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ORIGINAL ARTICLE

Phage-induced lysis enhances biofilm formation in *Shewanella oneidensis* MR-1

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Shewanella oneidensis **MR-1 is capable of forming highly structured surface-attached communities. By DNase I treatment, we demonstrated that extracellular DNA (eDNA) serves as a structural component in all stages of biofilm formation under static and hydrodynamic conditions. We determined whether eDNA is released through cell lysis mediated by the three prophages LambdaSo, MuSo1 and MuSo2 that are harbored in the genome of *S. oneidensis* MR-1. Mutant analyses and infection studies revealed that all three prophages may individually lead to cell lysis. However, only LambdaSo and MuSo2 form infectious phage particles. Phage release and cell lysis already occur during early stages of static incubation. A mutant devoid of the prophages was significantly less prone to lysis in pure culture. In addition, the phage-less mutant was severely impaired in biofilm formation through all stages of development, and three-dimensional growth occurred independently of eDNA as a structural component. Thus, we suggest that in *S. oneidensis* MR-1 prophage-mediated lysis results in the release of crucial biofilm-promoting factors, in particular eDNA.**
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Introduction

Shewanella oneidensis MR-1 belongs to the Gram-negative γ-proteobacteria and is characterized by an enormous respiratory versatility, which allows this species to use an impressive variety of organic and inorganic compounds as alternative terminal electron acceptors when growing under anaerobic conditions (Myers and Nealson, 1988; Venkateswaran *et al.*, 1999). The group of alternative electron acceptors includes metal ions such as Fe(III) and Mn(IV), which are highly abundant in soils and sediments. In addition, a number of radionucleotide oxides can be reduced (Myers and Nealson, 1988; Nealson and Scott, 2003; Icopini *et al.*, 2009). Thus, bacteria such as *Shewanella* significantly impact biogeochemical cycling processes and are of particular interest with regard to bioremediation processes (Heidelberg *et al.*, 2002; Nealson *et al.*, 2002; Lovley *et al.*, 2004; Ward *et al.*, 2004; Hau and Gralnick, 2007). It has been hypothesized that direct interaction of *Shewanella* cells with, or close proximity to, an appropriate surface facilitates the deposition of electrons (Das and Caccavo, 2000; Gorby *et al.*, 2006; McLean *et al.*, 2010). In fact, *Shewanella* species have been demonstrated to adhere to various surfaces and form biofilms (Bagge *et al.*, 2001; Thormann *et al.*, 2004, 2005, 2006; Teal *et al.*, 2006; McLean *et al.*, 2008a; Zhang *et al.*, 2010).

Previous work has demonstrated that, under aerobic hydrodynamic conditions, *S. oneidensis* biofilm development proceeds via initial attachment of single cells, subsequent surface coverage and finally in the formation of pronounced three-dimensional structures (Thormann *et al.*, 2004; Teal *et al.*, 2006; McLean *et al.*, 2008a). Generally, formation of these structures is thought to rely on the release of extracellular polymeric substances, such as proteins, polysaccharides, lipids or extracellular DNA (eDNA) (Sutherland, 2001; Branda *et al.*, 2005; Flemming *et al.*, 2007). The identity and composition of the extracellular components that are critical for *S. oneidensis* MR-1 community architecture is still unknown. Mutant analyses have identified several factors whose absence leads to aberrant biofilm formation. One of these factors is type IV pili, which were demonstrated to be required for initial surface attachment and are also implicated in mediating tight cell–cell interactions (Thormann *et al.*, 2004; McLean *et al.*, 2008b; Saville *et al.*, 2010). A similar role has been attributed to the outer membrane protein AggA, which is thought to represent a component of a protein transporter system similar to the *Pseudomonas fluorescens* Lap system (De Vriendt *et al.*, 2005; De Windt *et al.*, 2006; McLean *et al.*, 2008b; Theunissen *et al.*, 2009). In addition, a four-gene locus, termed *mxdABCD*,

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was identified to be critical for three-dimensional growth of the community and is assumed to be involved in the production and/or maintenance of the extracellular matrix (Thormann *et al.*, 2006; Saville *et al.*, 2010). MxdB most likely is a glycosyl transferase, suggesting that *mxd* might contribute to matrix formation by synthesis of a polysaccharide.

It has long been established that proteins and polysaccharides have an important structural role in bacterial biofilms. In contrast, the significance of eDNA for cellular attachment and structural integrity has more recently been recognized for an increasing number of Gram-negative and Gram-positive species (Whitchurch *et al.*, 2002; Steinberger and Holden, 2005; Allesen-Holm *et al.*, 2006; Moscoso *et al.*, 2006; Jurcisek and Bakaletz, 2007; Qin *et al.*, 2007; Izano *et al.*, 2008; Thomas *et al.*, 2008; Heijstra *et al.*, 2009; Vilain *et al.*, 2009; Harmsen *et al.*, 2010; Lappann *et al.*, 2010). Release of DNA in bacterial biofilms has mainly been attributed to the lysis of a cellular subpopulation, mediated by the activity of autolysis systems (Allesen-Holm *et al.*, 2006; Rice *et al.*, 2007; Thomas *et al.*, 2008, 2009; Mann *et al.*, 2009). The analysis of aggregates formed by *S. oneidensis* MR-1 in planktonic cultures indicated the presence of proteins, α-D-mannose or α-D-glucose containing exopolysaccharides, and substantial amounts of eDNA (McLean *et al.*, 2008b). This finding prompted us to determine whether eDNA is also a critical component of *S. oneidensis* biofilms.

In this study, we demonstrate that eDNA is, in fact, an important factor through all stages of *S. oneidensis* biofilm development. Based on mutant studies, we postulate that, in *S. oneidensis* MR-1, eDNA originates from lysis through induction of three genome-encoded prophages. Our study strongly indicates that phage-mediated lysis during early stages of biofilm formation may have beneficial consequences for subsequent structure formation.

Materials and methods

Growth conditions and media

Bacterial strains used in this study are summarized in Table 1. *Escherichia coli* strains were routinely grown in LB medium at 37 °C. For strain WM3064, 2,6-diamino-pimelic acid was added to the medium to a final concentration of 300 μM. *S. oneidensis* strains were routinely grown at 30 °C in LB. For solidification, agar was added to a final concentration of 1.5% (w/v).

Media were supplemented with 6 μg ml^{-1} chloramphenicol, 30 μg ml^{-1} kanamycin and/or 2 μg ml^{-1} tetracycline, where necessary. Biofilms of *S. oneidensis* were cultivated in LM medium (Paulick *et al.*, 2009) without antibiotics containing 0.5 mM lactate. DNase I (Serva Electrophoresis GmbH, Heidelberg, Germany) was used at a concentration of 30 μg ml^{-1} in medium supplemented with 5 mM $MgCl_2$. DDAO (7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)) was used at a concentration of 4 μM to stain eDNA in biofilms grown under hydrodynamic conditions.

Vector and strain constructions

DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989) or following the manufacturer's instructions. Kits for the isolation of chromosomal DNA, the isolation of plasmids and the purification of polymerase chain reaction (PCR) products were purchased from HISS Diagnostics GmbH (Freiburg, Germany). Enzymes were purchased from New England Biolabs (Frankfurt, Germany) and Fermentas (St Leon-Rot, Germany). Strains and plasmids used in this study are summarized in Table 1.

In-frame deletion mutants of *S. oneidensis* MR-1 were constructed essentially as reported earlier (Lassak *et al.*, 2010) using the suicide vector pNTPS-138-R6K and appropriate primer pairs, as summarized in Supplementary Table 1.

