Energy-Dependent Stability of *Shewanella oneidensis* MR-1 Biofilms[▽]

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Stability and resistance to dissolution are key features of microbial biofilms. How these macroscopic properties are determined by the physiological state of individual biofilm cells in their local physical-chemical and cellular environment is largely unknown. In order to obtain molecular and energetic insight into biofilm stability, we investigated whether maintenance of biofilm stability is an energy-dependent process and whether transcription and/or translation is required for biofilm dissolution. We found that in 12-hour-old *Shewanella oneidensis* MR-1 biofilms, a reduction in cellular ATP concentration, induced either by oxygen deprivation or by addition of the inhibitor of oxidative phosphorylation carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), dinitrophenol (DNP), or CN[−], resulted in massive dissolution. In 60-hour-old biofilms, the extent of uncoupler-induced cell loss was strongly attenuated, indicating that the integrity of older biofilms is maintained by means other than those operating in younger biofilms. In experiments with 12-hour-old biofilms, the transcriptional and translational inhibitors rifampin, tetracycline, and erythromycin were found to be ineffective in preventing energy starvation-induced detachment, suggesting that neither transcription nor translation is required for this process. Biofilms of *Vibrio cholerae* were also induced to dissolve upon CCCP addition to an extent similar to that in *S. oneidensis*. However, *Pseudomonas aeruginosa* and *P. putida* biofilms remained insensitive to CCCP addition. Collectively, our data show that metabolic energy is directly or indirectly required for maintaining cell attachment, and this may represent a common but not ubiquitous mechanism for stability of microbial biofilms.

Microbial biofilms are surface-associated, dynamic communities that respond to changing external and internal environments, enabling continued acclimation of the biofilm population (8). In biofilms, individual cells are associated with either other biofilm cells or a matrix, which generally consists of exopolysaccharides, proteins, and DNA, and are thereby retained in biofilms that form under hydrodynamic conditions (5). Under such conditions, two opposite processes determine the stability of microbial biofilms: cellular attachment and detachment. Both processes are mechanistically linked in that those factors that mediate attachment of cells need to be inactivated when cells detach.

Cell detachment in “young” biofilms occurs in response to environmental or cellular changes and often involves external or population-induced nutrient limitation, including starvation for carbon, nitrogen, or oxygen (2, 7, 9, 15, 25, 27, 33). For example, rapid dispersal of *Pseudomonas aeruginosa* and *P. putida* biofilms were reported in response to limitation in carbon substrate and oxygen (9, 25). In these cases, most of the biofilm dissolution occurred within a few minutes after onset of nutrient deprivation. In the former case, there was also a correlation between protein dephosphorylation and extent of detachment (25). Also, *Vibrio cholerae* biofilms have been found to naturally dissolve to a large extent in response to metabolic cues (22). *Shewanella oneidensis* MR-1 is a Gram-negative facultative gamma-pro-

teobacterium capable of unprecedented respiratory diversity under anoxic conditions, including reduction of insoluble Fe(III) and Mn(IV) oxides (23). We previously showed that the molecular mechanisms that control *S. oneidensis* MR-1 biofilm formation are primarily enabled by the mannose-sensitive hemagglutinin (MSHA) and *mxd* gene sets (26). The *msh* genes encode a type IV pilus apparatus while the *mxd* genes putatively encode a carbohydrate-containing cell-associated component. Moreover, oxygen depletion induced rapid dissolution of aerobically growing biofilms (31, 33). We also showed that intracellular cyclic di-GMP (c-di-GMP) influences biofilm formation in this microorganism by increasing biofilm biomass and by rendering biofilms resistant to detachment (31). Overexpression of a cyclic-di-GMP-specific phosphodiesterase in *S. oneidensis* reduced intracellular cyclic di-GMP, inhibited biofilm formation by reducing adhesion of single cells, and induced detachment in established biofilms. However, the molecular basis of the association between a biofilm cell and other cells or a biofilm cell and the biofilm matrix is still insufficiently understood.

