Environmental Microbiology (2007) **9**(11), 2844–2854 doi:10.1111/j.1462-2920.2007.01398.x

Biofilm formation in *Desulfovibrio vulgaris* Hildenborough is dependent upon protein filaments

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Summary

Desulfovibrio vulgaris **Hildenborough is a Gram-negative sulfate-reducing bacterium (SRB), and the physiology of SRBs can impact many anaerobic environments including radionuclide waste sites, oil reservoirs and metal pipelines. In an attempt to understand *D. vulgaris* as a population that can adhere to surfaces, *D. vulgaris* cultures were grown in a defined medium and analysed for carbohydrate production, motility and biofilm formation. *Desulfovibrio vulgaris* wild-type cells had increasing amounts of carbohydrate into stationary phase and approximately half of the carbohydrate remained internal. In comparison, a mutant that lacked the 200 kb megaplasmid, strain ΔMP, produced less carbohydrate and the majority of carbohydrate remained internal of the cell proper. To assess the possibility of carbohydrate re-allocation, biofilm formation was investigated. Wild-type cells produced approximately threefold more biofilm on glass slides compared with ΔMP; however, wild-type biofilm did not contain significant levels of exopolysaccharide. In addition, stains specific for extracellular carbohydrate did not reveal polysaccharide material within the biofilm. *Desulfovibrio vulgaris* wild-type biofilms contained long filaments as observed with scanning electron microscopy (SEM), and the biofilm-deficient ΔMP strain was also deficient in motility. Biofilms grown directly on silica oxide transmission electron microscopy (TEM) grids did not contain significant levels of an exopolysaccharide matrix when viewed with TEM and SEM, and samples stained with ammonium molybdate also showed long filaments that resembled flagella. Biofilms subjected to protease treatments were degraded, and different proteases that were added at the time of inoculation inhibited biofilm formation. The data indicated that *D. vulgaris* did not produce an extensive exopolysaccharide matrix, used protein filaments to form biofilm between cells and silica oxide surfaces, and the filaments appeared to be flagella. It is likely that *D. vulgaris* used flagella for more than a means of locomotion to a surface, but also used flagella, or modified flagella, to establish and/or maintain biofilm structure.**

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Introduction

Desulfovibrio vulgaris Hildenborough is a Gram-negative sulfate-reducing bacterium (SRB), and the genome has been sequenced (Heidelberg *et al*., 2004). Although much research has been conducted with respect to the ability of *D. vulgaris* to reduce heavy metals (Lovley and Phillips, 1994; Chardin *et al*., 2002; Lloyd, 2003; Goulhen *et al*., 2005; Cabrera *et al*., 2006), little research has been done on the structure and function of surface-adhered populations. Biofilms are typically defined as cells adhered to surfaces that are embedded within an exopolymer matrix, and matrices of well-studied biofilms (i.e. *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*) are usually composed of exopolysaccharides (EPS) (Branda *et al*., 2005). In addition, other microorganisms can produce biofilms that contain protein, DNA, lipids and various appendages (i.e. pili, fimbriae) within the matrix (Sutherland, 2001; Branda *et al*., 2005), but less is known about the possible roles of the different constituents. The paradigm thus far has been the biofilm of *Pseudomonas* spp. that produce a carbohydrate-based matrix that can also contain various polymers within the polysaccharide matrix (O'Toole *et al*., 2000; Sutherland, 2001; Sauer *et al*., 2002; Branda *et al*., 2005; Vasseur *et al*., 2005). *Escherichia coli* biofilms are typically composed of a thick colanic acid EPS layer, with structure similar to *Pseudomonas* biofilms (Danese *et al*., 2000; O'Toole *et al*., 2000).

Sulfate-reducing bacteria are frequently observed within biofilms that can cause biocorrosion, or microbial induced corrosion (MIC), and SRB populations, particularly those in the *Desulfovibrio* genus, have been observed within biofilm communities formed on interior surfaces of pipelines and metals (Zhu *et al*., 2003; Jan-Roblero *et al*., 2004; Neria-Gonzalez *et al*., 2006; Liu *et al*., 2007). Although the *Desulfovibrio* populations within these biofilms are small, the role they play could be key for MIC due to sulfide production and/or electron transfer mechanisms (Zhu *et al*., 2003; Jan-Roblero *et al*., 2004; Neria-Gonzalez *et al*., 2006). Other populations, including enteric, acid-forming bacteria, may play a direct role in corrosion or may possibly serve as support for more destructive SRBs (Jan-Roblero *et al*., 2004). The actual matrix of the biofilm, including EPS and proteins, may also act as reducing agents that could contribute to metal corrosion (Chan *et al*., 2002; Beech and Sunner, 2004).

In contrast to the less desired roles of the SRB in metal corrosion, SRBs can play beneficial roles in heavy metal and radionuclide bioremediation. Metals and metalloids such as Fe(III), V(V), Cr(VI), Mo(VI), Pd(II), Mn(IV), As(V), Se(VI), Te(IV), and radionuclides U(VI) and Tc(VII) can be reduced by various SRBs (Lloyd, 2003). Reduction can occur directly via hydrogenases or type I tetrahaem cytochrome c_3 or indirectly by the production of hydrogen sulfide (Lovley *et al*., 1993; Lovley and Phillips, 1994; Lloyd, 2003). Recent studies with SRB biofilms have indicated that the reduction and precipitation of zinc sulfide and sulfur-selenium occurs within the biofilm matrix (Hockin and Gadd, 2003; Labrenz and Banfield, 2004). In addition, Beyenal and Lewandowski (2004) demonstrated the reduction and stabilization of lead within *Desulfovibrio desulfuricans* biofilms.