For biofilm studies, *S. oneidensis* MR-1 strains constitutively expressing *gfp* were constructed by using a modified Tn*7* delivery system (see Supplementary Material). To construct pME-P_{mot}-lacZ, *lacZ* was PCR amplified from pBAD-*lacZ* and cloned into the *Hin*dIII/*Xho*I sites of the broad-host-range vector pME6031. To generate a translational fusion of the *S. oneidensis* MR-1 *motAB* promoter region to *lacZ*, the corresponding region was amplified from *S. oneidensis* MR-1 chromosomal DNA and cloned into the *Bam*HI/*Hin*dIII sites. The resulting vector was introduced into *S. oneidensis* MR-1 by electroporation (Myers and Myers, 1997).

Cultivation of S. oneidensis *MR-1 biofilms*

Static conditions. Biofilm cultivation in polystyrene microtitre plates (Sarstedt, Newton, NC, USA) was carried out essentially as previously described (Thormann *et al.*, 2004). Briefly, overnight cultures of *S. oneidensis* MR-1 strains grown in LM medium were diluted 1:35 in LM medium. The diluted cultures were transferred to wells of polystyrene microtitre plates (170 μl per well) and incubated for the desired time at 30 °C. When required, DNase I (30 μg ml^{-1}) was added to the wells at different time points (0, 4 and 24 h). To serve as control, DNase I was heat inactivated by a 10-min incubation at 65 °C. Prior to processing, the density of the planktonic population in the wells was determined at 600 nm. Afterwards, 10 μl crystal violet (0.5% (w/v)) were added to the wells, followed by incubation for 10 min. The wells were then washed with 200 μl distilled water to remove loosely attached biomass. Subsequently, the crystal violet retained by the cells was redissolved in 200 μl ethanol (96% (w/v)), and the absorbance was determined at 570 nm using an Infinite M200 plate

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	*Relevant genotype*	*Source or reference*
Bacterial strains		
Escherichia coli		
DH5α λpir	Φ80*dlacZ*ΔM15 Δ(*lacZYA-argF*) U196 *recA1 hsdR17 deoR thi-l supE44 gyrA96 relA1/ëpir*	Miller and Mekalanos (1988)
WM3064	*ThrB1*004 *pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)567 Δ*dapA*1341::[*erm pir*(wt)]	W Metacalf, University of Illinois, Urbana
Shewanella oneidensis		
MR-1	*Shewanella oneidensis* MR-1 wild type	Venkateswaran *et al.* (1999)
S198	MR-1, tagged with eGfp in a mini-Tn*7* construct, Cm^r	This work
S176	MR-1, tagged with Cfp in a mini-Tn*7* construct, Cm^r	This work
S199	MR-1, tagged with Yfp in a mini-Tn*7* construct, Cm^r	This work
S1407	MR-1 ΔMuSo1, deletion of gene cluster SO0641–SO0685	This work
S1412	MR-1 ΔMuSo1, tagged with eGfp in a mini-Tn*7* construct, Cm^r	This work
S1381	MR-1 ΔMuSo2, deletion of gene cluster SO2651–SO2704	This work
S1392	MR-1 ΔMuSo2, tagged with eGfp in a mini-Tn*7* construct, Cm^r	This work
S1387	MR-1 ΔLambdaSo, deletion of gene cluster SO2939–SO3013	This work
S1393	MR-1 ΔLambdaSo, tagged with eGfp in a mini-Tn*7* construct, Cm^r	This work
S1421	MR-1 ΔLambdaSo ΔMuSo1	This work
S1426	MR-1 ΔLambdaSo ΔMuSo2	This work
S1422	MR-1 ΔMuSo1 ΔMuSo2	This work
S1419	MR-1 ΔLambdaSo ΔMuSo2 ΔMuSo1	This work
S1461	MR-1 ΔlambdaSo ΔMuSo2 ΔMuSo1, tagged with eGfp in a mini-Tn*7* construct, Cm^r	This work
Plasmids		
pNTPS-138-R6K	Ori-R6K *sacB*, suicide plasmid for generating in-frame deletions, Km^r	Lassak *et al.* (2010)
pNTPS-R6K-dMu1	Fragment for in-frame deletion of the gene region SO0641–SO0685 in pNTPS-R6K	This work
pNTPS-R6K-dMu2	Fragment for in-frame deletion of the gene region SO2651–SO2704 in pNTPS-R6K	This work
pNTPS-R6K-dlambda	Fragment for in-frame deletion of the gene region SO2939–SO3013 in pNTPS-R6K	This work
pME6031	*repA oriV*$_{pVS1}$ *oriV*$_{p15A}$ *oriT*, Tc^r, broad-host-range plasmid	Heeb *et al.* (2000)
pME6031-P_{mot}-lacZ	*motAB* promoter fused to *lacZ*, Tc^r	This work
pUC18-R6KT-miniTn*7*T	ori-R6K, Tn*7* recognition sites, Ap^r	Choi *et al.* (2005)
pTNS2	ori-R6K; encodes the TnsABC+D specific transposition pathway, Ap^r	Choi *et al.* (2005)
pBK-miniTn*7*-*gfp3*	ori-ColE1, *gfp*3, Ap^r, Cm^r, Gm^r	Lambertsen *et al.* (2005)
miniTn*7*(Gm)$P_{A1/04//03}$-*eyfp-a*	ori-ColE1, *eyfp*, Ap^r, Cm^r, Gm^r	Lambertsen *et al.* (2005)
miniTn*7*(Gm)$P_{A1/04//03}$-*ecfp-a*	ori-ColE1, *ecfp*, Ap^r, Cm^r, Gm^r	Lambertsen *et al.* (2005)
pUC18-R6KT-miniTn*7*T-egfp	NotI-egfp-Cm^r-NotI fragment from pBK-miniTn*7*-*gfp3* in pUC18-R6KT-miniTn*7*T	This work
pUC18-R6KT-miniTn*7*T-ecfp	NotI-ecfp-Cm^r-NotI fragment from miniTn*7*(Gm)$P_{A1/04//03}$-*ecfp-a* in pUC18-R6KT-miniTn*7*T	This work
pUC18-R6KT-miniTn*7*T-eyfp	NotI-eyfp-Cm^r-NotI fragment from miniTn*7*(Gm)$P_{A1/04//03}$-*eyfp-a* in pUC18-R6KT-miniTn*7*T	This work
pASK-IBA3plus	expression vector, Ap^r, Strep-TagII epitope	IBA GmbH, Göttingen, Germany
pASK-SO_2963	SO_2963 in pASK-IBA3plus	This work
pASK-SO_2685	SO_2685 in pASK-IBA3plus	This work

Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Tc^r, tetracyclin resistance.

reader (Tecan, Männedorf, Switzerland). The relative amount of surface attachment was normalized to that of the wild type. The assay was repeated in at least three independent experiments.

Hydrodynamic conditions. Biofilms were cultivated at room temperature in LM medium in three-channel flow cells with individual channel dimensions of 1 by 4 by 40 mm. Microscope cover slips (Roth, Germany) were used as a colonization surface, glued onto the channels with silicone (Sista-Henkel, Germany) and left to dry for 24 h at room temperature prior to use. Assembly, sterilization and inoculation of the flow system were performed essentially as previously described (Thormann *et al.*, 2004). Analyses were carried out in triplicate in at least two independent experiments. For treatment with DNase I, the enzyme was added to the inflow medium reservoir at a concentration of 30 $\mu g\,ml^{-1}$. For DDAO staining, the

flow was arrested briefly, and DDAO was added to the medium in the bubble trap and the upstream tubing. This process took no longer than 1 min, and control channels, in which the medium flow was stopped in parallel, ensured that the short arrest did not affect biofilm development. The biofilm cells were incubated with the dye for 1 h. Microscopic visualization using an inverted CLSM was performed at defined spots close to the inflow before and after the treatment.