In this work, we investigated the energetic and gene expression requirements for biofilm stability primarily in *S. oneidensis* but also extended our key findings to *Vibrio cholerae* and *Pseudomonas* sp., which are two of the most studied biofilm “model” systems. The data presented here suggest that maintenance of the stability of young biofilms requires metabolic energy and that control of detachment is primarily posttranslational. The latter finding is also particularly important, as subpopulations of biofilm microbes are gen-

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TABLE 1. Strains used in this study

Strain	Relevant genotype and phenotype or characteristics	Source or reference
<i>Shewanella oneidensis</i> AS93	<i>S. oneidensis</i> MR-1 tagged with <i>egfp</i> (constitutively expressed) in a mini-Tn7 construct; Gm ^r Cm ^r	32
AS130	<i>S. oneidensis</i> MR-1 tagged with <i>egfp</i> under inducible control of the Lac promoter in a mini-Tn7 construct; Gm ^r Cm ^r	
AS141	AS93 Δ <i>mxdB</i> (SO4179); Gm ^r Cm ^r	31
AS645	AS93 Δ <i>pilD</i> (SO0414); Gm ^r Cm ^r	26
AS647	AS93 Δ <i>pilT</i> (SO3351); Gm ^r Cm ^r	26
AS648	AS93 Δ <i>mshA</i> (SO4105); Gm ^r Cm ^r	26
<i>Vibrio cholerae</i> A1552	<i>Vibrio cholerae</i> El Tor, Inaba, wild type, smooth, tagged with GFPmut3b* using a mini-Tn7 construct; Rif ^r Sm ^r Gm ^r	22
<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i> PAO1 tagged with a mini-Tn7 GFPmut3b* cassette inserted into the chromosome; Gm ^r	20
<i>Pseudomonas putida</i>	<i>P. putida</i> R1 with a mini-Tn5-Km- <i>rrnBP1-gfp</i> [mut3b*]-T ₀ -T ₁ cassette inserted into the chromosome; Nal ^r Km ^r	28

erally considered to be resistant to conventional antibiotic treatment (1, 6, 21).

MATERIALS AND METHODS

Growth conditions and media. The strains used in this study are summarized in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C, and *Shewanella oneidensis* MR-1 strains were grown at 30°C in LB, lactate medium (LM) (32), or minimal medium (MM) (31). If required, the medium was solidified with 1.5% (wt/vol) agar or 0.3% (wt/vol) agar for motility plates and supplemented with 10 µg/ml gentamicin and/or 25 µg/ml kanamycin. Gene induction from the *lacI^{q1}-P_{Lac}-gfp* construct, which expresses green fluorescent protein (GFP) upon chemical induction, was achieved by addition of 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Strain construction in *S. oneidensis* MR-1. All genetic work was carried out according to standard protocols or by following the manufacturer's instructions. Kits for the isolation and/or purification of plasmid DNA were obtained from Qiagen (Valencia, CA), and enzymes were purchased from New England BioLabs (Beverly, MA). In order to construct a system for controlled gene induction in *S. oneidensis* MR-1, chromosomal insertion of a *lacI^{q1}-P_{Lac}-gfp* fusion was carried out using a Tn7 delivery system by four-parental mating using pSM2360 as a delivery plasmid as described for *S. oneidensis* MR-1 (32).

Biofilm cultivation and image acquisition. Biofilms were cultivated at 30°C in LM in three-channel flow cells with individual channel dimensions of 1 by 4 by 40 mm. Microscope coverslips (Fisher Scientific, Pittsburgh, PA) were used as a colonization surface, glued with silicone (GE Sealants & Adhesives, Huntersville, NC) onto the flow cells, and left to dry for 24 h at room temperature prior to use. Assembly, sterilization, and inoculation of the flow system were carried out essentially as described previously (32).

For switching medium, the flow was arrested briefly, and the medium was exchanged in the bubble trap and the upstream tubing. This process took no longer than 1 min, and control channels, where the medium flow was stopped in parallel channels without changing the medium, ensured that the observed effects were not due to that short arrest of flow. Confocal laser scanning microscopy (CLSM) was performed at defined locations in the flow chamber close to the inflow before and after the treatment.

Stop-of-flow-induced detachment was carried out essentially as described earlier (33). Briefly, the medium flow was arrested for 15 min and subsequently resumed for the same amount of time. CLSM images were taken immediately before the stop of flow and after 15 min of flow.

