

a National Science Foundation Engineering Research Center in the MSU College of Engineering

## Abstract

One of our main objectives is the identification of key genes necessary for biofilm formation and maintenance, and these data will provide insight into cellular responses to heavy metals when cells are grown as surface-adhered populations. *Desulfovibrio* spp. are model SO<sub>4</sub>-reducing bacteria (SRB), and recent work has observed *Desulfovibrio* spp. at field sites contaminated with chromium and uranium. SRB biofilms have been shown to reduce heavy metals, but little is known about the cellular events that lead up to biofilm formation and development in SRB, including the cellular material and gene products used to promote and maintain cell adherence under sulfate-reducing conditions. Biofilm formation was observed on glass slides submerged in a CDC reactor that contained a defined medium and a dilution rate of approximately 0.09 h<sup>-1</sup>. Significant levels of carbohydrates were not detected in biofilms grown in continuous mode, and less than 2 µg/cm<sup>2</sup> of carbohydrate was detected at any stage of biofilm growth tested. Biofilms cultivated in batch and continuous culture appeared similar, and both biofilms contained long filaments interconnected between the cells and the surface. The images revealed a monolayer of cells, and the biofilm maintained a constant cell number throughout cultivation. The filaments remained throughout the cultivation of the biofilms, and our recent results suggested that the extracellular filaments were flagella. Transcriptomic analyses of the biofilm cells revealed that most up-expressed genes could be classified in the COG categories of energy production and conversion, followed by signal transduction mechanisms, cell motility, secretion, and hypothetical proteins. With respect to flagella, *D. vulgaris* has six putative flagellin proteins, but only one novel putative flagellin was up-expressed in biofilm cells compared to planktonic cells. *D. vulgaris* ATCC 29579 (wild-type) and three mutants, *ΔflaG*, *ΔflaA*, and *ΔMP* were grown in batch mode in a defined medium with lactate and sulfate and biofilms were allowed to form on glass slides. Initial results indicated that *ΔflaG* mutants were motile, while the *ΔMP* and *ΔflaA* mutants were deficient in motility. The filaments, possibly a form of modified flagella, were present within wild-type biofilms but fewer were seen in *ΔflaG*, and were almost completely lacking in the *ΔflaA* and *ΔMP* mutants. Crystal violet staining revealed that *ΔflaG*, *ΔflaA*, and *ΔMP* mutants produced 5-fold, 2-fold, and 3-fold less biofilm compared to the wild-type, respectively. In addition, transcriptomic analysis indicated that *flaG* was the only novel flagellin that was up-expressed in biofilm cells compared to planktonic cells. The data indicated that *D. vulgaris* Hildenborough biofilms had unique gene expression patterns compared to both exponential and stationary-phase cells, that biofilms maintained simple, monolayers, and that a significant carbohydrate matrix was not required for biofilm formation or maintenance. In addition to initial attachment, unique flagella appear to be involved in biofilm maturation and stability.

## INTRODUCTION

Biofilms are characteristically thought to be a microbial population embedded within an exopolysaccharide (EPS) matrix adhered to a surface. This concept is derived from well studied pathogenic organisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. Studies on the structure and function of biofilms have been limited and little research has been done on environmental isolates. Recent work has shown that surfaces are colonized when placed down-well at contaminated sites (Reardon et al., 2004), and these results indicated that *in situ* microorganisms have the ability to adhere to surfaces. Regardless of the terms used, subsurface sediments contain bacterial populations, and these populations are associated with the porous medium. In fact, multiple studies have shown that redox transformations of uranium and other metals are controlled by kinetic factors that are strongly influenced by microbial activity, and it is the microbes that are associated with the subsurface matrix that pose challenges in manipulating and/or predicting the mobility and fate of heavy metals in complex and heterogeneous environmental settings (Suzuki and Suko, 2006). Whether microbial growth associated with the subsurface sediments is desired (bioremediation) or deleterious (aquifer clogging), the surface-associated populations represent a portion of the system that we know the least about. Here we begin to reveal some of the structural features of the biofilm produced by the model SRB, *Desulfovibrio vulgaris*, under metal reducing conditions. Our results show that *D. vulgaris* produces a biofilm that is dependent upon protein and not an EPS. Data indicated that the protein filaments are flagella and these flagella appear to be important not only in biofilm initiation, but also in maturation.

## METHODS

**Growth:** *D. vulgaris* ATCC 29579 was grown on LS4D which contains 60mM lactate as the carbon and energy source and 50mM sulfate as the electron acceptor. Batch growth occurred in Hungate tubes filled with 10mL of LS4D. Biofilms were grown on glass slides or silicon oxide TEM grids that were submerged in the medium before sterilization. Continuous culture growth occurred in a CDC reactor containing 400 mL of LS4D that was continuously sparged with nitrogen. Cultures were started with 2.5% inocula. Cultures were allowed to grow for 24h. At 24h, LS4D was pumped into the system at a flow rate of 0.48l/min. Sterile glass slides were submerged into the culture at this time to allow biofilm to form. Samples were taken approximately 12h apart over the time course for carbohydrate measurements and SEM. Samples were taken at 70h for transcriptomic analysis.