Given the applicability of metal-reducing bacteria for bioremediation and the possible roles in metal corrosion, biofilm studies are needed to understand the cellular processes and mechanisms that are involved during the distinct physiological state of surface-attached growth. Until recently, the majority of biofilm research has been conducted with pathogenic microorganisms; therefore little is known about monoculture or community biofilms involved in bioremediation processes. Here we have examined the biofilm structure of *D. vulgaris* under anaerobic, sulfate-reducing conditions. The results demonstrated that the *D. vulgaris* biofilm contained very little EPS and was dependent upon proteins for biofilm formation and matrix stability. The characterization of the structure and function of *D. vulgaris* biofilms will provide insight into a novel growth mode for a diverse group of microorganisms with repercussions for both bioremediation and metal corrosion.

Results

Growth of D. vulgaris *and ΔMP with lactate and sulfate*

For the described studies, *D. vulgaris* Hildenborough ATCC 29579 was considered wild type, and a strain that lacked the 200 kb megaplasmid was referred to as ΔMP. The ΔMP strain was obtained via long cultivation in nitrogen complete medium in the laboratory. Planktonic generation times were approximately 5 h for both wild-type and ΔMP cultures under the tested growth conditions. When lactate (60 mM) and sulfate (50 mM) were the electron donor and acceptor, respectively, carbohydrate production increased during late exponential growth (up to 10 $\mu g\ ml^{-1}$) (data not shown). A portion of the carbohydrate (approximately half) was located internal to the cell proper. The internal carbohydrate to protein ratio (C : P) for wild-type cells 35 h after inoculation (stationary phase) was 0.02 $\mu g\ \mu g^{-1}$. In contrast, the ΔMP cells generated slightly less carbohydrate, but the internal C : P ratio was twofold higher than for wild-type cells in stationary phase. The increased C : P ratio for the ΔMP cells remained constant throughout stasis (approximately 70 h). In comparison, *Klebsiella pneumoniae*, a capsulated bacterium, had a C : P ratio that was ninefold higher (0.180 $\mu g\ \mu g^{-1}$) than that of *D. vulgaris* wild-type cells. Samples of spent medium contained a minimal amount of carbohydrate for either wild type or ΔMP. These data indicated that wild-type cells utilized or re-allocated internal carbohydrate during the transition to stationary phase; however, small amounts were detected extracellular to the cell proper. Due to the differences in carbohydrate levels, biofilm formation was determined for both wild type and ΔMP.

Biofilm formation in batch growth

Biofilm formation was noted on the culture tubes at approximately 15 h or greater post inoculation (mid-exponential growth phase). To further investigate biofilm formation, glass slides were inserted into tubes of LS4D medium prior to sterilization. Biofilms were allowed to form for 30 h post inoculation, and biofilms for wild-type and ΔMP cultures were analysed via crystal violet staining (Fig. 1). Wild-type biofilms retained more crystal violet, and crystal violet quantification revealed that ΔMP biofilm formation was threefold less than the wild type even though planktonic protein levels were similar. Scanning electron microscopy (SEM) images showed fewer ΔMP cells present on the glass slides than observed for wild-type biofilms (Fig. 2A and B). Wild-type biofilms also contained numerous filaments compared with planktonic cells observed via both SEM and transmission electron microscopy (TEM) (Figs 2C and D and 4, Figs S1 and S2).

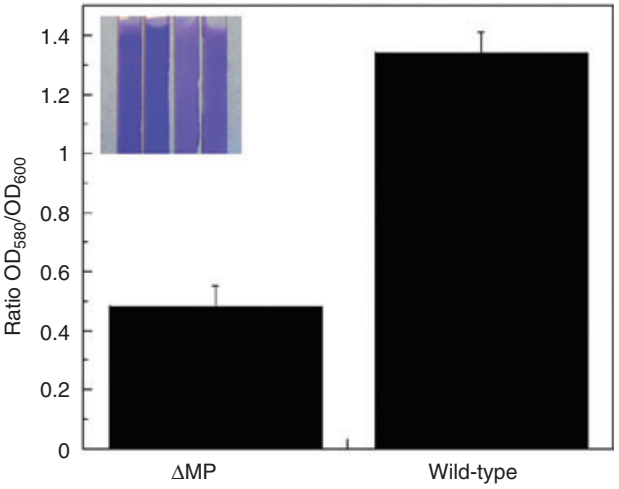

Fig. 1. Crystal violet staining (inset) and spectrophotometric quantification of *D. vulgaris* biofilms on glass slides. Values are expressed as a ratio of OD_{580}/OD_{600} normalized to cell density. The quantification was repeated six times.

Motility in wild-type and ΔMP cells

Due to the possible connection between motility and biofilm formation in other organisms, wild-type and ΔMP planktonic cells were analysed for motility by growth on LS4D motility agar plates. The motility agar plates contained either 1% agar or 0.7% (w/v) agar. Wild-type cells produced a diffuse halo around the colony on the 1% agar plate (approximately 24 mm) and a halo was not observed for ΔMP (data not shown). Wild-type cells on the 0.7% agar produced a confluent colony with a diameter of 34 mm compared with 23 mm for the ΔMP colony (Fig. 3). In addition, the ΔMP colony on 0.7% agar appeared sporadic with an uneven margin and distribution around the site of inoculation (Fig. 3). Extracellular structures were then analysed qualitatively with TEM. Flagella were observed in planktonic wild-type cells and most cells had a single, polar flagellum (Fig. 4A); however, a majority of the ΔMP cells did not possess a flagellum (Fig. 4B). The data suggested that the ΔMP strain was deficient in motility due to defective and/or absent flagella. A small amount of stained material was observed around wild-type cells in the TEM images; therefore, biofilms were analysed for carbohydrate content.