Induction of *gfp* expression was executed by switching the irrigating LM to LM containing 10 mM IPTG (Sigma). Inhibition of gene expression was executed by switching the irrigating LM to LM containing rifampin, erythromycin, or tetracycline (all from Sigma Chemical Company). The concentrations used were 100, 50, or 25 µg/ml, respectively. Metabolic inhibitor experiments were executed by switching the irrigating medium to LM containing carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma), sodium cyanide (Baker), or dinitrophenol (DNP) (Sigma). The concentration used was 20 µM, 150 mM, or 2 mM, respectively, for *S. oneidensis*. For the remaining organisms, 50 µM CCCP was used. These were determined to be the lowest concentrations of each uncoupler at

which detection of fluorescence was prevented under biofilm growth conditions. For stock solutions, solid CCCP (80 mM), DNP (1 M), and sodium cyanide (0.5 M) were dissolved in dimethyl sulfoxide (DMSO), ethanol, and water, respectively, prior to dilution into LM. Control channels were irrigated with LM containing the same added volume of each stock solvent only as used in experimental channels to test for solvent-dependent effects.

Microscopic visualization and image acquisition of biofilms were conducted at the Stanford Biofilm Research Center using an upright Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) equipped with a 63×/1.2-W objective. For displaying biofilm images, CLSM images were processed using the IMARIS software package (Bitplane AG, Zürich, Switzerland) and Adobe Photoshop. Biofilm parameters, such as biomass and average biofilm thickness, were quantified with the software program COMSTAT (10).

Nucleotide extraction from planktonic cultures. For CCCP and oxygen starvation experiments, planktonic cultures were grown in LM to an optical density at 600 nm (OD₆₀₀) of approximately 0.3, and either the metabolic activity was inhibited with 40 µM CCCP or the culture was deprived of oxygen. Control flasks were grown in parallel and were either untreated or treated with equivalent volumes of DMSO. CCCP was dissolved in DMSO to 80 mM, and this was added to planktonic cultures to give a final CCCP concentration of 40 µM. This concentration of CCCP was determined to be the lowest at which growth and fluorescence detection were blocked under planktonic conditions. Oxygen starvation was induced by removing the flask from the shaker, flushing the headspace with nitrogen, and placing a rubber stopper in the flask neck. In a control experiment, oxygen concentration was measured in similarly treated cultures with an oxygen-sensitive electrode. The concentration decreased below the level of detection within 12 min. Four samples (525 µl) from each culture were taken at designated time points and flash frozen on liquid nitrogen for subsequent nucleotide assays.

Cyclic-di-GMP and ATP determination. For ATP determination, culture samples were thawed on ice and immediately assayed. Intracellular ATP was quantified using the BacTiterGlo reagent (Promega PR-G8230) according to the manufacturer's protocol, with a lysis time of 2.5 min. Luminescence was measured with a Mithras LB940 luminometer (Berthold Technologies). ATP solutions used to establish the standard curve were made in the culture medium.

Extraction of cyclic di-GMP was accomplished as described previously by the heat and ethanol addition method (31). After extraction, supernatants were dried on a Thermo Savant SpeedVac SPD (Thermo Fisher Scientific, Waltham, MA) and then stored at -20°C for subsequent liquid chromatography-mass spectrometry (LC-MS) quantitation of the cyclic di-GMP (see below).

Cyclic di-GMP was quantified by LC-MS as previously described (31). All samples were analyzed on a Shimadzu high-performance liquid chromatography (HPLC) system (Columbia, MD) coupled to a Sciex API 3000 triple quadrupole mass spectrometer (MDS Sciex, Ontario) operating in positive electrospray ionization multiple reaction monitoring (MRM) mode. The high-performance liquid chromatography system was equipped with an autosampler and degasser, which were used for solvent delivery and sample introduction. Triplicate samples were injected twice.

