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ORIGINAL PAPER

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

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

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Abbreviations CCCP: Carbonyl cyanide *m*-chlorophenyl hydrazone · CFU: Colony forming units · CTC: 5-Cyano-2,3-ditolyl tetrazolium chloride · OD_{600}: Optical density at 600 nm · SDS: Sodium dodecyl sulfate · ROS: Reactive oxygen species

Introduction

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

biofilm formation as a defensive reaction to the presence of aminoglycoside antibiotics has been shown in *P. aeruginosa* (Hoffman et al. 2005).

We are interested in the role of bacterial aggregates in the degradation of toxic chemicals, in particular anionic detergents. Sulfate ester detergents like sodium dodecyl sulfate (SDS) are considered as readily biodegradable (Scott and Jones 2000). Several publications have described SDS degradation by pure cultures of *Pseudomonas* strains (Payne and Feisal 1963; Stavskaia et al. 1989; Marchesi et al. 1994; Ellis et al. 2002). Degradation is started by an alkyl sulfatase which hydrolyses SDS to sulfate and 1-dodecanol. This primary alcohol is oxidized to lauric acid and further degraded by β-oxidation to acetyl-CoA residues (Thomas and White 1989). Degradation of SDS is a major challenge for bacteria because this detergent solubilizes biological membranes and denatures proteins (Helenius and Simons 1975). However, no publication dealing with SDS degradation has addressed the toxicity of SDS so far. Several resistance mechanisms against anionic detergents like diffusion barriers (Nikaido and Vaara 1985), multidrug efflux pumps (Poole 2004), or Clp-proteases (Rajagopal et al. 2002) have been described. All these resistance mechanisms require energy and have been shown to protect cells which grow in the presence of detergents (Nickerson and Aspedon 1992). Bacteria using detergents for growth face an additional challenge. They have to invest part of their energy into protection, while taking an increased risk of damage because they have to take up the toxic detergents to metabolize them. So far it is not known whether bacteria that utilize detergents as growth substrates require additional strategies to protect themselves. We hypothesize that formation of cell aggregates would be a feasible strategy for this purpose.

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

Materials and methods

Cultivation and growth experiments

Pseudomonas aeruginosa strain PAO1 (Holloway Collection) was maintained on solid (1.5% w/v agar) Luria-Bertani (LB) medium. A *rpoS* mutant of the same strain (Diggle et al. 2002) and a *cioB* insertion mutant of [32522] derived from strain MPAO1 (Jacobs et al. 2003) provided by the Washington genome center (http://www.genome.washington.edu/UWGC) were maintained on solid LB medium containing 50 μg/ml kanamycin or 60 μg/ml tetracycline, respectively. For cultivation in liquid media, the LB medium and a modified M9 medium (Sambrook et al. 1989) were used. The M9 medium contained the following components (final concentration in mM): Na_2HPO_4 (47.6), KH_2PO_4 (22), NaCl (8.6), NH_4Cl (18.6), $MgSO_4$ (2), $CaCl_2$ (0.1), $FeCl_2$ (0.03), and the trace element solution SL10 (Widdel and Pfennig 1981). SDS (3.5 mM) or Na_2-succinate (10 mM) was used as carbon and energy sources. Growth was measured as optical density at 600 nm (OD_{600}) in a spectrophotometer. For growth experiments, a test tube with 5 ml LB medium was inoculated with strain PAO1 from a LB-plate and incubated on a rotary shaker (Orbital Incubator S 150; Stuart Scientific) at 150 rpm for 10–14 h at 30°C. This pre-culture was used to inoculate 100 ml of M9 medium in a 500 ml Erlenmeyer flask without baffles at $OD_{600} = 0.01$. These flasks were then incubated on a rotary shaker at 200 rpm at 30°C. Immediately after inoculation and at regular intervals thereafter, samples were withdrawn from cultures to measure bacterial growth and substrate degradation. Samples for substrate measurements were centrifuged in plastic tubes at 18,500*g* for 10 min at room temperature. Supernatants were transferred into new plastic tubes and stored at −20°C until further analysis.