Carbohydrate content in wild-type biofilms

Wild-type cells were analysed for the presence of EPS. Wild-type biofilm samples were collected and tested for the presence of pentoses, hexoses and uronic acids using three separate colorimetric assays. Wild-type biofilms contained 1.8 μg cm^{-2} or less of pentoses, hexoses or

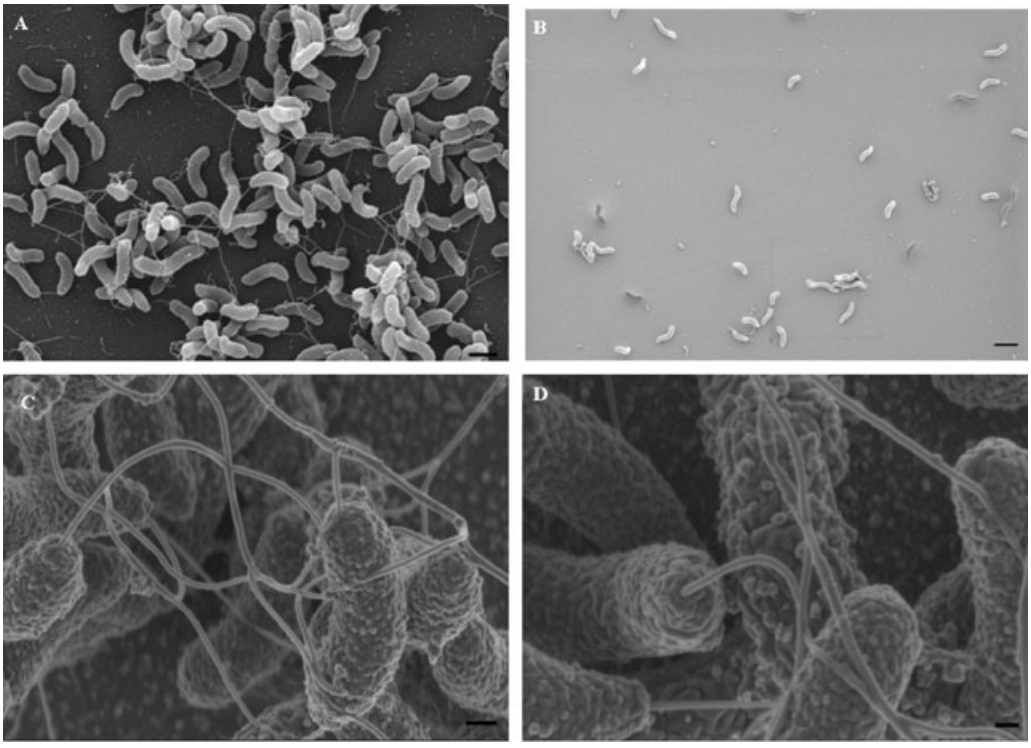

Fig. 2. Scanning electron images of *D. vulgaris* wild-type (A, C and D) and ΔMP (B) cells attached to glass slides. Images (A) and (B) were taken on a Zeiss Supra 35 FEG-VP scanning electron microscope and the magnification bar is 1 μm (A) and 2 μm (B). Images (C) and (D) were taken with a Zeiss Supra 55VP PGT/HKL field emission scanning electron microscope and the magnification bar is 200 nm (C) and 100 nm (D).

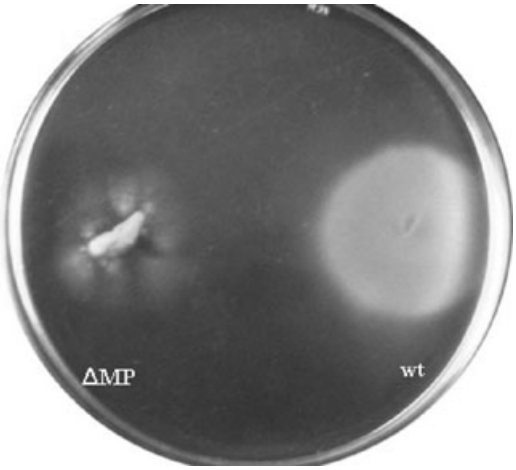

Fig. 3. Motility of *D. vulgaris* wild-type and ΔMP cells in LS4D medium that contained 0.7% agar.

uronic acids per slide (Fig. 5). Planktonic and biofilm cells contained 0.045 and 0.032 μg of hexoses per μg of protein, respectively, and this result indicated that biofilm cells had a lower C : P ratio than planktonic cells under the tested growth conditions. Wild-type biofilms were also stained with calcofluor white, concanavalin A and Congo red to analyse for possible EPS. The calcofluor white stain did not reveal significant amounts of carbohydrate within the wild-type biofilm (Fig. 5). The calcofluor white did seem to highlight crystalline structures that were also observed in ΔMP cultures as well as TEM images, and this result suggested that the fluorescence was most likely a consequence of spent medium constituents. In addition, spent medium was visualized via SEM, and similar crystalline structures were observed. For comparison, the biofilm of *Shewanella oneidensis* was stained in a similar fashion, and the presence of carbohydrate was observed under the tested conditions (data not shown). Concanavalin A and Congo red stains revealed similar results (data not shown), and the data indicated that *D. vulgaris* did not direct significant amounts of carbohydrate external to the cell proper within a biofilm matrix under the tested growth conditions.