RESULTS

Metabolic energy is required for maintenance of biofilm stability. We previously showed that oxygen depletion or a stop in medium flow, which imposes conditions of oxygen depletion in a hydrodynamic, aerobic biofilm, leads to massive detachment of single cells (33). We hypothesized that this oxygen limitation likely impacts the cellular ATP concentration and that this could affect the availability of cellular energy of individual cells and thus the stability of biofilms. In order to determine whether or not metabolic energy is required for biofilm stability, and particularly for biofilm detachment, we subjected *Shewanella oneidensis* MR-1 (AS93) to three metabolic inhibitors that specifically affect ATP synthesis in respiring microorganisms. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and dinitrophenol (DNP) are membrane-permeable, weak acids and act with high specificity as protonophores to collapse the electrochemical proton potential across the cytoplasmic membrane (11, 13). As a consequence, no ATP synthesis occurs via a chemiosmotic mechanism when a bacterial culture is amended with these protonophores. Sodium cyanide (NaCN) inhibits cellular respiration by binding to cytochrome oxidase of the electron transport chain, thus preventing electron flux and consequently the generation of an electrochemical proton potential and ATP synthesis via electron transport phosphorylation. When these metabolic inhibitors were tested in 12-hour-old *S. oneidensis* MR-1 biofilms, massive biofilm dissolution occurred immediately after their addition to the irrigating medium (Fig. 1A). This uncoupler-induced detachment resulted in loss of individual cells from the biofilm rather than of large cell clusters, which is similar to the detachment observed in response to a stop-of-flow treatment (33). To test whether the residual biofilm was still sensitive to a stop-of-flow-induced detachment, after 60 min of inhibitor treatment, biofilms were subjected to a stop-of-flow assay as described previously (33), and 30 min later, images were again recorded by CLSM. Quantification of these images revealed that no further biomass was lost from the uncoupler-treated biofilms while untreated control biofilms were fully detachable (Fig. 1B).

In order to show that these inhibitors act as such in our hydrodynamic biofilm system, we conducted control experiments to test whether the inhibitors are active *in situ* in biofilm cells. We used *S. oneidensis* MR-1 strain AS130 containing a chromosomally inserted reporter gene construct consisting of *gfp* under the control of P_{tac} , which is repressed by the also chromosomally encoded LacI^{q1} protein. For biofilm cells to express *gfp* and to become fluorescent, transcription in response to IPTG addition as well as translation is necessary. Thus, monitoring expression of *gfp* enables us to observe these processes directly and *in vivo*. As both processes are strongly ATP dependent, *gfp* expression also served here as a qualitative surrogate for the availability of cellular ATP.

Biofilms of strain AS130 were grown under hydrodynamic conditions as described above. In the absence of IPTG, no detectable GFP fluorescence was observed, whereas upon addition of 10 mM IPTG, GFP fluorescence in cells throughout the biofilm was readily observed via CLSM (Fig. 2 and 3). After 1 h, nearly 20% of the maximum inducible GFP fluorescence was observed. In contrast, biofilms treated prior to induction

FIG. 1. Stability of 12-hour *S. oneidensis* MR-1 biofilms treated with metabolic inhibitors. (A) The graph represents quantified CLSM images generated by COMSTAT. Biofilms were exposed to media amended with indicated metabolic inhibitors for 90 min, and images were obtained at the designated time points. ◆, untreated; ×, 2 mM DNP; ●, 20 μM CCCP; +, 1 mM sodium cyanide. Each data point represents the mean value for four independent images monitored in two separate channels. Error bars represent 1 standard deviation. (B) The graph represents quantified CLSM images generated by COMSTAT. The degree of biomass detachment as a result of a 90-min exposure to medium amended with metabolic inhibitor alone (left panel) or followed by a stop of flow (right panel) is shown. White, untreated; black, 20 μM CCCP; dark gray, 2 mM DNP; light gray, 1 mM sodium cyanide. Each data point represents the mean value for four independent images monitored in two separate channels. Error bars represent 1 standard deviation.

with specific inhibitors of transcription (100 μg/ml rifampin) or translation (25 μg/ml tetracycline or 50 μg/ml erythromycin; see below) did not yield any detectable fluorescence after 1 h (Fig. 2). In separate experiments, we found that addition of the protonophores CCCP and DNP and of NaCN to AS130 biofilms prior to addition of IPTG resulted in no observable fluorescence (data not shown). These experiments showed that small molecules such as IPTG, rifampin, tetracycline, erythromycin, and the metabolic inhibitors CCCP, DNP, and NaCN could access the majority of cells in the biofilm and that diffusional barriers did not restrict mass transport of these compounds into the biofilm. Moreover, we found that these *in situ*-grown biofilm cells were competent for induction of gene expression as well as sensitive to inhibitors of transcription, translation, and energy conservation. These experiments were

FIG. 2. Gene expression in *S. oneidensis* MR-1 12-h biofilm cells as determined by GFP fluorescence in untreated and inhibitor-treated biofilms. The graph represents quantified CLSM images generated by COMSTAT. Biofilms of strain AS130 containing the inducible expression construct were grown as described in Materials and Methods, and *gfp* expression was induced upon addition of 10 mM IPTG, either in the absence of inhibitor or after 40 min (rifampin) or 20 min (erythromycin and tetracycline) of exposure to inhibitor. ◆, untreated; ■, rifampin treated; +, erythromycin treated; ×, tetracycline treated. Each data point represents the mean for six independent images taken from two channels. Error bars represent 1 standard deviation (in this graph, error bars are smaller than symbols).

also conducted with planktonic cultures, where we observed the same inhibitory effect on fluorescence (data not shown).