Characterization of macroscopic cell aggregates

Macroscopic cell aggregates of strain PAO1 from growing cultures or from SDS shock experiments (see below) were collected and washed twice in M9 medium. These aggregates were treated with DNase I (Type II, stock solution in water; Sigma) or alginate lyase (stock solution in water; Aldrich) in appropriate buffer solutions (50 mM Tris–HCl with 10 mM $MgCl_2$ at pH 7.2 with DNaseI and 50 mM Tris–HCl at pH 7.5 with alginate lyase) with shaking at 50 rpm at 37°C. Aggregates were stained with 0.1% (w/v) Alcian Blue 8GX (dissolved in M9; Fluka) in M9 medium with shaking at 50 rpm at 30°C, or with 2 mM CTC (5-cyano-2,3-ditolyl tetrazolium chloride, stock solution in M9 medium; Polysciences) in M9 medium with shaking at 50 rpm at 37°C. LIVE/DEAD staining (BacLight, 2× stock solution in M9 medium; Molecular Probes) of aggregates was performed in M9 medium without shaking in the

dark. For quantification of cells stained as live or dead after SDS shock (see below) and DNAse treatment, coverslips (Thermanox 13 mm; Nunc) were placed into cell suspensions in 24-well microtiter plates for 5 min and washed twice in 1 ml of M9 medium to remove SDS, which otherwise interfered with the fluorescent dyes of the LIVE/DEAD kit. Cells attached to the coverslips were stained, washed again, and counted by epifluorescence microscopy.

Preparation of cell suspensions

Cells were grown as described above and harvested in the late exponential phase by centrifugation in sterile 50 ml plastic tubes at 12,850*g* for 15 min at 20°C. Cells were washed twice in 20 ml M9 medium without carbon source. After final resuspension, the cell suspension still contained cell aggregates that were removed by filtration through a sterile polycarbonate membrane filter (25 mm diameter; Nuclepore) with 5 μm pore size. These filtrates containing only freely suspended cells were adjusted to OD_{600} = 1 with M9 medium. For substrate degradation and respiration experiments, cell aggregates remaining after the final resuspension were removed by centrifugation at 50*g* for 5 min at 20°C.

SDS shock experiments

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

For comparing survival rates of suspended cells and cells within aggregates, SDS shock experiments were modified. Cells growing with SDS were separated into two fractions of aggregates and suspended cells by centrifugation at 80*g* for 5 min. Both fractions were washed twice with M9 medium by centrifugation at either at 80*g* (aggregates) or 10,000*g* (suspended cells), finally suspended in 10 ml M9 medium in 50 ml plastic tubes, and shocked with SDS for 45 min at 30°C with shaking at 50 rpm. After 45 min, 40 ml M9 was added to the tubes. Cells were harvested by centrifugation at 10,000*g* for 10 min, washed twice in M9 medium, and finally suspended in 5 ml DNase buffer. After treatment with 10 U/ml DNase for 30 min at 37°C with shaking at 200 rpm, CFU counts were performed as described above.

Substrate degradation and oxygen uptake experiments

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

Preparation of cell-free extracts and alkyl sulfatase assay

Cells were grown in M9 medium as described above and harvested by centrifugation at 10,000*g* for 10 min at 4°C. Cells were washed twice with 50 mM Tris–HCl, pH 7.0 at 4°C, and finally resuspended in a small volume of this buffer. Cells were broken by three passages through a pre-cooled French Press (SLM Aminco; SLM Instruments) at 136 MPa. The homogenates were centrifuged at 20,800*g* for 10 min at 4°C. The supernatants (cell-free extract) were transferred to a plastic tube and either used directly for the sulfatase assay or stored at −20°C. Alkyl sulfatase was measured discontinuously by determination of sulfate. Assays were performed in plastic tubes in a final volume of 1 ml at 30°C, containing 50 mM Tris–HCl pH 7.0, cell-free extract (ca. 0.5 mg protein), and were started by the addition of SDS (1 mM). Immediately after start of the assay and at regular intervals thereafter, samples (100 µl) were withdrawn and diluted with 100 µl 1 M NaOH to stop the reaction. These samples were stored at −20°C until further analysis.

Detection of oxidized proteins in cell-free extracts

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

Protein and DNA quantification

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

SDS quantification

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

Succinate and sulfate quantification

Succinate was measured by ion-exclusion HPLC. The HPLC-system consisted of a high-pressure pump (Sykam), an Aminex HPX-87H column (300 × 7.8 mm^2; BioRad) at 40°C, a refraction index detector (ERC-7512, Sykam) and an autoinjector (Gilson 234; Abimed). The eluent used was 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. Sulfate was measured by ion chromatography. The HPLC-system (Sykam) consisted of a high-pressure pump (S 4260), an ion-exchange column (LCA-A03) at 30°C, a conductivity detector (S 3110), and an autoinjector (S 5200). As the eluent, a solution containing 5 mM Na_2CO_3, 50 mg/l 4-hydroxybenzonitrile, and 200 ml/l acetonitrile at a flow rate of 1.5 ml/min was used. The column was regenerated intermittently with 0.2 M H_2SO_4.