Microscopy and image acquisition
Microscopic visualization of biofilms and image acquisition were conducted using an inverted Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with $\times 10/0.3$. Plan-Neofluar and $\times 63/1.2$ W C-Apochromate objectives. For displaying biofilm images, CLSM images were processed using the IMARIS software package (Bitplane AG, Zürich, Switzerland) and Adobe Photoshop. For the quantification of the surface coverage, the image of the confocal plane displaying the cell layer attached to surface was selected. The amount of surface-attached biomass was determined by the amount of green pixels (cells) in relation to that of the background (black) using Adobe Photoshop. For each data point, at least four individual images from at least two independent experiments were analyzed.

Measurements of β-galactosidase activity in culture supernatants
The activity of extracellular β-galactosidase in statically grown biofilms was determined as previously described (Steinmoen *et al*., 2002). Overnight cultures of *S. oneidensis* MR-1 strains grown in LM medium were diluted to an $OD_{600\,nm}$ of 0.05. The diluted cultures were transferred into Petri dishes and incubated at room temperature for 1, 4 and 24 h. As a control, the supernatant of a 24-h culture incubated at room temperature was used. To obtain cell-free supernatant, the samples were centrifuged at $2\,500 \times g$ for 5 min and subsequently filtered (0.2 μm filter). β-Galactosidase assays on supernatants were carried out in Eppendorf tubes at 30 °C according to standard protocols (Miller, 1972). The β-galactosidase activity was normalized to the overall amount of protein in the sample as quantified with the BCA protein assay reagent (Thermo Scientific, Schwerte, Germany).

Isolation of eDNA from biofilm supernatants
S. oneidensis MR-1 biofilms were grown in Petri dishes as described above. After 1, 4 and 24 h of incubation, 500 μl of the supernatant was collected, centrifuged (13 000*g*, 3 min) and passed through a membrane filter (0.45 μm) to remove all cell material. The supernatant was transferred to a new eppendorf tube. NaCl was added to a concentration of 0.25 M, and the eDNA was precipitated by adding 2:1 volume of ethanol (96% (w/v)). The precipitated eDNA was dissolved in TE (10 mM Tris (pH 8.0); 1 mM EDTA) buffer, and the DNA concentration was determined by spectrophotometry using a NanoDrop ND-1000 (Peqlab, Erlangen, Germany).

Quantification of eDNA in culture supernatants
Sterile supernatants of statically grown *S. oneidensis* MR-1 biofilms were used in a DNA release assay modified after Hamilton *et al*. (2001). The supernatants were collected after 1, 4 and 24 h of incubation and filter sterilized. A measure of 100 μl of a 1:200 dilution of PicoGreen fluorescent dye (Molecular Probes; Invitrogen, Darmstadt, Germany) was added to 100 μl of biofilm supernatant. DNA release was immediately measured fluorometrically at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Tecan Infinite M200 reader (Tecan). Concentration of eDNA was then calculated using DNA reference standards prepared in culture medium.

Quantitative RT–PCR
Isolated eDNA was used as template for quantitative RT–PCR (Real Time 7300 PCR Machine, Applied Biosystems, Darmstadt, Germany) using the Sybr Green detection system (Applied Biosystems). The signals were standardized to *recA*, with the CT (cycle threshold) determined automatically by the Real Time 7300 PCR software (Applied Biosystems), and the total number of cycles was set to 40. Samples were assayed in duplicate. The efficiency of each primer pair was determined using four different concentrations of *S. oneidensis* MR-1 chromosomal DNA ($10\,ng\,l^{-1}$, $1.0\,ng\,l^{-1}$, $0.1\,ng\,l^{-1}$ and $0.01\,ng\,l^{-1}$) as a template in quantitative PCRs. 0.1 ng of isolated eDNA was used to quantify the ratio of phage DNA to chromosomal DNA.

Immunoblot analyses
Rabbit polyclonal antibodies raised against the heterologously produced phage proteins SO_2685 and SO_2963 (see Supplementary Material) were generated by Eurogentec (Seraing, Belgium).

S. oneidensis MR-1 protein lysates for western blot analyses were prepared from statically grown biofilm cells harvested via scraping. Cells corresponding to an $OD_{600\,nm}$ of 0.25 were harvested by centrifugation, resuspended in 10 μl sample buffer, heated at 99 °C for 5 min and stored at −20 °C. For immunoblot analysis, 10 μl of the sample was resolved by denaturing SDS–polyacrylamide gel electrophoresis on 13% acrylamide gels. Subsequently, proteins were transferred to polyvinylidene difluoride membrane by semidry transfer. For detection of the proteins, polyclonal antibodies against SO_2685 and SO_2963 were used at a dilution of 1:5000 and 1:20 000, respectively. Secondary G-horseradish peroxidase-conjugated

antibody anti-rabbit immunoglobulin (Thermo Fisher Scientific, Schwerte, Germany) was used at a dilution of 1:20 000, and signals were detected using the SuperSignal West Pico Chemoluminescent Substrate (Thermo Fisher Scientific) followed by exposure to autoradiography film. Representative immunoblot patterns are shown, but similar patterns were obtained from at least two biological replicates.

Determination of phages's lytic activity in biofilm supernatants
To analyze the lytic activity of phage particles in *S. oneidensis* MR-1 biofilms, the supernatants of 24 h statically grown biofilms or 30 h planktonic cultures were collected, centrifuged and filter sterilized using a 0.45-μm filter (Sarstedt). In parallel, *S. oneidensis* MR-1 cells of the appropriate strain were cultivated overnight, freshly diluted and grown to an $OD_{600\,nm}$ of 1. A volume of 200 μl of the cell suspension was mixed with 40 ml LB soft agar (0.3% (w/v)) and poured into Petri dishes. Cell-free supernatants were spotted onto the cooled soft agar plates. The plates were incubated overnight at 30 °C and checked for plaque formation.

Determination of relative living cell number in biofilms
To determine the relative living cell number in *S. oneidensis* MR-1 biofilms, cells were grown statically in Petri dishes and collected at certain time points via scraping. The total cell number of each fraction was determined using a Thoma counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). In parallel, aliquots were plated onto LB agar to quantify the number of colony forming units.

Results

eDNA is an important structural component of S. oneidensis *biofilms*
To determine whether eDNA serves as a structural component in the formation of surface-attached communities by *S. oneidensis* MR-1, we applied DNase I to biofilms in different developmental stages. Biofilms of *S. oneidensis* MR-1 were grown either in static microtitre dishes or in the hydrodynamic flow chamber system. At appropriate time points, DNase I was added to the cultures in concentration that was previously determined not to interfere with the bacterial growth rate (data not shown). Addition of DNase I to static biofilm cultures of *S. oneidensis* MR-1 resulted in dissolution of the community within 15–30 min to 52–62% of the mass determined for untreated biofilms, independent of the developmental stage (Figure 1). In contrast, addition of heat-inactivated DNase I had no effect on the biofilm structure. Communities grown in the hydrodynamic flow chamber system also released large amounts of biomass within 60 minutes after exposure to DNase I, and the surface coverage of a 24-h biofilm decreased from 83% to 33%. Dispersal from the biofilm mainly occurred from the less structured areas, whereas the