Biofilm TEM

Biofilms were grown on silicon oxide grids submerged in LS4D medium. Grids were removed 30 h after inoculation and stained with ammonium molybdate. Transmission electron microscopy and SEM images showed similar cell density to biofilms grown on glass slides (Fig. 6A and C). The matrix contained minimal amounts of EPS material located immediately external to the cell proper (Fig. 6A), and this observation coincided with the measurement of low carbohydrate levels and little fluorescence with lectin fluorophores. The biofilm contained numerous flagella-like filaments that connected cells as well as other filaments. The air-dried grid was also analysed via SEM (Fig. 6C). The image showed a minimal amount of EPS material that surrounded the cell, and the flagella-like filaments were intertwined between and among the cells. For comparison, biofilms grown on silicon oxide grids were also processed similar to biofilms prepared for SEM. Transmission electron microscopy and SEM images were taken of the fixed, dehydrated and critical point-dried grid. Transmission electron microscopy images (not stained with

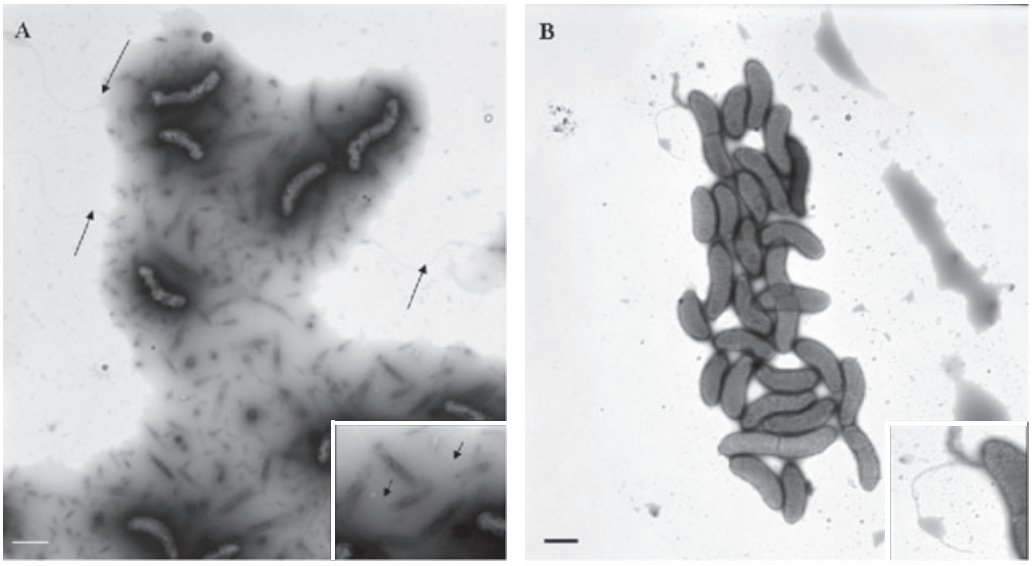

Fig. 4. Transmission electron images of *D. vulgaris* mid-log phase planktonic cells grown in LS4D medium. Wild-type (A) and ΔMP (B) cells were stained with ammonium molybdate. Arrows point to flagella, and the magnification bar = 1 μm. Inset shows close-up of cell ends with and without flagella.

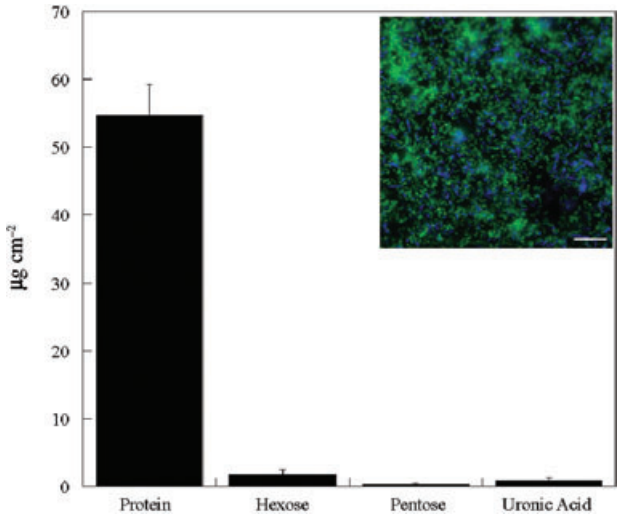

Fig. 5. Analysis of *D. vulgaris* wild-type biofilm carbohydrate content. Samples were analysed for protein, hexose, pentose and uronic acid by colorimetric assays. Analyses were performed in duplicate. Inset: Epifluorescence microscopy of *D. vulgaris* cells attached to glass slides. Wild-type cells were rinsed with anoxic PBS and stained with calcofluor white (blue) to stain exopolysaccharide and acridine orange (green) to stain nucleic acids. Magnification bar = 10 μm.

ammonium molybdate) revealed cells that were still interconnected with flagella, even though the filaments lacked the typical 'sinusoidal wave' appearance (Fig. 6B). Images also revealed that the flagella were interconnected and were strong enough to remain intact between cells even when the silicon oxide film beneath had been broken and displaced (data not shown). Scanning electron microscopy images of the grid were very similar to those taken of biofilms formed on glass slides (Fig. 6D). Similar filaments were observed in 4-day-old biofilms, and this result indicated that the observed filaments were not just needed for biofilm initiation but were essential structures as the biofilm matured (Fig. S3). These results indicated that flagella-like filaments were observed in the biofilms in all electron microscopy observation methods.

Biofilm susceptibility to protease treatment

Protease treatments were used to determine the role of extracellular proteins in biofilm formation. Susceptibility of the biofilm during initial attachment and after formation was tested with trypsin, chymotrypsin and proteinase K. When each protease was added at the time of inoculation biofilm development was inhibited approximately twofold (Fig. 7). Protease addition did not affect the growth rate of planktonic cells and all cultures reached similar optical densities irrespective of the presence of protease at the tested concentrations (data not shown). Proteases not only affected initial attachment of the cells but also disrupted the stability of the biofilm. Glass slides with wild-type biofilms were incubated in buffer that contained the tested proteases. Short incubation periods (15 min) showed minimal biofilm disruption (data not shown); however, biofilm degradation was noted for longer incubations (1 h) compared with the no protease control (Fig. 7). When the protease concentrations were increased to 1.0, 75 and 30 U ml^{-1} for proteinase K, trypsin and chymotrypsin, respectively, biofilm levels were decreased approximately threefold (data not shown).