**Carbohydrate and Protein Measurements:** Colorimetric assay was used to measure levels of protein and carbohydrate in planktonic and biofilm samples. The Lowry assay was used to determine protein levels with BSA as the standard. Hexoses, pentoses, and uronic acid was measured by the cysteine-sulfuric acid assay, the xylose assay, and the carboxase method, respectively.

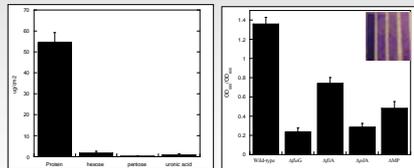
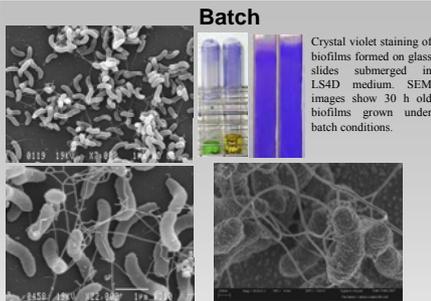
**Biofilm Visualization:** Biofilms were quantified using 0.1% crystal violet solution and destained with an ethanol acetone (80:20) solution. The OD<sub>600</sub> was determined from this solution and the ratio of OD<sub>600</sub>/OD<sub>600</sub> was calculated.

**Direct Count:** Biofilm samples were removed over time, rinsed IX with 50mM PBS pH 7.2, and scrapped into a 2% w/vol formaldehyde solution. Samples were placed in a filter tower and incubated with acetone (1) for 4 min. Samples were then vacuumed onto a black Nuclepore polycarbonate filter (0.2µm) and rinsed once with 3 ml of ddH<sub>2</sub>O. Filter was transferred to a microscope slide and viewed with an Olympus AX-70 Multimode Microscopy System.

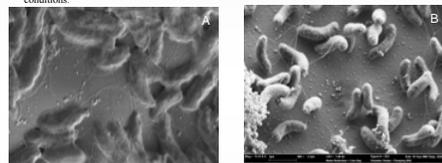
**Transcriptomic analysis:** Three biofilm samples (3 slides each) were scrapped into 1ml ice cold PBS pH 7.2, pelleted via centrifugation and snap frozen with liquid nitrogen. Three Planktonic samples (60ml each) were collected, chilled to 20°C, pelleted via centrifugation, and snap frozen with liquid nitrogen. These samples were used for transcriptomic analysis using whole genome chips for *Desulfovibrio vulgaris* (done by our collaborators at the University of Oklahoma). Results are based against planktonic expression.

**Scanning electron microscopy (SEM):** SEM was done on biofilms from batch and continuous culture growth. Glass slides with adhered biofilms were rinsed IX with 50mM PBS pH 7.2 and then aldehyde fixed (2.5% w/vol glutaraldehyde, 2.0% w/vol paraformaldehyde, 0.05M sodium cacodylate buffer pH 7.0 overnight). Samples were fixed 4X with ddH<sub>2</sub>O and ethanol dehydrated. Sample were then dried at the critical point with CO<sub>2</sub> and sputter coated with gold (20nm). Silicon oxide TEM grids containing biofilm growth were also analyzed. One grid was prepared in described above. A separate grid with biofilm growth was rinsed IX with 50mM PBS pH 7.2 and stained with ammonium molybdate (1.5% w/vol) and viewed. Samples were viewed on a Zeiss Supra 35 FEI-VP SEM.

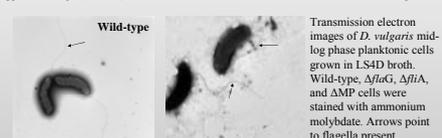
**Transmission electron microscopy (TEM):** TEM was done on planktonic cells and on biofilms grown upon silicon oxide TEM grids. Planktonic cells (1 µl) was dropped on collodion coated nickel TEM grids and allowed to settle. Grids were then rinsed IX with 1 µl H<sub>2</sub>O and stained with either ammonium molybdate (1.5% w/vol) or uranyl acetate (2% w/vol). Silicon oxide TEM grids with biofilm formation were process as described above. Samples were viewed on a JEOL 100S TEM.



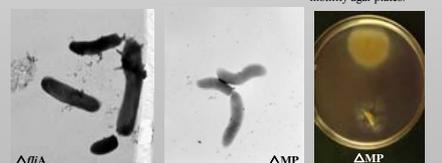
*D. vulgaris* biofilms were analyzed for the presence of various sugars. Little carbohydrate is measured or observed within wild-type biofilms. Biofilms were 30 h old under batch conditions.