Intracellular concentrations of ATP and c-di-GMP. The most likely mode of action of CCCP and DNP is to act as protonophores by collapsing the chemiosmotic membrane potential, thus reducing ATP synthesis under respiratory conditions. The cellular ATP level affects the cellular c-di-GMP level as both nucleotide pools are interconnected by the activities of guanylate kinases and nucleoside diphosphate kinases; c-di-GMP is synthesized from two molecules of GTP, which is derived from two molecules of ATP. In order to directly test whether the intracellular ATP and c-di-GMP concentrations were reduced upon both uncoupler treatment and rapid oxygen starvation, we reverted to planktonically grown cells in order to obtain sufficient biomass for subsequent nucleotide measurements as well as to allow multiple samples to be taken from the same culture (before and after 20 min of treatment). The latter point in particular was critical for these measurements due to stochastic variation in culture physiology and the sensitivity of the assays. We therefore exposed early-log-phase ($OD_{600} = 0.3$) cells that were grown aerobically in shake flasks either to oxygen starvation or to the metabolic inhibitor CCCP (40 μ M). Samples from all flasks were taken before and during CCCP treatment and assayed for cellular ATP and cyclic-di-GMP levels. After 20 min, oxygen-deprived or CCCP-treated cells contained only 81% and 13%, respectively, of the intracellular ATP concentration relative to untreated cells (Fig. 4A). Under conditions of CCCP treatment, the global cellular c-di-GMP pool was also reduced by nearly 24% relative to that in untreated samples (Fig. 4B). These data show that both uncoupler treatment and oxygen limitation reduce cellular ATP level. In addition, the global cellular level of c-di-GMP is measurably reduced upon CCCP treatment. The modest re-

duction in c-di-GMP level, relative to ATP level, might be due to a generally slower turnover of this nucleotide, including hydrolysis, which is supported by the observed low activities of diguanylate cyclases or phosphodiesterases involved in c-di-GMP metabolism and/or localized cellular pools.

Effect of CCCP on older biofilms. In the experiments described above, collapse of the proton motive force in biofilm cells by exposure to metabolic inhibitors resulted in approximately 90% removal of biofilm within 2 h of exposure. This indicates that the dominant population in 12-hour biofilms requires metabolic energy in some form in order to mediate biofilm stability. In contrast, a stop-of-flow-induced detachment in similarly aged biofilms resulted in approximately 50% removal of biofilm. Moreover, older and thicker *S. oneidensis* MR-1 biofilms (18 and 48 h) were even less responsive to stop-of-flow treatment (less biomass detached from these biofilms). We speculated that this detachability of cells may be related to their degree of metabolic activity since the cells closest to the irrigating medium, and therefore with the access to high-nutrient concentration, detach more readily than those in the layers closer to the substratum (33). To test this hypothesis, we examined uncoupler-induced detachment in older biofilms. Control experiments were performed with biofilms of strain AS130, containing the IPTG-inducible GFP reporter construct, as described above to verify that the uncoupler was effective in older biofilms. These biofilms were grown for 60 h to an average height of 60 μ m. Expression of *gfp* could be induced in these biofilm cells upon IPTG addition (Fig. 3A, B, and D) and could be blocked by CCCP addition (Fig. 3C). Apparently, the thicker biofilms did not present a significant diffusion barrier to either IPTG or CCCP. Notably, this observation indicates that even cells close to the substratum surface are inducible by IPTG and are thus metabolically active. This is consistent with previously reported findings that even very thick biofilms, although lacking actively growing cells near the substratum, have subpopulations that are metabolically active (30). We then examined the response of 60-hour *S. oneidensis* AS93 biofilms to a 140-min exposure to CCCP. As shown in Fig. 3D and E, and in contrast to younger biofilms, in 60-hour biofilms only a fraction of the total biomass was removed by the CCCP treatment of the 60-hour biofilms. These experiments revealed that although most cells near the substratum surface of the older biofilm were metabolically active and capable of gene expression, those cells did not respond to CCCP addition with detachment to the same degree as that observed in younger biofilm cells. Thus, older biofilms may be dominated by adhesion mechanisms that are different from those found in younger biofilms. They may contain more physiologically heterogeneous subpopulations, where only the medium-exposed cells require metabolic energy for attachment, perhaps due to a more constraining matrix composition in the thicker regions. The fact that the same concentration of CCCP that prevented *gfp* expression (Fig. 3C) was ineffective in inducing detachment indicates that the absence of detachment of those cells was not due to a limiting concentration of the inhibitor.