Wild-type biofilms incubated for 1 h with each protease were also analysed with SEM. The treated biofilms contained far fewer cells when compared with the control (Fig. S4). The filamentous strands also appeared to be degraded in the protease-treated cells compared with the control. It should also be noted that biofilms treated with chymotrypsin resulted in fewer cells adhered to the surface, and this result corresponded to the crystal violet assays. The results supported the idea that the filamentous strands were composed of protein, and that the degradation of the filament network disrupted biofilm integrity.

Discussion

It is becoming increasingly clear that a mode of attached growth more closely resembles *in situ* conditions for many microorganisms in different environments and might likely be a universal feature (Kolter, 2005). However, when compared with the information available for aerobic and/or pathogenic biofilms (i.e. *P. aeruginosa*, *E. coli*, *Streptococcus pyogenes*, etc.), relatively little is known about anaerobic non-pathogenic biofilms, particularly the structure and behaviour (Dunsmore *et al*., 2002). The potential of biofilm communities for bioremediation is becoming increasingly clear because of increased biomass, the capacity for immobilization of compounds of interest (Singh *et al*., 2006), and the possible effects on hydrodynamic flow paths. However, little is known about relevant populations during the physiological state as surface-adhered populations.

The data presented here suggested that *D. vulgaris* does not produce an extensive carbohydrate-based matrix under the tested growth conditions. Different sulfate reducers have been shown to produce varying degrees of EPS (Beech and Cheung, 1995) and have been isolated from biofilm communities within mine drainage systems, activated-sludge basins and oil pipelines (Santegoeds *et al*., 1998; Labrenz and Banfield, 2004; Neria-Gonzalez *et al*., 2006). It is possible and likely that *D. vulgaris* responds differently to various biofilm conditions, for example, the presence of other microbial populations; therefore, extracellular structures may vary. Preliminary data suggest that *D. vulgaris* altered the

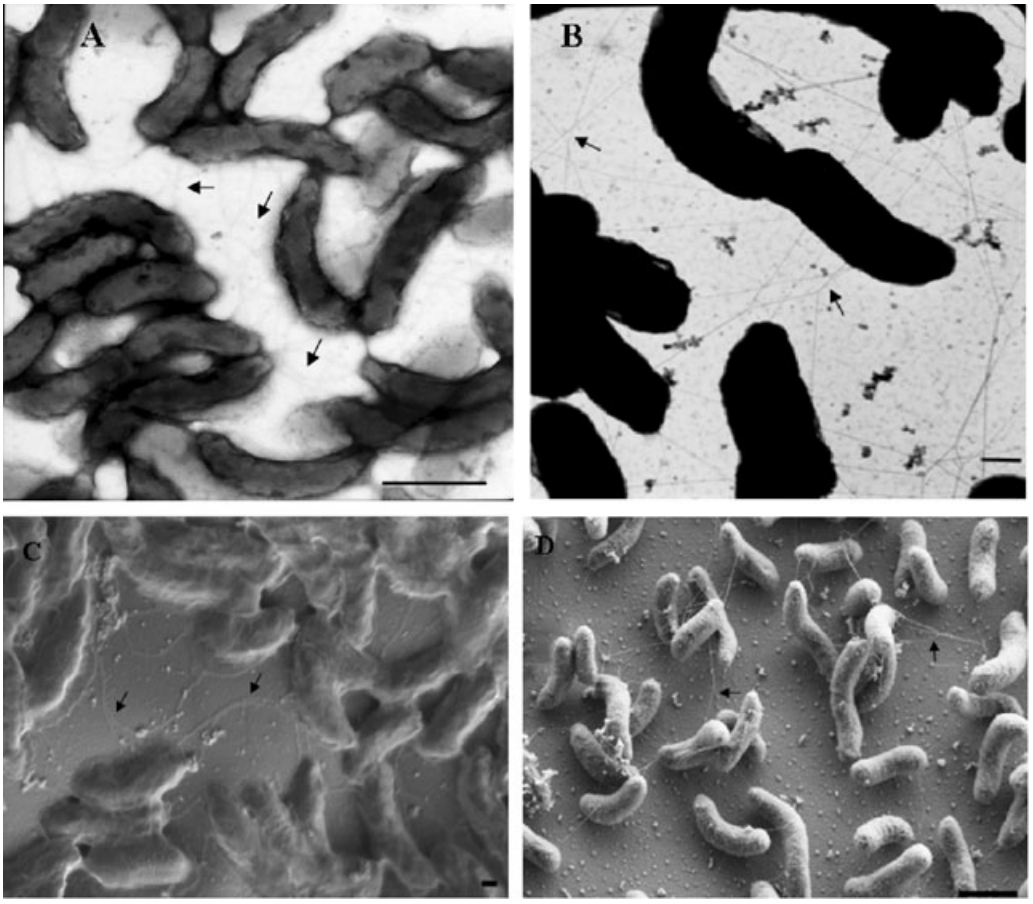

Fig. 6. Transmission and scanning electron images of *D. vulgaris* biofilms grown on silicon oxide TEM grids. Cultures were grown in LS4D medium at 30°C that contained the silicon oxide TEM grids. Transmission electron microscopy (A) and SEM (C) images of a silicon oxide grid stained with ammonium molybdate. Transmission electron microscopy (B) and SEM (D) images of a silicon oxide grid that was fixed with gluteraldehyde and paraformaldehyde, ethanol dehydrated and critically point-dried. Magnification bar = 1 μm (A), 100 nm (B), 200 nm (C), 1 μm (D). The arrows denote observed filaments.