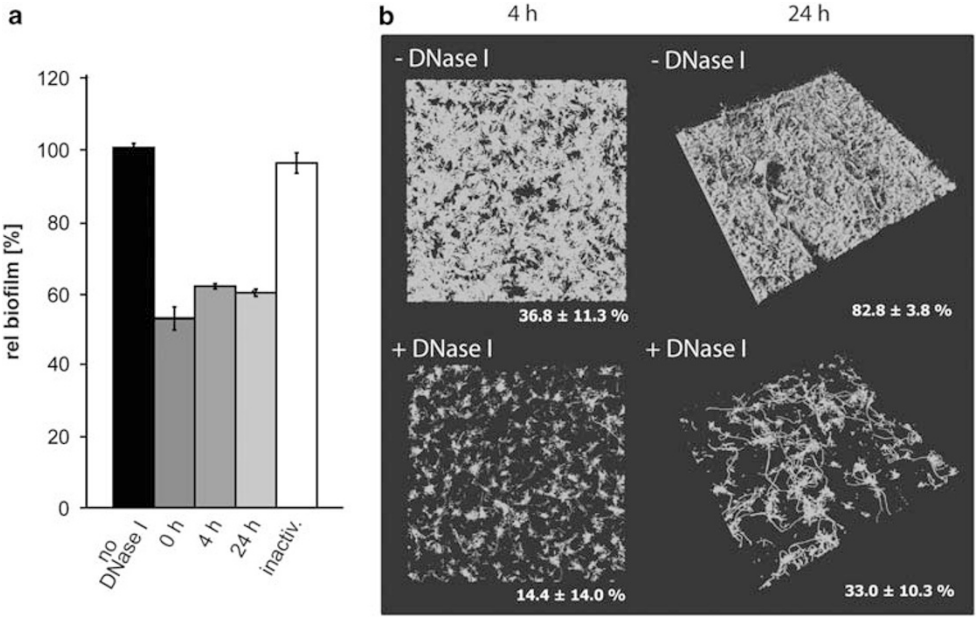

Figure 1 Effect of DNase I on *S. oneidensis* MR-1 biofilms grown under static and hydrodynamic conditions. (**a**) Wild-type cells were incubated in microtitre plates and DNase I was added directly (0), or after 4 and 24 h of incubation. As a control, a 24-h biofilm was treated with heat-inactivated DNase I. The values are means of three replicates, and the s.d. is displayed by error bars. (**b**) Biofilms formed by Gfp-tagged wild-type cells were cultivated under hydrodynamic conditions. After 4 and 24 h of incubation, DNase I was added to the medium. The biofilms were irrigated with DNase I-containing medium for 2 h prior to analysis by CLSM (lower panel). The lateral edge of a micrograph is 250 μm. The numbers display the average surface coverage of at least four individual scans from at least two independent experiments.

more densely packed three-dimensional structures that started to form after 24 h were more resistant to DNase I treatment and rarely dissolved. Treatment of cells subsequent to initial attachment released the majority of cells directly from the surface, and cultures pretreated with DNase I prior to incubation were significantly deficient in initiating surface contact (Figure 1).

To visualize eDNA, biofilms grown under hydrodynamic conditions were treated with cell membrane-impermeable DNA stain DDAO. eDNA appeared as a faint haze with string-like structures pervading the community (Figure 2). Notably, elevated amounts of eDNA were observed in the towering three-dimensional structures, even though these were more resistant against DNase I treatment. We determined an eDNA concentration of up to 3.5 ng μl^{-1} in the supernatant of static *S. oneidensis* MR-1 cultures after 24 h of incubation. However, eDNA directly associated with cells might have been lost during sample preparation, thus, local DNA concentrations within the community may be much higher. Isolated eDNA from cell-free supernatants of static *S. oneidensis* MR-1 biofilms was used as template in PCR reactions with different primer pairs that covered distant loci of the genome. All reactions gave rise to distinct products (Supplementary Figure S1), indicating that the DNA isolated from the supernatant represents the full chromosome. We further determined whether the addition of eDNA stimulates initial attachment or biofilm formation of *S. oneidensis* MR-1. To that end, the culture medium was supplemented with purified chromosomal DNA or herring sperm DNA. However, no stimulation of initial attachment occurred with either type of DNA, and further biofilm development was not affected (data not shown).

These data provide evidence that eDNA is a significant factor through all stages of *S. oneidensis* MR-1 biofilm formation and enhances initial surface attachment. However, as addition of DNase I never resulted in complete biofilm dissolution, additional structural components, such as proteins or polysaccharides, must be important for biofilm development. Moreover, we conclude that auxiliary factors may be required for the eDNA to mediate cell–cell and cell–surface interactions.

A role for the LambdaSo and MuSo prophages in cell lysis

A number of bacterial species was demonstrated to release eDNA by lysis of a subpopulation of the cells. So far, potential autolysis systems have not been characterized in *Shewanella* species. However, according to the genome data, *S. oneidensis* MR-1 harbors three prophages, designated LambdaSo (SO_2939-SO_3013; 50.84 kbp), MuSo1 (SO_0641-SO_0683; 34.55 kbp) and MuSo2 (SO_2652-SO_2704; 34.53 kbp) (Heidelberg *et al*., 2002). All three prophages have previously been shown

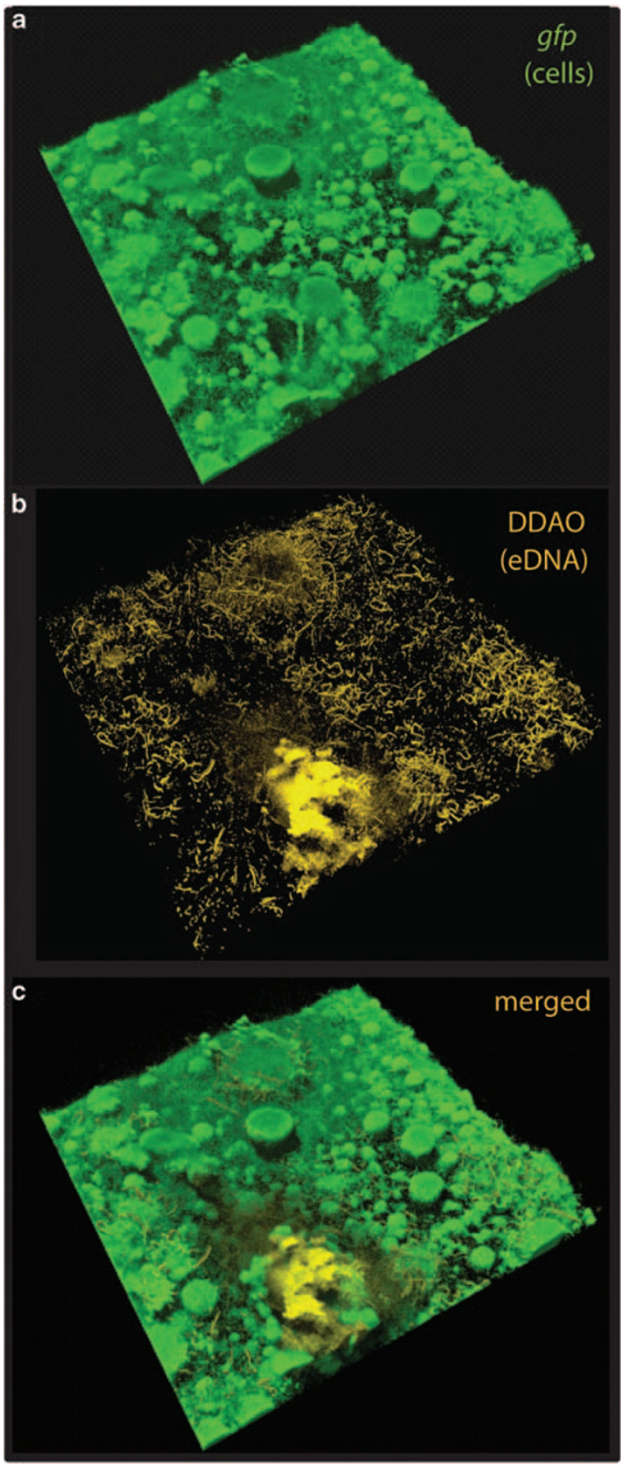

Figure 2 Extracellular DNA is a structural component of the matrix in *S. oneidensis* MR-1 biofilms. Shown are projections of a 72-h-old biofilm of Gfp-tagged *S. oneidensis* cells grown under hydrodynamic conditions that was subjected to DDAO staining. The upper image (**a**) displays the Gfp-tagged cells, the middle image (**b**, yellow) reveals the spatial distribution of extracellular DNA within the biofilm. The lower image (**c**) displays a merged projection of both upper images. The lateral edge of the micrograph is 250 μm.