Biofilms mediated by either MSHA or *mxd* are detachment competent. Previous work in our laboratory demonstrates that *S. oneidensis* biofilms are mediated by the MSHA and *mxd* gene systems (26, 32). In order to determine whether

FIG. 3. Gene expression and stability of 60-h *S. oneidensis* MR-1 biofilms. Biofilms of strain AS130 containing IPTG-inducible *gfp* were grown in a flow chamber as described in Materials and Methods, and *gfp* expression was induced upon addition of 10 mM IPTG, either in the absence of (A) or after exposure to (C) CCCP. (A) Cross-section of 60-hour biofilm exposed to IPTG for final 2 h of growth; (B) same cross section, stained with SYTO63 to visualize total biofilm; (C) 60-hour biofilm treated with the metabolic inhibitor CCCP (20 μ M) for 10 min prior to and during a 2-h IPTG exposure; (D) cross section of 60-hour AS93 (constitutive *gfp* expression) biofilms prior to CCCP exposure; (E) same cross section after 140 min of CCCP exposure (20 μ M). Scale bars represent 50 μ m.

the MSHA or the *mxl* biofilm system mediates the energy-dependent biofilm attachment, we applied the stop-of-flow assay to 12-h biofilms of relevant mutants. Figure 5 illustrates that biofilms formed by the mutant lacking the *mxlB* gene (Δ *mxlB*) and the MSHA pilus (Δ *mshA* Δ *pilD* Δ *pilT*) are capable of stop-of-flow-induced detachment. These data collectively show that both the MSHA and the *mxl* gene systems require metabolic energy for maintenance of attachment.

Detachment of *S. oneidensis* MR-1 cells from biofilms is controlled posttranslationally. As we had demonstrated that both uncoupler treatment and oxygen limitation rapidly affected cellular ATP and partially c-di-GMP concentration (Fig. 4), and that CCCP also inhibited transcription (Fig. 2), we wanted to test whether the role of cellular energy in biofilm dissolution was indirect, e.g., acting at the transcriptional level, or direct, acting posttranslationally (e.g., on some type of cellular adhesion machinery). Control experiments similar to those described above, testing the effectiveness of these inhibitors, revealed that rifampin, tetracycline, and erythromycin, which are inhibitors of transcription or translation, respectively, functioned as such under the biofilm conditions applied here in that they inhibited the expression of *gfp* as quantified by CLSM in 12-hour biofilms of strain AS130 (Fig. 2). We then treated 12-hour wild-type biofilms with rifampin, which caused no loss of biomass within the time frame of this experiment (data not shown), and subsequently examined whether these biofilms were competent to undergo detachment upon a stop of flow. Biofilms treated with the translational inhibitor erythromycin or tetracycline (again, no loss of biomass occurred from this treatment) also detached upon a stop of flow (Fig. 6). Alternatively, if rifampin-treated cells were exposed to CCCP rather than a stop of flow for 90 min, this exposure caused detachment of 75% of the biomass (data not shown). These experiments indicate that transcription and protein synthesis are not required for the rapid oxygen starvation-dependent detachment response or for the uncoupler-mediated detachment. This suggests that the requirement for cellular energy in biofilm stability is direct.

FIG. 4. Intracellular ATP and c-di-GMP concentrations in planktonic *S. oneidensis* MR-1 cells. Cultures were grown aerobically in a shaking flask to an optical density of 0.3 (600 nm), and either CCCP was added or oxygenation was prevented by stopping the shaking as described in Materials and Methods. At the indicated time points after initiation of treatment, samples were removed and ATP (A) and cyclic di-GMP (B) were assayed. Values expressed are percentages of initial values ($\mu\text{M}/\text{OD}_{600}$). White, untreated control; black, 40 μM CCCP; gray, oxygen deprived. Each data point represents the mean value for six samples measured twice from two separate experiments. Error bars represent 1 standard deviation.