Microscopy, photography, and image processing

For microscopic studies, an epifluorescence microscope (Axiophot; Zeiss) equipped with a cooled CCD camera (Magnafire; Intas) and respective filter sets for fluorescent dyes (HQ 480/40, Q 505 LP, HQ 527/30 for SYTO9; HQ 545/30, Q 570 LP, HQ 610/75 for propidium iodide) was used. Photographs of microtiter plates and macroscopic aggregates were acquired with a digital camera. All images were processed with the computer software Magnafire and Paint Shop Pro 4.

Results

Growth with and in the presence of SDS

Pseudomonas aeruginosa strain PAO1 degraded SDS concomitantly with growth (Fig. 1a). When the maximal

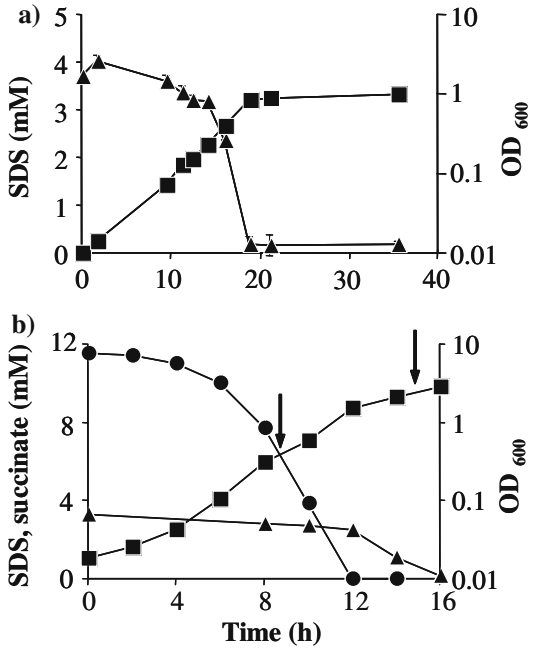

Fig. 1 Growth of *Pseudomonas aeruginosa* strain PAO1 with SDS (*filled triangle*) (**a**) or with a mixture of SDS (*filled triangle*) and succinate (*filled circle*) (**b**). Growth was measured as OD_{600} (*filled square*). *Arrows* indicate the time points when samples were taken for determination of alkyl sulfatase activity

turbidity was reached, SDS was not detectable in the culture supernatant. About 2 h after inoculation, we observed an increasing number of whitish aggregates in the culture. Within the next 4 h, some of these aggregates grew larger (up to 1 cm in diameter). After 8 h, the turbidity of the culture increased and suspended cells started to grow exponentially. In this growth phase, biofilms were formed at the air–liquid interface and at the bottom of the flask. Obviously, the biomass formed during SDS degradation was not homogenously distributed. During sampling, we avoided removing large aggregates from the culture, and, thus, OD_{600} does not reflect growth correctly. However, SDS degradation at the highest rate coincided with the increase of OD_{600} during exponential growth. In cell-free extracts of SDS-grown cells, we detected SDS-dependent release of sulfate at a specific activity of 30 mU/mg protein. This alkyl sulfatase activity was not detectable in cell-free extracts of succinate-grown cells and in heat-inactivated extracts of SDS-grown cells.

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

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

Characterization of macroscopic aggregates

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

SDS shock experiments

A first step to elucidate the function of these cell aggregates during degradation of SDS was to investigate the sensitivity of *P. aeruginosa* to SDS. We tested how suspensions of SDS- and succinate-grown cells responded to the addition of SDS (3.5 mM) under different conditions. SDS caused a decrease of OD_{600} from 1 to about 0.2 within 30 min to suspensions of SDS-grown cells during static incubations (Fig. 3a, panel 1). The OD_{600} in the control suspension without SDS remained constant. During the experiment, the SDS-treated cell suspensions became more viscous. DNA (Fig. 3b, panel 1) and protein (Fig. 3c, panel 1) concentrations in the supernatant increased over time to about 2 and 200 μg/ml, respectively. In control suspensions without SDS, the DNA concentration was below the detection limit (0.01 μg/ml), and the protein concentration remained constant below 10 μg/ml. After 45 min, cells were plated to determine CFU counts. SDS caused about 80% reduction of CFU compared to the control without SDS (Fig. 4a). Microscopic examination of cell suspensions after SDS shock revealed the formation of cell aggregates. Removing these aggregates by passing the cell suspensions through a filter with 5 μm pore size reduced the CFU counts of SDS-treated