to be upregulated upon exposure to environmental stresses such as UV and ionizing radiation (Qiu *et al.*, 2005, 2006). Consistent with this finding, cell death upon UV radiation has been attributed to lysis mainly caused by LambdaSo as demonstrated by the occurrence of phage particles in the supernatant (Qiu *et al.*, 2005). We therefore hypothesized that one or more of the prophages may be involved in cell lysis and eDNA release during *S. oneidensis* MR-1 biofilm formation. To identify a potential role of the prophages in cell lysis, we generated *S. oneidensis* MR-1 mutants that harbored a single prophage (ΔλΔMu2, ΔλΔMu1 and ΔMu1ΔMu2, respectively) and a mutant in which all three prophages were deleted (ΔΔΔ). To that end, the full prophage-encoding sequence was removed by double homologous recombination. By PCR and Southern blot analyses, we confirmed that no additional integrated or non-integrated copy of the phage genome remained (data not shown). As large gene regions including potential integration sites for the phages were deleted, these mutants could not be complemented by ectopic expression or reintegration of the deleted fragment. Therefore, we tested strains that had restored the original genotype after the second recombination step of the deletion procedure. These strains phenotypically equaled the corresponding predecessor strains, and we concluded that the phenotypes associated with the loss of the prophage genomes were not due to spontaneous mutations.

We then determined whether the presence of the prophages influenced cell physiology during growth in complex media. The mutant devoid of all prophages reached a significantly higher final optical density than the wild type (7.4 versus 4.9 after 31 h; Figure 3), indicating that the prophages may induce cellular lysis under appropriate conditions. To further demonstrate that a prophage-less mutant is less prone to autolysis than the wild type under static biofilm growth conditions, β-galactosidase was constitutively produced from a plasmid in the mutant and the wild-type strain. To that end, the strains were grown in static cultures, and the extent of cellular lysis was assessed by measuring the activity of β-galactosidase in the cell-free supernatant relative to that of the cells after 1, 4 and 24 h (Figure 3). A ratio of 0.7 was measured for the wild type after 24 h, whereas that of the phage-less mutant was significantly lower (0.3). As a second indicator for cell lysis, we determined the concentration of DNA in the medium supernatant.

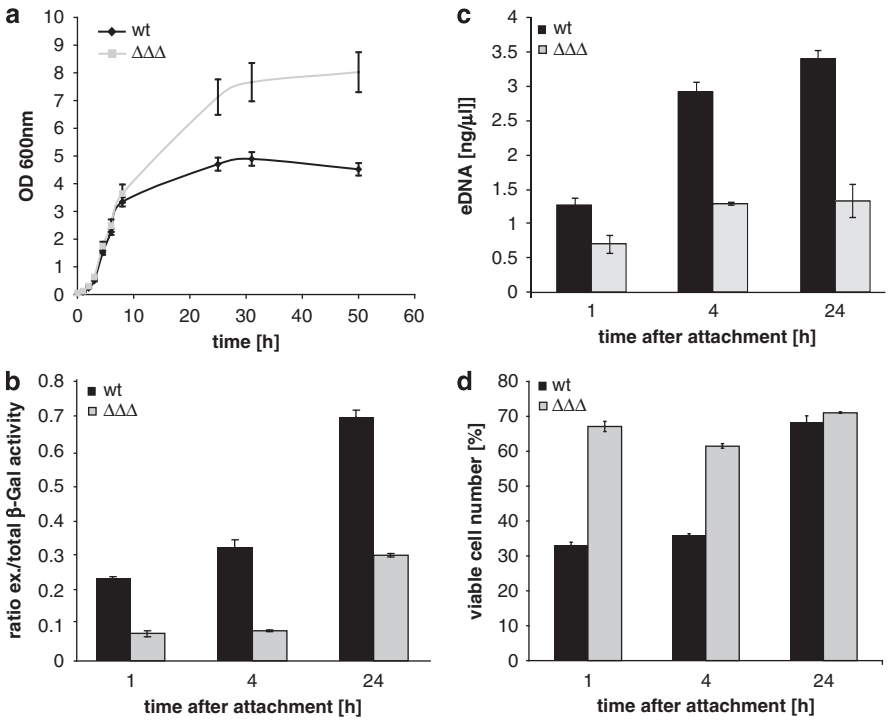

Figure 3 The role of prophages in cell lysis in *S. oneidensis* MR-1 cultures. (**a**) Growth analysis of batch cultures of wild-type (wt, black) and phage-less mutant ΔLambdaSoΔMuSo2ΔMuSo1 (ΔΔΔ, light gray) in LB medium. (**b**) Extracellular β-galactosidase activity in cell-free biofilm culture supernatants of the wild-type (black) and a phage-less mutant (light gray) transformed with vector pME-P_{mot}-lacZ. Cells were grown in Petri dishes and supernatant was collected after 1, 4 and 24 h of incubation. The average and associated s.d. of three replicates are shown. (**c**) Concentration of eDNA in cell-free supernatants (as obtained for β-galactosidase activity measurements) of the wild-type (black) and the phage-less mutant (ΔΔΔ, light gray). (**d**) Determination of the relative live cell number in static biofilm cultures of the wild-type (black) and phage-less mutant (light gray) strains. Cells were incubated in Petri dishes and harvested 1, 4 and 24 h after attachment. The bars represent the ratio of colony forming units of each fraction to the total cell number. Average values and s.d. displayed by error bars resulted from three replicates.

Up to 3.4 ng ml^{-1} was measured for the wild type after 24 h, compared with 1.34 ng ml^{-1} released by the mutant strain. In parallel, the relative number of colony forming units was determined. The relative amount of colony forming units of the phage-less mutant was almost twice as high as that of wild-type cells after 1 h (67–33%) and 4 h (61–36%). After 24 h, the viable cell number of the mutant and wild type was similar (~70%). Taken together, the prophage-less mutant released significantly lower amounts of β-galactosidase and DNA at all time points and, after 1 and 4 h of incubation, the relative amount of colony forming units was significantly higher than for the wild type. From these results, we concluded that loss of the prophages results in decreased cell lysis and increased cell viability.