biofilm morphology when grown on different surfaces (M.E. Clark and M.W. Fields, unpubl. results), and this point highlights the plausibility that the biofilm growth state can vary depending upon different factors. The results indicated that *D. vulgaris* utilized internal carbohydrate as planktonic cells transitioned to stationary phase, but the carbohydrate was not simply *trans*-located to the external cell proper but most likely utilized for carbon and energy. The fact that *D. vulgaris* biofilms did not contain significant amounts of carbohydrate might suggest that anaerobic bacteria do not have surplus energy to expend on the production and translocation of extensive amounts of carbohydrate to the external cell proper. Other recent examples of protein-based biofilms exist for some pathogens when interacting with host cells (Giron *et al.* 2002; Wright *et al*., 2005) and the hyperthermophilic, archaeon, *Pyrococcus furiosus* (Nather *et al*., 2006). Further work is needed to determine if the nature of the biofilm matrix can change with respect to energy and carbon sources and stress responses.

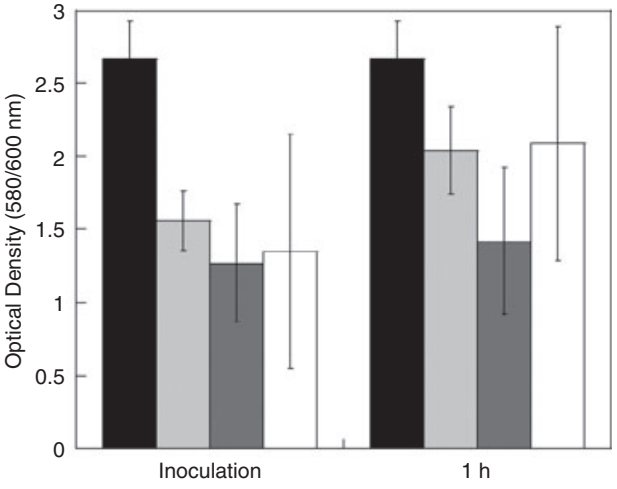

Fig. 7. Protease treatments of *D. vulgaris* wild-type biofilms grown on glass slides. Proteinase K (0.3 U/ml; □), trypsin (25 U/ml; ■), and chymotrypsin (10 U/ml; □) were added individually at time of inoculation or incubated with formed biofilm for 1 h at 30°C and compared to a no protease control (■). The amount of present biofilm was determined via Crystal violet staining.

Another factor for biofilm formation is the ability of the cell to reach the surface of interest. The primary mode of locomotion is flagella (O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Vatanyoopaisarn *et al*., 2000; Harshey, 2003; Kirov *et al*., 2004), but little is known about the role flagella may play after the cell has reached the surface. It has been shown that *P. aeruginosa* may use flagella for the maintenance of biofilm shape after maturation when grown on citrate (Klausen *et al*., 2003), and the architecture of mature *E. coli* biofilms also seems to be affected by the presence or absence of flagella (Wood *et al*., 2006). Previous work has also shown the roles of flagella in virulence of uropathogenic *E. coli* for adherence to host cells (Giron *et al*., 2002; Wright *et al*., 2005). Recent work in the pathogens *Campylobacter jejuni* and *Stenotrophomonas maltophilia* showed a possible role for flagella in biofilm matrices (de Oliveira-Garcia *et al*., 2002; Kalmokoff *et al*., 2006). In the *Archaea* domain, a recent study with *P. furiosus* showed that flagellin-containing filaments were used for cell–cell interactions and surface adhesion (Nather *et al*., 2006). These results allude to the fact that flagella may play alternative roles within the mature biofilm and not just a role of locomotion. In contrast, a *P. aeruginosa* study showed that flagella were not involved in attachment (Klausen *et al*., 2003), and overexpression of flagellin in *E. coli*, *Pseudomonas putida* and *P. aeruginosa* resulted in reduced surface adhesion and biofilm formation (Landini and Zehnder, 2002; Choy *et al*., 2004). These results suggest that the exact role of flagella in biofilm formation and maintenance is not completely understood and highlight the differences between microorganisms for the role of protein filaments in biofilm growth and maintenance.

The ΔMP cells did not utilize intracellular carbon during the transition into stationary phase to the same extent as wild-type cells, and the C : P ratio was two- to threefold higher compared with wild type. Although this result appeared to suggest that external carbohydrate could explain the deficient ΔMP biofilm, the wild-type biofilm did not contain significant carbohydrate but did contain long protein filaments. The ΔMP strain was also deficient in motility and these results suggested a correlation between flagella-based motility and biofilm formation. The megaplasmid does not contain any putative structural genes for flagella, but does contain several putative type III secretion system components that may be involved in secretion. Interestingly, an EAF plasmid that encoded for type III secretion in uropathogenic *E. coli* was required for adherence to epithelial cells (Giron *et al*., 2002). Whether ΔMP was affected in biofilm formation due to an inability to concentrate at the surface or because non-functional flagella hindered biofilm integrity is not known. While it is also possible that the megaplsmid encodes for additional proteins that are needed for surface-adhered growth, the putative type III secretion systems may be important for the translocation of biofilm proteins. The results with the ΔMP strain suggested a link between motility, flagella and biofilm formation, and future work is planned to elucidate the possible individual roles of plasmid encoded genes involved with biofilm formation and maintenance.