Effect of metabolic inhibitors on *Vibrio cholerae* and *Pseudomonas aeruginosa* biofilms. In order to test whether the observed sensitivity to uncouplers and metabolic inhibitors in *S. oneidensis* is particular to this microorganism or a more general phenomenon, we tested *Vibrio cholerae* and *Pseudomonas aeruginosa* biofilms for uncoupler-mediated detachment. Biofilms of *V. cholerae* (A1552) and *P. aeruginosa* PAO1 were grown in glycerol and glucose minimal medium in flow cells, respectively, and treated with 50 μM CCCP as described above. As indicated in Fig. 7, biofilms of *V. cholerae* readily detached upon addition of CCCP in a response similar to that observed in *S. oneidensis*, while *P. aeruginosa* biofilms did not decrease in biomass. We also tested *P. aeruginosa* Δpel as well as *P. putida* and again did not observe detachment (data not shown). Collectively, these observations showed that the uncoupler-mediated detachment is not unique to *S. oneidensis* MR-1 but is a microorganism-specific rather than a general biofilm characteristic.

FIG. 5. Detachment of *S. oneidensis* MR-1 biofilm mutant cells in 12-h biofilms. The graph represents quantified CLSM images generated by COMSTAT. Biofilms of the respective in-frame deletion mutants were grown in flow chambers, and stop of flow (SOF) was applied to induce detachment. Each data point is the mean value for at least four independent images taken from two channels. Error bars represent 1 standard deviation.

DISCUSSION

The data shown here provide some mechanistic insights into the stability and dissolution of biofilms. Since biofilms have been defined as consisting of cells that are generally fixed within a “sticky” matrix, one of the noteworthy findings reported in this study is that biofilm stability actually requires metabolic energy (Fig. 1 and 7) (29). Treatment of 12-hour *S. oneidensis* MR-1 biofilms with metabolic inhibitors CCCP, DNP, and NaCN resulted in rapid detachment of individual cells. Notably, the extent of this dissolution in young biofilms was even greater than the stop-of-flow-induced detachment (10% versus 50% biomass retention). The facts that detachment was observed within 15 min after the addition of the

FIG. 6. Transcription/translation-independent detachment of *S. oneidensis*. Stop of flow induced detachment in inhibitor-treated biofilms. The graph represents quantified CLSM images generated by COMSTAT. Biofilms were incubated in the flowthrough medium amended with inhibitors for 40 (rifampin) or 20 (erythromycin and tetracycline) minutes prior to application of a stop-of-flow detachment assay as described in Materials and Methods. Each data point represents the mean for four independent images taken from two separate channels. Error bars represent 1 standard deviation.

FIG. 7. Effect of CCCP on stability of *Vibrio cholerae* and *Pseudomonas aeruginosa* biofilms. Biofilms of *Vibrio cholerae* (A1552) and *Pseudomonas aeruginosa* (PAO1) were grown in a flow chamber and treated with CCCP as described in Materials and Methods. The graph represents quantified CLSM images generated by COMSTAT.

inhibitors and that stop-of-flow-induced detachment was not dependent on transcription and translation argue that the maintenance of cellular attachment itself is energy dependent. The dependence on metabolic energy to maintain biofilm stability is novel and interesting in light of the previously reported findings that a sudden decrease in molecular oxygen triggers massive cell detachment in *Shewanella oneidensis* MR-1 biofilms (33). Here, we showed that both CCCP treatment and oxygen starvation lead to a decrease in intracellular ATP (Fig. 4A). Therefore, it is conceivable that the metabolic inhibitor-dependent and oxygen-induced detachment have a common mechanism, which is based on cellular depletion of metabolic energy. Our experiments did not address whether the required energy is in the form of a proton (or other cation) motive force (PMF) across the cytoplasmic membrane or of ATP or whether PMF consumption or ATP hydrolysis is required. Attempts to treat cells with dicyclocarbonyldiimide (DCCD), which specifically inhibits the F_0 subunit of ATPase but does not affect PMF, were inconclusive, as the control strain showed induced fluorescence after treatment with DCCD (data not shown).