LambdaSo and MuSo2 produce infectious phage particles

To further determine whether all three phages are able to form infectious virus particles during biofilm formation and planktonic growth, cell-free supernatants were harvested from static biofilm cultures of the wild type and the mutants bearing a single prophage that were incubated for 24 h. Supernatants from the planktonic cultures were harvested after 30 h. Small aliquots were spotted onto soft-agar lawns of either the wild type and the prophage-less mutant (ΔΔΔ). Supernatants from mutants harboring only the LambdaSo and the MuSo2 prophage produced plaques in this assay, indicating the production of infectious LambdaSo and MuSo2 virus particles (Figure 4; Supplementary Figure S2). In contrast, supernatants from a MuSo1-harboring strain did not lead to plaque formation, suggesting that this prophage might still exert detrimental effects on its host cell but is not able to assemble a functional virus particle. This finding is in agreement with an earlier prophage gene analysis that reported the absence of several tail genes and insertions in the MuSo1 gene cluster encoding the head subunits (Canchaya *et al.*, 2003). We determined whether MuSo1 is excised during the process of biofilm formation. To that end, PCR was performed using DNA as template that was purified from 24 h biofilms (Supplementary Figure S3). No product was obtained with primer pairs bracketing the MuSo1 locus, strongly indicating that the phage genome is not excised. In contrast to the triple mutant cleared of all the prophages, the wild type was not lysed by supernatants of any mutant or the wild-type strain. Given the higher growth rate of the triple mutant, it cannot be excluded that the mutant strain is in a different physiological stage that renders it more susceptible for phage infection. However, we propose that cell lysis under the conditions tested is rather due to induction of the prophage's lytic cycle and subsequent lysis of the host cell, but not to reinfection and lysis of other prophage-bearing wild-type cells by the released phage particles. This would suggest that the presence of the prophages protects the cells from reinfection.

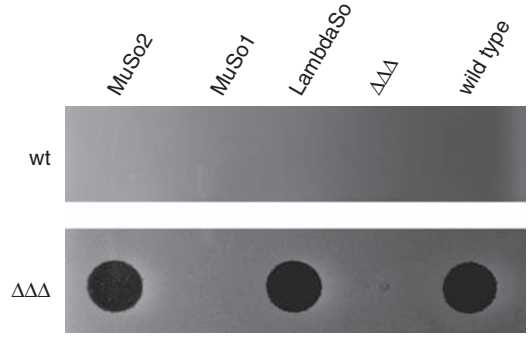

Figure 4 Detection of phages in the supernatant of statically grown biofilms of *S. oneidensis* MR-1. Cell-free supernatants of 24 h-old biofilms formed by mutants exclusively harboring MuSo2 (ΔMu1Δλ, lane 1), MuSo1 (ΔMu2Δλ, lane 2) and LambdaSo (ΔMu1ΔMu2, lane 3), by the phage-less mutant (ΔΔΔ, lane 4) and the by wild type (lane 5) were spotted on soft agar lawns of wild-type cells (upper panel) and phage-less mutant cells (lower panel).

To determine the timing of phage release, supernatants and biofilm cells of static biofilm cultures were tested after 1, 4 and 24 h of incubation for the occurrence of phage proteins (Figure 5) using antibodies raised against the predicted major head subunit of MuSo2 (SO_2685) and the predicted major capsid protein of LambdaSo (SO_2963). Substantial amounts of the MuSo2 major head protein were already detected after 1 h incubation and after prolonged incubation for 24 h. Notably, after 4 h, the concentration of the protein was significantly lower. In contrast, the corresponding protein of LambdaSo was only detected at low concentrations after 4 h of incubation but occurred in substantial amounts in later stages of biofilm formation. Accordingly, quantification of phage DNA in the total eDNA by quantitative RT–PCR revealed an enrichment of MuSo2 DNA during early biofilm formation. An increase in the occurrence of LambdaSo DNA was observed after 4 h of incubation (Figure 5). However, after 24 h, the relative amount of phage DNA dropped, suggesting that, at a certain stage, no more phage particles are produced although the bacterial culture continuous to grow. This is in accordance with the finding that the living cell number of the phage-less mutant and wild type is similar and suggests that induction of the phages and phage-mediated cell lysis decreases between 8 and 24 h. The copy number of MuSo1 DNA equaled that of the control gene *recA* (data not shown). This is indicating that the phage genome is not replicated and is consistent with the finding that MuSo1 does not produce infectious particles.

Taken together, we demonstrated that at least two of the three *S. oneidensis* MR-1 prophages are capable of mediating cell lysis and, thus, may contribute to the release of eDNA in the process of

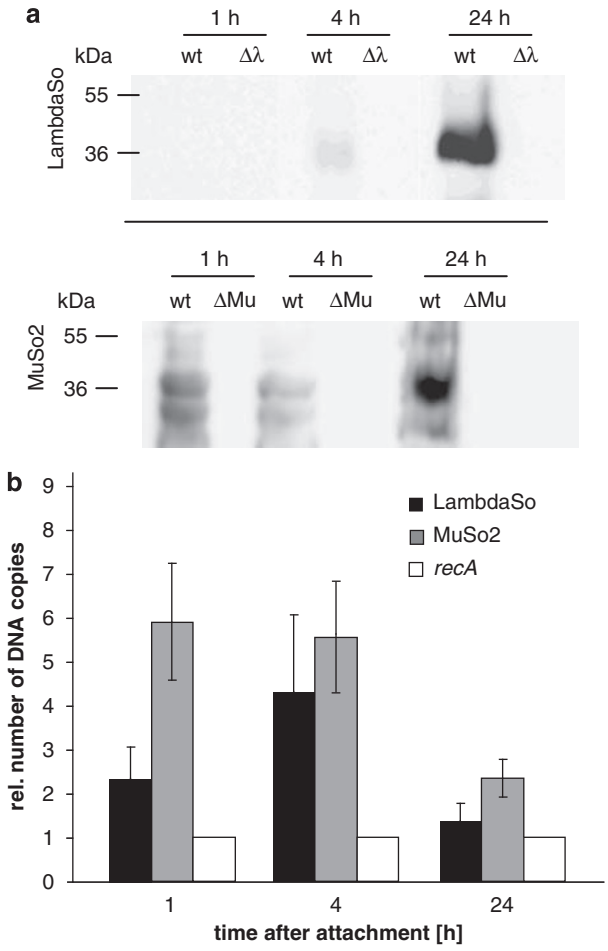

Figure 5 Phage release during attachment and biofilm formation of *S. oneidensis* MR-1. (**a**) Presence of phages LambdaSo (upper panel) and MuSo2 (lower panel) in wild-type cells (wt) and appropriate deletion mutant cells (Δλ and ΔMuSo2). Cells were grown under static conditions and harvested 1, 4 and 24 h after attachment. Cell lysates were subjected to SDS–polyacrylamide gel electrophoresis followed by western blotting and detection with specific antisera against the LambdaSo and MuSo2 phage major head proteins. (**b**) Determination of the relative DNA copy number of the phages LambdaSo (black) and MuSo2 (light gray) compared with *recA* (representing the chromosomal DNA (white) in biofilm supernatants). Extracellular DNA was isolated from the supernatant of statically grown biofilms 1, 4 and 24 h after attachment. DNA levels were analyzed via quantitative RT–PCR. Amplification of *recA* was used as control to determine the number of chromosomal DNA copies. The values are means of three replicates. Error bars display the s.d.

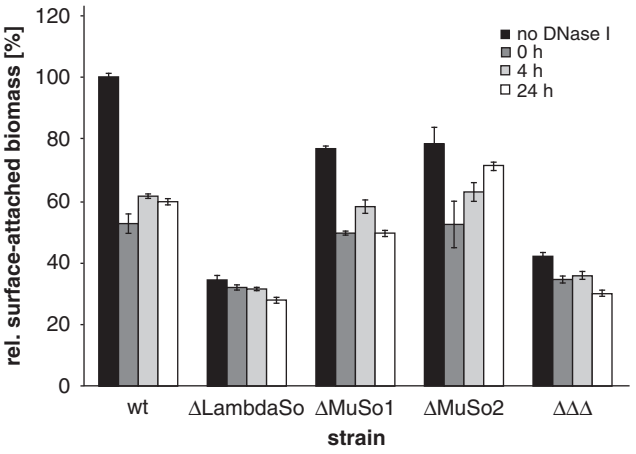

Figure 6 Effects of DNase I treatments on statically grown biofilms formed by phage mutants. *S. oneidensis* MR-1 wild-type and mutant cells were incubated in microtitre plates and DNase I was added after 0, 4 or 24 h of incubation. The biofilm was quantified using a crystal violet assay. The values are means of three replicates, and the s.d. are displayed by error bars.

biofilm formation. MuSo2 and LambdaSo are induced during different stages of biofilm development. We therefore speculated that loss of the prophages and subsequent decrease in cell lysis might influence *S. oneidensis* biofilm formation due to a lack of eDNA.