Desulfovibrio vulgaris biofilms contained little extracellular carbohydrate as observed via electron microscopy, epifluorescent microscopy and biochemical analyses. The data indicated that *D. vulgaris* relied more on protein filaments than on typical exopolysaccharide matrix, not only for initial attachment, but also for biofilm formation and stability. The data also showed that *Desulfovibrio* biofilms were susceptible to protease treatment, and this result corroborated the notion that the filaments were protein. In addition, TEM demonstrated that the filaments were flagella-like. The whip-like feature may become a more rigid filament after fixation and dehydration for SEM preparation. It is known that critical point and air dehydration will cause shrinkage within a sample even after aldehyde fixation (Crang and Klomparens, 1988). This suggests that flagella, when fixed, will stretch out and appear more rigid after a dehydration process and may account for the differences between the aldehyde-fixed and non-fixed samples.

The protein nature of the filaments was confirmed by protease susceptibility, and protease treatment could potentially serve as a means to control SRB biofilm growth, although the interactions with other microbes in a mixed biofilm may alter the outcome and large-scale application would have to be further tested. Our preliminary transcriptomic analysis of mature *D. vulgaris* biofilms indicate the up-expression of flagellins compared with planktonic cells (unpublished results), but other extracellular proteins are also likely important for biofilm formation in *D. vulgaris*. The presented work demonstrated that *D. vulgaris* biofilms were not dependent upon a carbohydrate matrix, and future work includes the identification of specific extracellular proteins and the elucidation of roles important for biofilm growth and maintenance.

Experimental procedures

Growth

Desulfovibrio vulgaris ATCC 29579 wild-type cells were obtained from Dr Terry Hazen (Lawrence Berkeley National Laboratory). The ATCC 29579 strain contains a 204 kb megaplasmid, and when the cells were continuously subcultured in nitrogen replete medium the cells were cured of the plasmid. Plasmid DNA could not be extracted from the cured cells, and three plasmid loci could not be detected via PCR amplification (data not shown). The megaplasmid-less strain is referred to as ΔMP. *Desulfovibrio vulgaris* cells were grown in defined medium (LS4D) as previously described (Clark *et al*., 2006). Cultures were started with 6.25% inocula from the

mid-log phase of batch grown cells. For carbohydrate analyses, planktonic growth occurred in an open system at 30°C, sparged continuously with nitrogen at an approximate rate of 1 ml min^{-1}, and stirred continuously (Clark *et al*., 2006). For total carbohydrate, protein and lactate determination, four samples (1 ml each) were collected at various time points from each culture (wild type and ΔMP) throughout growth. Each sample was centrifuged at 9300 *g* for 8 min at 4°C, and pellets and supernatants were frozen at −20°C. Analysis of intracellular carbohydrate levels was also performed at each time point. To separate external polysaccharide, a modified form of the centrifugation technique described by Zhang and colleagues (1999) was used. Briefly, cell pellets were collected by centrifugation from 30 ml of cultures at 3500 *g* for 10 min at room temperature. The pellet was then re-suspended in 20 ml of dH_2O and vortexed vigorously for 1 min. External polysaccharide was then removed by centrifugation for 10 min at 33 000 *g* at 4°C. The pellet was re-suspended in 2 ml of dH_2O and transferred to a microcentrifuge tube, and the suspension centrifuged at 9300 *g* for 8 min at 4°C. The supernatant was discarded and the pellets were stored at −20°C.

Biofilm formation and crystal violet staining

Biofilm formation occurred on glass slides (cut in half) submerged in an anaerobic tube that contained 10 ml of LS4D medium. Biofilm formation peaked at approximately 30 h post inoculations (starting OD 0.03). At this time, liquid cultures were removed and the slide was rinsed with an anaerobic solution of 50 mM phosphate-buffered saline (PBS, pH 7.2). The slides were then submerged in 10 ml of 0.1% (w/v) crystal violet for 10 min at room temperature. Slides were rinsed with water and destained with an ethanol/acetone solution (80:20) overnight with shaking. The destain solution was measured at OD_{580}, the OD_{600} was measured for the original planktonic cell suspension, and the ratio of OD_{580} to OD_{600} was calculated to determine relative biofilm formation.

Determination of protein and carbohydrate levels

Planktonic cell pellets were collected as described above and were re-suspended in 1 ml of 0.7% NaCl (w/v). Protein concentrations were determined with the Lowry assay and bovine serum albumin (Pierce Biochemicals) as the standard (Lowry *et al*., 1951). Hexose sugars were measured using the colorimetric cysteine-sulfuric acid method (Chaplin, 1986) with glucose as the standard. Culture supernatant samples were also analysed for hexose sugars. Samples were measured in duplicate and the entire growth experiment was repeated.

For biofilm samples, glass slides with biofilm growth were rinsed once in 50 mM PBS (pH 7.2) approximately 30 h post inoculation. Slides were scraped in order to remove the biomass. Biofilm cell material was re-suspended in 1 ml of 50 mM PBS and used to measure protein and carbohydrate (hexose) levels. Pentose sugars were also measured by the xylose method (Chaplin, 1986), and uronic acid levels were measured via the carbazole method (Chaplin, 1986) with lactone as the standard.