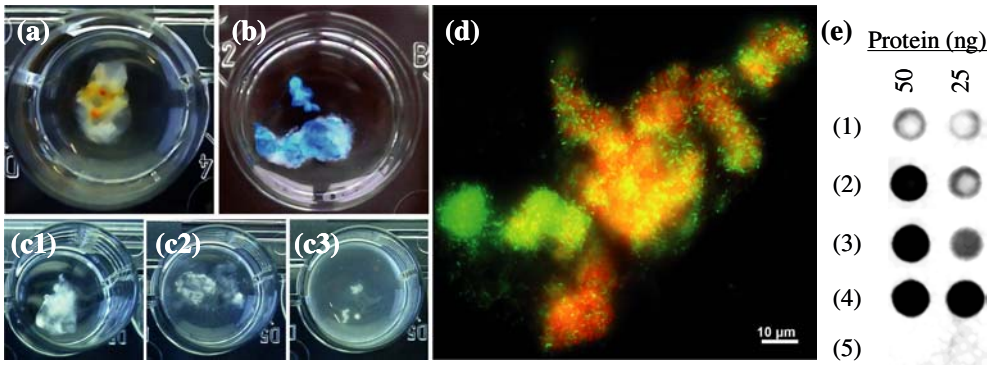

Fig. 2 Characterization of cell aggregates of *P. aeruginosa* strain PAO1 formed in SDS shock experiments. **a** Staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). **b** Staining with a 0.1% (w/v) Alcian Blue solution. **c** Incubation with DNase (10 U/ml). Pictures were taken after 0 min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