If we assume that consumption of metabolic energy is required, the question remains as to the identity of the energy-requiring molecular mechanism(s) in control of biofilm stability. One mode of energy requirement for attachment could be via direct energy use, for example, by a molecular machinery that mechanically mediates biofilm stability. For instance, the activity of bacterial type IV pili, such as the *S. oneidensis* MSHA pilus (26, 32), requires ATP hydrolysis via the motor proteins PilB and PilT (17). It is assumed that ATP hydrolysis by these enzymes drives the controlled polymerization and depolymerization of the pilus from and into the pilin monomeric subunits. Indeed, *S. oneidensis* Δmxd cells, which form biofilms mediated solely by the MSHA system, detach upon a stop of flow and addition of CCCP (Fig. 5 and data not shown). In the absence of ATP, the pilus motor proteins are predicted to cease to function, and consequently, cell surface adhesion within a biofilm mediated by the MSHA pilus would be diminished.

An indirect mechanism requiring metabolic energy could involve ATP or PMF as a regulator of enzyme activity, where

hydrolysis of ATP or consumption of PMF is not necessary. A second indirect mechanism may be one in which metabolic energy is used, such as by posttranslational modulation of an energy-dependent signal transduction cascade or by a secretion process involved in biofilm stability. One candidate signaling molecule involved in biofilm-related processes is cyclic di-GMP. Synthesis of c-di-GMP by diguanylate cyclases requires two molecules of GTP as a substrate. Intracellular GTP is synthesized from GMP, the end product of complete c-di-GMP hydrolysis, by transphosphorylation from ATP by guanylate kinase and nucleoside diphosphate kinases. Indeed, a decreased ATP pool, as a consequence of CCCP treatment, resulted in a reduced c-di-GMP pool in *S. oneidensis* (Fig. 4). An altered general or localized c-di-GMP pool could constitute a signal for detachment. Indeed, as shown in Fig. 6, detachment requires neither transcription nor translation, ruling out a FleQ-type transcriptional mechanism (12). The absence of a measured decrease in c-di-GMP upon oxygen starvation may reflect a localized pool and be consistent with the lesser detachment observed under that condition than for the CCCP-treated biofilm (Fig. 1). It is possible that a large decrease in c-di-GMP was not observed upon oxygen deprivation, because of the low specific activities of c-di-GMP-specific phosphodiesterases. Alternatively, the role of c-di-GMP in *S. oneidensis* biofilm formation may be confined to biomass accumulation (31), and the detachment response could be attributed mainly to the cell's energetic state.

In considering the former possibility, there are several known posttranslational mechanisms through which c-di-GMP could act. In *P. aeruginosa*, FimX was identified as a protein controlling type IV pilus activity (14) and was shown to have cyclic-di-GMP-specific phosphodiesterase activity that may be controlled by a GTP-binding regulatory domain with sequence similarity to the characteristic diguanylate cyclase domain (19). Although FimX homologs are found in several biofilm-forming bacterial species, a FimX homolog in *S. oneidensis* MR-1 has not been identified, and the potential role of cyclic-di-GMP signaling in controlling pilus activity in this organism remains unclear. Also, YcgR controls speed and direction of bacterial flagellar rotation via c-di-GMP (3). However, flagellar biosynthesis is typically downregulated in biofilms.

The observed energy dependence of biofilm stability appears to be a more general but not a ubiquitous physiological trait. Biofilms of the closely related species *V. cholerae* showed detachment behavior similar to that of *S. oneidensis* (Fig. 6). On the other hand, biofilms of *P. aeruginosa* and *P. putida* were, under the conditions tested, insensitive to CCCP, suggesting that either different or additional modes of cellular adhesion are active in those microorganisms. These additional modes may be dominant and may mask an energy-dependent mode conferring biofilm stability, if such a mechanism is present in *P. aeruginosa*. Notably, in *Pseudomonas fluorescens* and *P. putida*, carbon but not oxygen starvation was shown to cause biofilm dissolution (7, 9, 15). Also, in some species, secretion of extracellular degradative enzymes causes biofilm detachment (4, 16, 18, 24). The findings reported here are significant because an accepted model of biofilms describes the cells as "fixed" in an extracellular matrix that must be actively degraded in order for the cells to be released (29). Our observation underscores that microbial biofilms in general, although morphologically simi-

lar, can be maintained by different mechanisms or combinations thereof.

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