Mutants lacking the prophages are defective in biofilm formation

To determine whether the prophages are involved in biofilm formation of *S. oneidensis* MR-1 by release of eDNA, we tested the biofilm-forming capacities of strains in which one of the three prophages was deleted and that of the triple mutant lacking all three prophages. In the static microtitre plate assay, deletion of the MuSo1 or MuSo2 prophage resulted in a decrease of surface-attached biomass to ~80% of that of the wild-type level (Figure 6). Addition of DNase I further released biomass to similar levels observed for wild-type biofilms that were equally treated with DNase I (~50%). A mutant lacking the LambdaSo prophage was drastically impaired in biofilm formation under static conditions (Figure 6). Compared with the wild type, it accumulated only ~40% surface-associated biomass, which could not be further dispersed by treatment with DNase I. The mutant lacking all three prophages displayed a biofilm phenotype similar to that of the LambdaSo deletion strain. When grown in the hydrodynamic flow chamber system, mutants lacking MuSo1 and, in particular, MuSo2 were delayed in biofilm formation (Figure 7). However, after prolonged incubation for 24 and 48 h, both mutants were able to cover the surface (70% and 50% after 24 h, respectively) and to develop pronounced distinct three-dimensional structures. In contrast, a LambdaSo mutant was not affected in the initial stages of biofilm formation. The mutant, however, was unable to cover the surface (30% after 24 h) and to form the distinct three-dimensional structures after 24 h (Figure 7). The triple-mutant lacking all three prophages displayed an additive biofilm-deficient phenotype. Very small amounts of surface-attached biomass were detected even after 48 h of incubation. The appearance of the biofilm formed by the triple mutant equaled that of wild-type cells treated with DNase I (Figure 1). As has been observed in the static microtitre plate assay, the biofilm structures formed by the triple-prophage

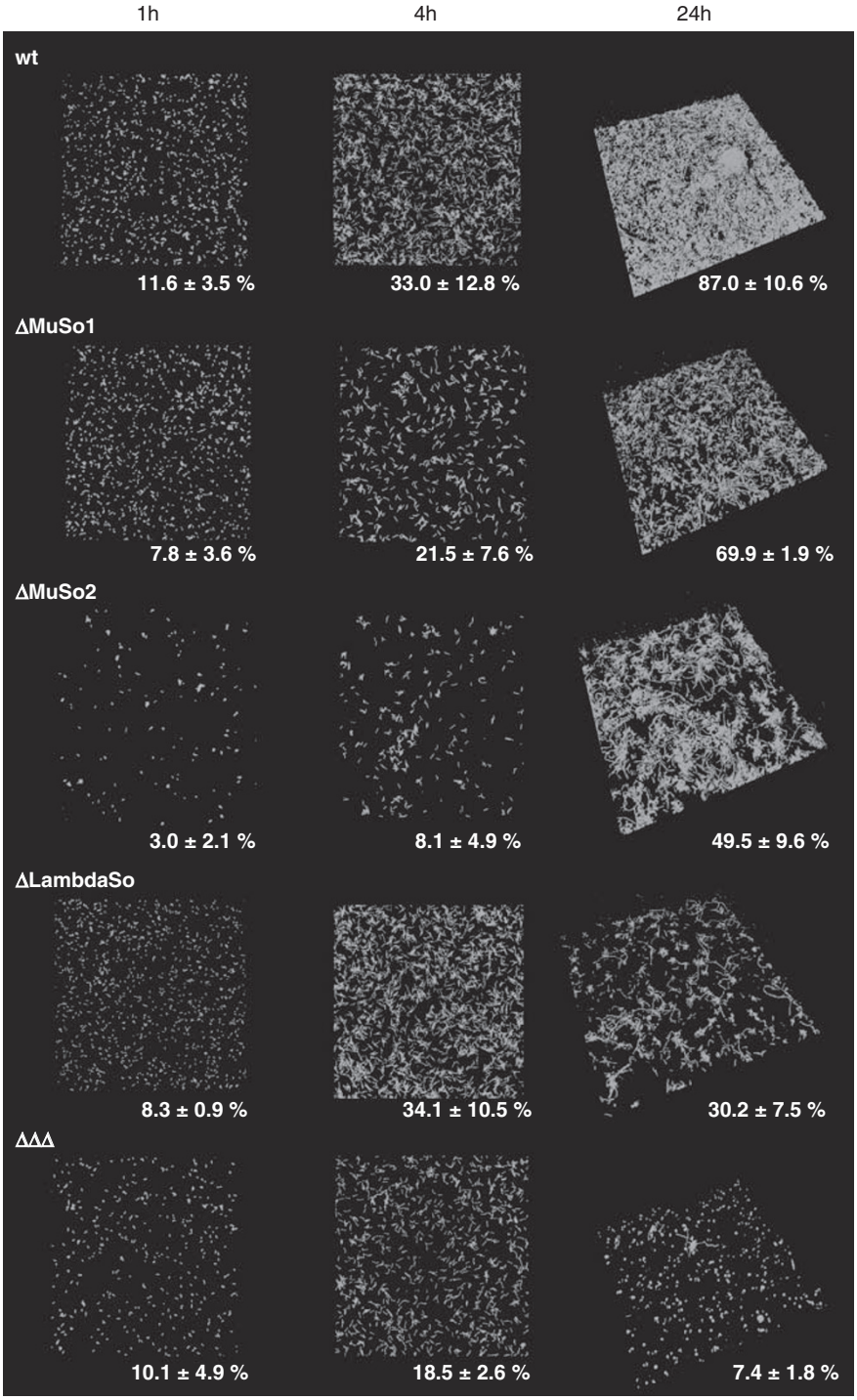

Figure 7 Involvement of prophages MuSo1, MuSo2 and LambdaSo in biofilm formation under hydrodynamic conditions. Gfp-tagged *S. oneidensis* MR-1 wild-type and mutant cells were incubated in flow chambers, and biofilm formation was microscopically analyzed via CLSM after 1 (left panel), 4 (middle panel) and 24 h (right panel) of attachment. Displayed are three-dimensional shadow projections. The numbers represent the average surface coverage. The lateral edge of each micrograph is 250 μm in length.

mutant could not be dispersed by addition of DNase I (Supplementary Figure S4). In addition, no eDNA was visualized in biofilms formed by the triple mutant by DDAO staining. Notably, biofilm formation of phage mutants was not restored upon addition of herring sperm DNA or *S. oneidensis* MR-1 chromosomal DNA (data not shown). From the biofilm analysis, we concluded that all three prophages contribute to biofilm formation, most likely by lysis of a subpopulation of cells and release of cytoplasmic factors such as eDNA. The LambdaSo phage appears to have a predominant role during that process, particularly at later stages of biofilm formation.