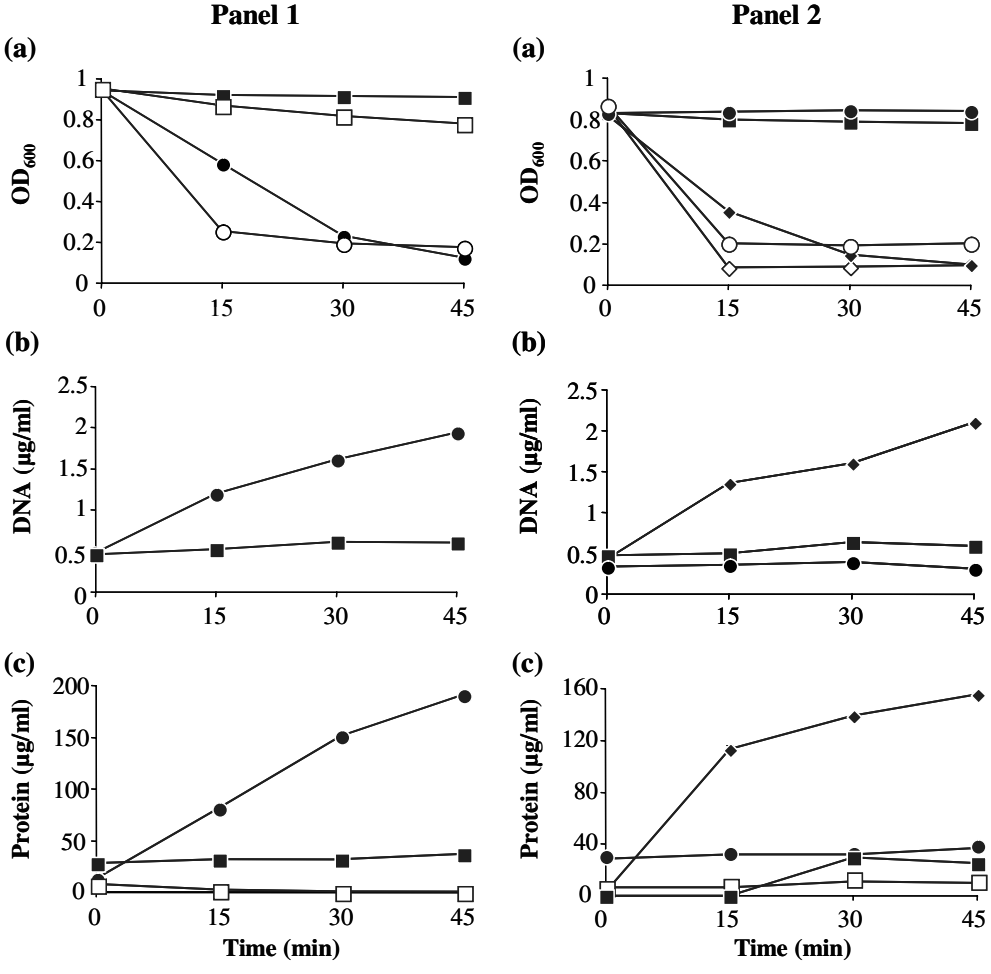

Fig. 3 Effect of SDS on optical density (**a**), DNA release (**b**), and protein release (**c**) of cell suspensions of *P. aeruginosa* strain PAO1 in the presence or absence of inhibitors. *Panel 1*: Suspensions of SDS-grown cells were supplied with SDS in the absence (*filled circle*) or in the presence of KCN (*filled square*) or CCCP (*open circle*). Controls (*open square*) did not contain SDS or inhibitors *Panel 2*: Suspensions of succinate-grown cell were supplied with SDS (*filled circle*), SDS in the presence of CCCP (*open diamond*), SDS plus succinate in the absence (*filled diamond*), or in the presence of KCN (*filled square*) or CCCP (*open circle*). Controls (*open square*) did not contain SDS or inhibitors. Concentrations of SDS, succinate, and inhibitors are given under Materials and methods. In the absence of SDS, inhibitors and their respective solvents did not affect OD_{600} and release of DNA or protein

cells further, while the control suspension without SDS was not affected by this treatment (Fig. 4a). If SDS-grown cell suspensions shocked with SDS were shaken (150 rpm), macroscopic aggregates formed that looked very similar to the aforementioned aggregates in growing cultures (Fig. 5a). After 60 min of incubation, a

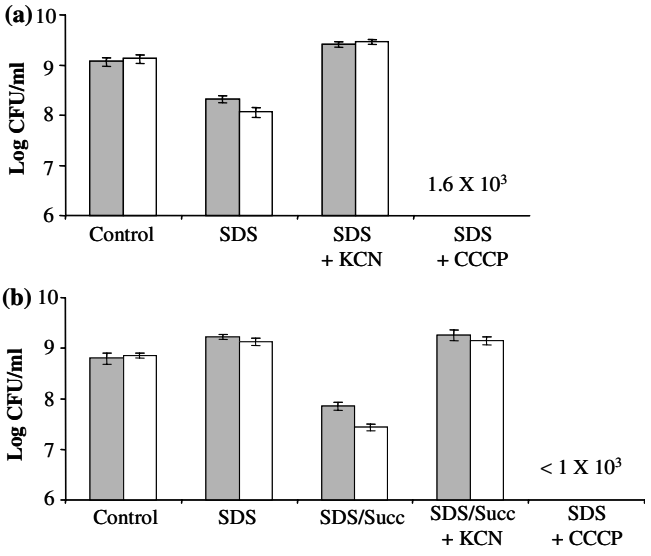

Fig. 4 Effect of SDS on CFU counts of cell suspensions of *P. aeruginosa* strain PAO1 in the presence or absence of inhibitors. After 45 min incubation, cell suspensions were either plated directly (*grey bars*) or after filtration through a polycarbonate membrane filter of 5 μm pore size to remove microscopic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

large aggregate formed, and the turbidity of the surrounding liquid decreased strongly. In controls without SDS, no such aggregation or clearance was observed.

To succinate-grown cell suspensions, SDS caused no or only a slight decrease in OD_{600} (Fig. 3a, panel 2) and reduction in CFU/ml (Fig. 4b). The free DNA concentration in the SDS-treated cell suspensions was constant around 0.3–0.4 μg/ml, while it was below the detection limit in the control without SDS (Fig. 3b, panel 2). The free protein concentration (30 μg/ml) was higher than in the control without SDS (below 10 μg/ml), but remained constant over time (Fig. 3c, panel 2). If SDS was added together with succinate the cells reacted in a similar manner as SDS-grown cells reacted to addition of SDS (Fig. 3a–c, panel 2; Fig. 4b), including aggregation. The SDS-induced reduction of OD_{600} was observed at SDS concentrations of 3.4 and 1.7 mM for both SDS- and succinate-grown cells (in the presence of succinate), but not at 0.34 mM SDS. With 0.34 mM SDS, the cells did also not aggregate.

