chloroform phase was transferred to a new tube and subsequently mixed with 1 ml of 0.2 M HCl. Pyocyanine was determined by measuring the absorbance of the aqueous phase at 520 nm (A_{520}).

For nucleotide extraction, cell suspensions (9 ml, OD₆₀₀ = 1.5) were shocked with SDS as described above. After 1 h, cells were supplied with 10 ml of ice cold DNase buffer, centrifuged at 15 000 g for 1 min at 4°C, and immediately extracted either with 1 M formic acid or by boiling in 65% ethanol (v/v). Extracts were separated from cell debris by centrifugation, lyophilized, and finally dissolved 200 µl in distilled water. Extracts were analysed with a reversed-phase HPLC system (Philipp *et al.*, 2006) using 100 mM sodium-phosphate buffer pH 5.8 (eluent A) and acetonitrile (eluent B) at a flow rate of 0.7 ml min⁻¹ at room temperature. For separation of nucleotides a gradient was applied starting with 5% B for 10 min, then rising to 70% B within 10 min and returning to 5% B within 1 min followed by 7 min equilibration. A standard of c-di-GMP (BIOLOG Life Science) eluted after about 15 min.

CLSM and macroscopic images

Macroscopic aggregates of *P. aeruginosa* cells labelled with CFP and YFP were placed on glass slides and fixed by embedding them in polyacrylamide (13%). Microscopic investigation was performed either directly or after staining of the bacterial aggregates with the DNA binding fluorochrome SYTO 9 (Molecular Probes) and the lectin concanavalin A labelled with the fluorescent dye tetramethyl rhodamine isothiocyanate (TRITC-ConA, Sigma). Samples were stained by layering a solution containing TRITC-ConA (10 µg ml⁻¹) and SYTO 9 (1.5 µl ml⁻¹) in deionized water on top of the aggregates, incubating them statically for 20 min in the dark and washing them twice in deionized water. Microscopic examination was performed with a LSM 510 confocal laser scanning microscope (Zeiss, Germany), consisting of a laser scanning module that was mounted on an Axiovert 100 M BP inverted microscope (Zeiss) equipped with the following objectives (Zeiss): Plan-Neofluar 10×/0.30, LD-Achroplan 40×/0.60 Korr and LD-Achroplan 63×/0.75 Korr Ph2. An argon laser and a helium-neon laser were applied for the analysis of the samples. Images of CFP-labelled cells were recorded at an excitation wavelength of 458 nm and an emission wavelength of 475–515 nm; images of YFP-labelled cells were recorded at an excitation wavelength of 488 nm and emission wavelength > 515 nm, using a LP 505 nm long-pass detection filter. Aggregates stained with SYTO 9 and TRITC-ConA were visualized at an excitation wavelength of 488 nm and an emission wavelength range of 505 nm–550 nm provided by a BP 505–550 band-pass filter (SYTO 9) and at an excitation wavelength of 543 nm and an emission wavelength > 570 nm using an LP 560 long-pass filter (TRITC-ConA). Image recording of optical thin sections was performed with the LSM software (version 3.2 SP2, Zeiss). Image analysis including data processing and three-dimensional reconstruction was performed using the AxioVision software version 3.1 (Zeiss). Macroscopic images of colonies, SDS-induced aggregation and swarming plates were taken with a Canon Powershot G6 camera. Images were processed with Paint Shop Pro 4.

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