Epifluorescent microscopy

Biofilm samples were also analysed for carbohydrate by fluorescent stains and epifluorescent microscopy with the following procedures. Biofilms were grown on glass slides and rinsed as described above. Biofilms were stained with a calcofluor white solution (10 mg ml^{-1}) to determine the presence of β-1,6-linked polymers, with concanavalin A (5.0 μg ml^{-1}) that targeted non-reducing mannosyl groups, and with Congo red (0.1% w/v) for β-1,4 glucans. The calcofluor white solution was domed on top of the biofilm covered glass slide. The slide was incubated at room temperature in the dark for 15 min. During the last 5 min, approximately 200 μl of an acridine orange solution (10 mg ml^{-1}) was added to the slide. The biofilm was then rinsed three times with 50 mM PBS (pH 7.2) and coverslips were placed over the biofilm for microscopy. Concanavalin A staining was performed following a protocol outlined by Magnuson and colleagues (2004). Briefly, biofilm slides were domed with the concanavalin A solution along with DAPI (2.0 μg ml^{-1}) and incubated at room temperature for 1 h. Slides were rinsed twice with 50 mM PBS (pH 7.2) and viewed. For staining with Congo red, the biofilm-covered glass slide was covered with the solution and incubated at room temperature for 40 min. Slides were then washed twice with 50 mM PBS (pH 7.2) and observed. Biofilms were viewed with an Olympus AX-70 Multimode Microscopy System and appropriate filters.

Ion chromatography

Supernatants from planktonic samples were analysed for lactate, acetate and sulfate concentrations throughout growth via ion chromatography (Metrohm-Peak) as previously described (Clark *et al*., 2006). Lactate and acetate levels were monitored with a Metrosep organic acid column and sulfate levels were measured with a Metrosep Anion Supp 5 column. All measurements were performed in duplicate.

Scanning electron microscopy

Biofilm samples were grown on glass slides for approximately 30 h. Slides were rinsed once with 50 mM PBS (pH 7.2) and then placed in a fixative that contained 2.5% glutaraldehyde (w/v), 2.0% paraformaldehyde (w/v) and 0.05 mM sodium cocadylate buffer (pH 7.0). The biofilms were fixed overnight and were then washed four times (20 min each) with ddH_2O. Slides were dehydrated by incubation in increasing concentrations of ethanol and then dried at the critical point with a CO_2 critical point drier (Tousimis). Samples were sputter coated with gold (20 nm) and viewed on Zeiss Supra 35 FEG-VP or Supra 55VP PGT/HKL field emission scanning electron microscopes.

Transmission electron microscopy

Thirty-hour planktonic samples were collected for analysis of flagella via TEM. Samples (1 μl) were dropped onto collodion-coated nickel TEM grids. Samples were incubated with ammonium molybdate (1.5% w/v) for 15 min at room temperature. Specimens were then viewed with a JEOL 100S

TEM. Biofilm samples were also analysed with TEM. Biofilms were grown directly on silicon oxide thin-film (50 nm) TEM grids (Structure Probe) that were submerged in LS4D medium. Biofilms were allowed to form for approximately 30 h. The cell culture was carefully removed from the tube and the grid was gently rinsed with 50 mM PBS (pH 7.2). Grids were incubated with ammonium molybdate and air dried. Biofilms were then viewed with both TEM and SEM without any further preparation. To ensure that similar biofilm structure had formed on these grids, a separate sample was prepared following the SEM fixation, dehydration and critical point drying procedures as described above. The sample was not gold coated so that TEM was still possible and was also observed via low voltage (1 Kev) SEM.

Motility

Desulfovibrio vulgaris wild-type and ΔMP mutant cells were analysed for motility on LS4D medium solidified with 0.7% or 1.0% (w/v) agar. Plates were stab inoculated from broth cultures and were incubated at 30°C in an anaerobic glove bag. Plates were visually inspected 48 h after inoculation for differences in motility halos between the wild-type and mutant strain.

Protease treatments

Biofilm samples were harvested 30 h post inoculation to analyse for protease susceptibility. Samples were rinsed with 60 mM PIPES (10 ml, pH 7.0) and placed in enough 50 mM PBS (pH 7.2) to cover the glass slide. The PBS contained 10 μg ml^{-1} or 30 μg ml^{-1} proteinase K, trypsin, chymotrypsin or no protease (control), and the biofilm samples were incubated at 30°C for 15 min or 1 h. After incubation, slides were inserted directly into a crystal violet solution for biofilm quantification or fixed for SEM preparation as described above. Proteases were added to the LS4D medium to test for inhibition of biofilm growth. Proteases (10 μg ml^{-1}) were added at the time of inoculation. Growth was monitored for 30 h to ensure cells lysis did not occur, and then slides were stained with crystal violet or prepped for SEM as described above.

Acknowledgements

The research was supported by the United States Department of Energy, Office of Science under the Environmental Remediation Science Program (DOE-ER64125). The authors thank the laboratory of Dr L.A. Actis for helpful discussions.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Scanning electron microscopy (SEM) images of *D. vulgaris* planktonic (A) and biofilm (B) cells. Planktonic cells were permitted to settle upon a poly L-lysine-coated coverslip and aldehyde fixed. Biofilms cells were grown in batch mode for 30 h, rinsed with 50 mM PBS pH 7.2 and aldehyde fixed. Both samples were ethanol dehydrated and critical point dried. Magnification bar = 1 μm.

Fig. S2. Transmission electron microscopy (TEM) image of *D. vulgaris* biofilm cells grown upon a silicon oxide TEM grid. Cells were stained with ammonium molybdate and observed. Magnification bar = 100 nm.

Fig. S3. The images show 4-day-old *D. vulgaris* biofilm cells grown in continuous culture mode at 10 000 (A) and 3000 (B)

magnification. Samples were rinsed with 50 mM PBS pH 7.2, aldehyde fixed, ethanol dehydrated and critical point dried. Magnification bar = 1 μm.

Fig. S4. Scanning electron images of *D. vulgaris* wild-type biofilms after protease treatment. Biofilm samples were rinsed once with anoxic PBS and then treated with 10 μg ml^{-1} protease for 1 h. Images are as follows: (A) control, (B) proteinase K, (C) chymotrypsin and (D) trypsin, and magnification bar equals 1 μm.

This material is available as part of the online article from http://www.blackwell-synergy.com