Characterization of cells from aggregates

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

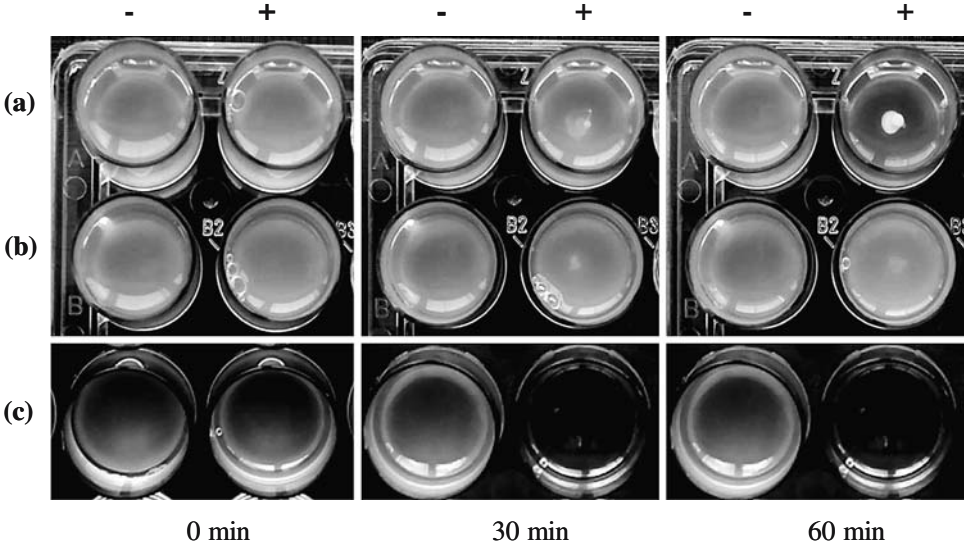

Fig. 5 Formation of macroscopic cell aggregates by SDS-grown cells of *P. aeruginosa* strain PAO1 (**a**, **b**) and a *cioB* mutant [32522] (**c**) derived from strain MPAO1 after SDS shock with shaking (150 rpm) in 24-well microtiter plates (15 mm in diameter; Nunc). Cell suspensions were supplied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

compared to cell-free extracts of cells supplied with succinate only (Fig. 2e, 1–2). In addition, we compared cells released from aggregates with those cells that had remained in suspension within the same experiment. Very clearly, the extracts of cells from the aggregates contained more oxidized proteins than the extracts of suspended cells (Fig. 2e, 3–4).

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

In order to investigate the influence of energy supply on the response of strain PAO1 to SDS, we performed SDS shock experiments in the presence of inhibitors of respiratory ATP synthesis. In suspensions of SDS-grown cells exposed to SDS and in suspensions of succinate-grown cells exposed to SDS plus succinate, the presence of KCN (2 mM) prevented all SDS-dependent effects, namely the decrease in OD_{600}, the release of DNA and protein over time (Fig. 3a–c, panels 1–2), and the decrease in CFU/ml (Fig. 4a, b). Importantly, KCN also inhibited the formation of microscopic and macroscopic aggregates (Fig. 5b). NaN_3 (2 mM) had a similar but weaker effect in SDS shock experiments (not shown). To verify that both inhibitors really interfered with the energy metabolism we tested their effects on respiration and substrate degradation. NaN_3 decreased the substrate-dependent oxygen consumption of SDS-grown and succinate-grown cells by about 50%, whereas KCN reduced the substrate-dependent oxygen uptake to a basal level that was measured in the absence of substrates (Table 1). Both inhibitors also reduced degradation of the respective substrates to the same extent as they inhibited respiration (not shown). To account for the influence of cyanide-insensitive respiration (Cunningham et al. 1997), we investigated a *cioB* mutant which is defective in the cyanide-insensitive cytochrome c oxidase Cio (Cooper et al. 2003). KCN inhibited the substrate-dependent oxygen uptake in this mutant completely (Table 1). In SDS shock experiments with this mutant strain, suspensions of SDS-grown cells responded with a decrease in OD_{600} and reduction of CFU in a similar manner as the parental strain (Fig. 6a, b). Addition of KCN in the presence of SDS caused a rapid decrease in OD_{600} and in CFU counts without aggregation (Figs. 5c, 6a, b). In the absence of SDS, KCN had no effect on OD_{600} or CFU counts of this mutant.