Discussion

eDNA occurs in significant amounts in terrestrial and aquatic environments where it may serve as important nutrient reservoirs, particularly for nitrogen and phosphorus (Deflaun *et al.*, 1986; Paul *et al.*, 1991; Niemeyer and Gessler, 2002; Dell'Anno and Danovaro, 2005). Accordingly, a previous study showed that *Shewanella* species are capable of using DNA as source of phosphorus, nitrogen, carbon and energy (Pinchuk *et al.*, 2008). Here we demonstrate that, in addition, eDNA has a major role in surface attachment and development of three-dimensional structures during *S. oneidensis* MR-1 biofilm formation. First conclusive evidence for eDNA as an important factor in the structural integrity of microbial biofilms was presented for *Pseudomonas aeruginosa* (Whitchurch *et al.*, 2002; Allesen-Holm *et al.*, 2006). Since that time, there has been emerging evidence from numerous studies on different bacterial species that identify eDNA as a common structural component in biofilm formation, although its exact role still remains unknown. As demonstrated here for *S. oneidensis* MR-1, eDNA is already involved in early attachment events, similar to what has been reported for other species (Whitchurch *et al.*, 2002; Izano *et al.*, 2008; Vilain *et al.*, 2009; Harmsen *et al.*, 2010; Lappann *et al.*, 2010). Recent studies on *Bacillus cereus*, *Listeria monocytonogenes* and *Staphylococcus epidermidis* suggest that the bacterial cell surface may be decorated with DNA, resulting in acid–base interactions that increase the ability for either cell–cell and cell–surface interactions (Vilain *et al.*, 2009; Das *et al.*, 2010; Harmsen *et al.*, 2010). Furthermore, short DNA fragments smaller than 500 bp added to a DNA-free culture of *Listeria monocytogenes* were demonstrated to prevent initial adhesion. This lead to the hypothesis that high-molecular-weight DNA bound to a limited number of attachment sites is required to mediate cell–surface interactions (Harmsen *et al.*, 2010). It remains to be shown if a similar mechanism exists in *S. oneidensis* MR-1. Later multicellular stages of *S. oneidensis* biofilms were less prone to DNase I-induced dispersal, suggesting that eDNA is not the only structural component of the biofilm matrix. Further, but not complete, detachment of biomass could be achieved by additional exposure to proteases (Gödeke and Thormann, unpublished data). Thus, proteinaceous compounds and exopolysaccharides are likely involved in structural integrity. Notably, addition of DNA to the media did not stimulate biofilm formation of *S. oneidensis* MR-1 or complement biofilm formation of the phage mutants. We therefore hypothesize that an auxiliary factor, in addition to eDNA, is involved. For *L. monocytogenes*, it was demonstrated that peptidoglycan is required as an additional prerequisite for DNA-dependent biofilm formation (Harmsen *et al.*, 2010), and a similar factor might be required in *S. oneidensis* MR-1 biofilms as well.

A main question remaining is how eDNA release is mediated, and different mechanisms for DNA release in bacterial biofilms have been discussed. Species such as *Neisseria* are capable of active DNA export (Hamilton *et al.*, 2005) and also DNA transport through vesiculation has been suggested (Whitchurch *et al.*, 2002; Allesen-Holm *et al.*, 2006). However, as opposed to active transport, several studies provide evidence that, in biofilms of many bacterial species, eDNA rather originates from the lysis of a cellular subpopulation. In *Neisseria*, DNA release is thought to be mediated by lytic transglycosylases and *N*-acetylmuramyl-L-alanine amidase (Lappann *et al.*, 2010). Other factors implicated in cell lysis are toxin/antitoxin system that have been characterized, for example, in *Enterococcus faecalis* (Thomas *et al.*, 2008) and *Staphylococcus* sp. (Qin *et al.*, 2007; Rice *et al.*, 2007; Mann *et al.*, 2009). However, a role for toxin/antitoxin systems in biofilm formation is not necessarily directly linked to cell lysis, as has been demonstrated for *E. coli* (Kim *et al.*, 2009, 2010). Corresponding systems in *Shewanella* are yet to be characterized. A putative holin/antiholin autolysis system with homology to the *Staphylococcus cid* system was identified in *S. oneidensis* MR-1 (SO_1046-SO_1048) (Bayles, 2007). However, mutant analyses revealed that this system does not have a significant role in *S. oneidensis* biofilm formation (Gödeke and Thormann, unpublished data). Instead, we identified prophage-mediated cell lysis as a likely mechanism for DNA release.

Bacteriophages are highly abundant in all environments and are thought to outnumber prokaryotes in nature by a factor of 10 (Rohwer and Edwards, 2002; Rice *et al.*, 2009). As opposed to phages that predominantly lyse cells, temperent phages can integrate as prophage into host cell genomes in a way that may benefit both host and prophage (Weinbauer, 2004; Chen *et al.*, 2005). Genome analyses revealed the presence of prophage-like elements in almost all bacterial genomes (Canchaya *et al.*, 2003). By mutant analyses, we demonstrated for the first time that at least two out of three prophages previously identified in *S. oneidensis* MR-1 are capable of mediating cell lysis, and that two of the prophages, LambdaSo and MuSo2, form infectious phage particles. A mutant lacking all prophages is less prone to cell lysis and biofilm formation of such a mutant occurs independently of eDNA as a structural component. Thus, our study links cell lysis to the release of factors promoting cell–cell and cell–surface attachment, in particular eDNA. Our present results strongly suggest that, in *S. oneidensis*, phage-mediated cell lysis already affects early stages of biofilm formation, and complements previous reports on the role of prophages in bacterial biofilm formation. The best-studied example in this regard is the role of the filamentous phage Pf4 in *P. aeruginosa* biofilm formation. Mutants lacking the phage form smaller

colonies during the first days of biofilm formation and a potential role of phage-mediated cell lysis in eDNA release has been discussed but has not directly been demonstrated (Allesen-Holm *et al.*, 2006; Rice *et al.*, 2009). At later stages of biofilm development, Pf4 is thought to convert into a superinfective lytic form that causes cell death and hollowing of the structures, and, by that, significantly contributes to seeding dispersal of the community (Webb *et al.*, 2003; Rice *et al.*, 2009). In addition, Pf4 has been linked to phenotypic variations in the dispersed cells, leading to small-colony variants that are characterized by accelerated biofilm formation (Webb *et al.*, 2004; Rice *et al.*, 2009). In contrast to MuSo2 and LambdaSo, MuSo1 does not form functional phage particles under the conditions tested. However, a mutant lacking this phage still had a slightly delayed biofilm phenotype. Notably, cryptic prophages unable to produce infectious phage particles have recently been demonstrated to affect bacterial biofilm formation. In *E. coli*, the prophage CP4-57 excises its genome from the bacterial chromosome during early biofilm formation. Subsequent loss of the excised prophage results in the induction of genes related to flagella-mediated motility and cell lysis, two factors known to affect biofilm development (Wang *et al.*, 2009). So far, we have no indications that MuSo1 is excised from the chromosome, and further studies will address how MuSo1 might affect cellular functions.

Our results also indicate that the prophages may contribute differentially to biofilm formation: whereas the Mu-like phages affect early steps of development, LambdaSo is the major contributing factor to the formation of three-dimensional structures. Transcription of genes from all three prophages has previously been demonstrated to be upregulated upon stresses such as UVB and UVC irradiation and ionizing radiation (Qiu *et al.*, 2005, 2006). However, the nature of the signals that trigger the prophages to enter the lytic cycle during biofilm formation in *S. oneidensis* is thus far unknown. Notably, phage genes have been shown to be strongly upregulated in biofilms of several bacterial species (Whiteley *et al.*, 2001; Ren *et al.*, 2004; Domka *et al.*, 2007). It remains to be shown whether phage induction in *S. oneidensis* MR-1 is a direct response to surface attachment and/or nutrient limitations as indicated by cell lysis occurring in planktonic cultures in late exponential phase and whether the prophages are also involved in the dispersal of *S. oneidensis* biofilms.

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