In the presence of CCCP (1 mM), SDS caused a rapid decrease in OD_{600} and a dramatic decrease in CFU/ml to SDS-grown cells of strain PAO1 (Fig. 3a, panel 1; Fig. 4a). Succinate-grown cells were affected by CCCP and SDS in the same way, independent of the presence of succinate (Fig. 3a, panel 2). In CFU counts, no colonies could be detected in dilution steps higher than 10^{-2}. No macroscopic aggregates were formed when cell suspensions shocked with SDS in the presence of CCCP were shaken. The effect of CCCP could not be prevented by addition of KCN or NaN_3. In the absence of SDS, all inhibitors had no effect on OD_{600} or CFU counts.

Under anoxic conditions, SDS caused a fast decrease in OD_{600} and strong reduction of CFU counts to cells grown aerobically with SDS (Fig. 6c, d). Compared to oxic conditions, these effects were stronger and could not be prevented by the addition of KCN. Oxic control experiments with the same cells indicated that the increased sensitivity to SDS was not caused by the anoxic treatment. Macroscopic aggregates were never formed under anoxic conditions. Succinate-grown cells reacted with a strong decrease of OD_{600} and CFU counts, independent of the presence of succinate (not shown).

Table 1 Oxygen uptake rates of cell suspensions (OD_{600} = 1) of *P. aeruginosa* strain PAO1 and a *cioB* insertion mutant [32522] derived from strain MPAO1 measured with a Clark electrode

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

Cells were incubated with SDS (3.5 mM) or succinate (10 mM) at 30°C. KCN was added to a final concentration of 2 mM; ± indicates standard deviation (n = 3)

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

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

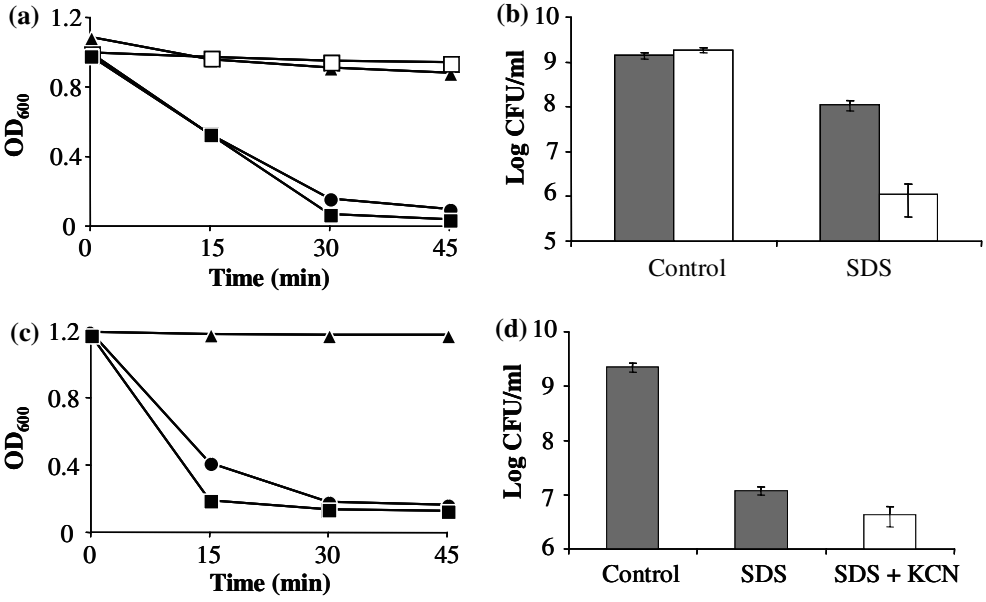

Fig. 6 Effects of SDS on suspensions of SDS-grown cells of a *cioB* insertion mutant [32522] derived from *P. aeruginosa* strain MPAO1 under oxic conditions (**a**, **b**), and of *P. aeruginosa* strain PAO1 under anoxic conditions (**c**, **d**). **a**, **c** OD_{600}: Cell suspensions were supplied with SDS (*filled circle*) or SDS in the presence of KCN (*filled square*). Controls did not contain SDS in the absence (*filled triangle*) or presence of KCN (*open square*). **b**, **d** CFU counts after 45 min of incubations in the absence (*grey bars*) or in the presence of KCN (*white bars*). *Error bars* indicate standard deviation ($n = 6$)

Discussion

The aim of our study was to investigate the function of macroscopic cell aggregates that were formed by *P. aeruginosa* strain PAO1 during growth with or in the presence of the toxic detergent SDS. Our hypothesis was that aggregation is an active process in response to the toxic effects of SDS. The key to test this hypothesis was to study aggregation of *P. aeruginosa* under defined conditions with cell suspension experiments. We found that the formation of these aggregates was strictly energy-dependent. *P. aeruginosa* formed aggregates only under conditions of high energy supply, but not at intermediate energy supply or when cells were completely deprived of energy. The latter situation was created by exposing aerobically grown cells of *P. aeruginosa* to SDS in the absence of oxygen or any other alternative electron acceptors like nitrate. In these experiments, the majority of the cells lysed as indicated by a rapid drop of OD_{600} and a strong decrease of CFU. This effect demonstrated the actual toxicity of SDS for *P. aeruginosa* and the requirement of energy-dependent resistance mechanisms to survive exposure to SDS. Rapid and strong lysis by SDS occurred also under oxic conditions if the uncoupling agent CCCP was present. This effect indicated the importance of the proton motive force and supported earlier observations that proton-dependent multidrug efflux pumps are involved in SDS resistance (Poole 2004). It must be emphasized that the formation of macroscopic aggregates was never observed with completely lysed cells because it demonstrates that these structures are not simply formed by cell debris agglutinated by cytoplasmatic DNA.

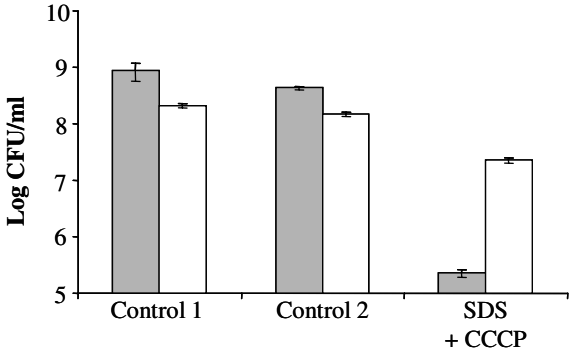

Fig. 7 Survival of suspended cells (*grey bars*) or cells within aggregates (*white bars*) of *P. aeruginosa* strain PAO1 after exposure to SDS (3.5 mM) plus CCCP (1 mM). Suspended and aggregated SDS-grown cells were submitted to a modified SDS shock experiment as described under Materials and methods.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

Conditions of intermediate energy supply were created by exposing succinate-grown cells, which were not induced for SDS-utilization, to SDS. Succinate-grown cells did not aggregate upon SDS-exposure, and they were also no indications of cell lysis. According to our oxygen uptake measurements, these cells were capable of basal respiration which, obviously, generated sufficient energy for operating resistance mechanisms against SDS. This conclusion is supported by the fact that succinate-grown cells lysed in the presence of SDS when they were completely deprived of energy.

Conditions of high energy supply were given if SDS-grown cells were exposed to SDS or succinate-grown cells were exposed to SDS plus succinate. In both cases, the cells formed the same kind of macroscopic aggregates that were also observed in growing cultures. Obviously, the formation of aggregates was energy-dependent. This conclusion is supported by the fact that KCN inhibited aggregation. In the presence of this

inhibitor, the cells respired at a low rate comparable to the level of intermediate energy supply described above. This basal respiration rate in the presence of cyanide was not observed in a *cioB* mutant indicating that respiration by cyanide-insensitive oxidases generated sufficient energy to survive exposure to SDS. Accordingly, the *cioB* mutant could not survive exposure to SDS when KCN was present.

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

DNA has been found in EPS of *P. aeruginosa* before but its origin has not been clarified so far (Whitchurch et al. 2002; Matsukawa and Greenberg 2004; Steinberger and Holden 2005). Delivery of DNA outside the cell could occur via the formation of membrane vesicles (Kadurugamuwa and Beveridge 1995; Beveridge 1999), and in electron micrographs of cells from macroscopic aggregates we actually observed such membrane vesicles (unpublished data). In addition, the DNA could originate from lysis or autolysis induced by stress (Webb et al. 2003). The acidic polysaccharides that we detected in the EPS were most probably not alginate because the aggregates could not be disintegrated by alginate lyase. Recently, it has been shown that *P. aeruginosa* strains are capable of producing other polysaccharides than alginate (Wozniak et al. 2003; Friedman and Kolter 2004; Matsukawa and Greenberg 2004).

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

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

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