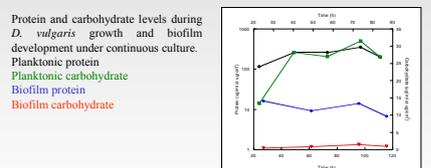
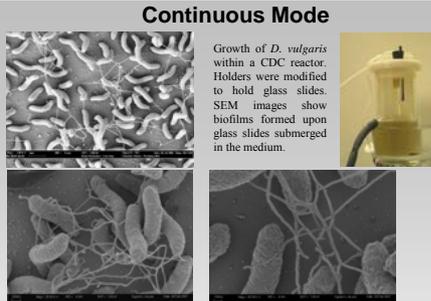
SEM and TEM images of *D. vulgaris* biofilms grown directly on silicon oxide TEM grids. Biofilms shown in A and C are stained with ammonium molybdate while B and D are biofilms that were aldehyde fixed and dehydrated with ethanol and critical point drying. Images A and C reveal the characteristic sinusoidal wave like structure of flagella. These filaments, when fixed and dehydrated, appear more rigid (B and D) and remain in tact even when the film beneath is broken and displaced.



Motility of wild-type and *ΔMP D. vulgaris* via motility agar plates.



## RESULTS

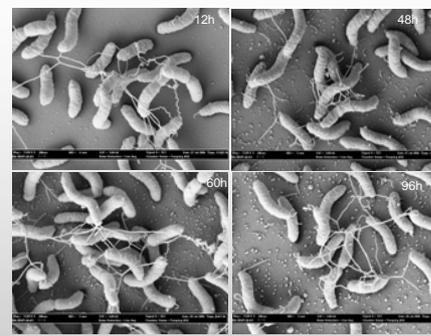


Protein and carbohydrate levels during *D. vulgaris* growth and biofilm development under continuous culture. Planktonic protein, Planktonic carbohydrate, Biofilm protein, Biofilm carbohydrate

**Protein and carbohydrate levels of cells grown under continuous culture conditions**

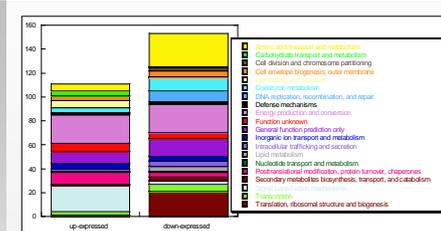
Time (h)	Protein (µg/ml) (Planktonic)	Carbohydrate (µg/ml) (Planktonic)	Time (h)	Protein (µg/cm <sup>2</sup> ) (Biofilm)	Carbohydrate (µg/cm <sup>2</sup> ) (Biofilm)
24	115.6	13.4	25	16.16	0.474
49	258.5	28.2	49	9.27	0.884
73	258.5	27	73	14.09	1.51
97	350.9	31.5	87	6.81	0.978
111	201.8	26.85			

SEM images of *D. vulgaris* biofilm grown in continuous culture mode. Samples were collected, fixed, and CPD every 12 h to monitor biofilm formation. Images represent part of the time course and show that *D. vulgaris* does maintain filamentous structures in later time points during chemostat growth.

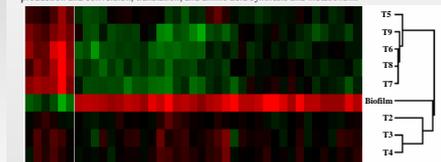


VIMSS	Gene ID	Name	Description	Expression	Z-score
206885	DVU1441	flaB1	Flagellin	0.55	0.93
206886	DVU1442	NA	flagellin FlaG, putative	1.34	2.36
207556	DVU2082	flaD	flagellin putative	0.36	0.58
207937	DVU2444	flaB3	flagellin	0.23	0.36
208458	ORF03856	NA	bacterial flagellin N-terminal domain protein	NA	NA
209458	DVU0520	NA	flagellar hook-associated protein	-0.02	-0.03

Expression of flagellin annotated genes within biofilm cells. Data indicated that one unique flagellin gene, *flaG*, is up-expressed.



Transcriptomic analysis of *D. vulgaris* biofilm cells compared to the corresponding planktonic cells. Colors represent different COG categories. Major up-expressed categories include energy production and conversion and signal transduction mechanisms. Major down-expressed categories include energy production and conversion, translation, and amino acid synthesis and metabolism.



Cluster analysis of transcriptomic expression patterns between biofilm cells and planktonic cells throughout growth when genes with expression ratios above or below 1.75 were considered. Based upon distances, the biofilm cells were just as different from stationary-phase cells as from exponential-phase cells. A representative subset is shown that displays the uniqueness of the biofilm expression patterns.

## Different electron transfer systems in biofilm cells

Gene	Name	Expression
DVU13025	por	-3.09
DVU13027	glcD	-2.06
DVU13030	ackA	-2.62
DVU12386	cooM	-4.30
DVU12388	cooL	-3.68
DVU12389	cooX	-3.00
DVU12390	cooY	-3.12
DVU12391	cooI	-3.13
DVU12391	hnpA	-1.97
DVU12393	cooF	-1.98
DVU1944	oorD	2.16
DVU1945	oorA	1.96
DVU1946	oorB	1.58
DVU1292	nrfC	1.97
DVU1293	nrfD	2.61
DVU1294	nrfE	2.20
DVU1295	nrfE	1.71
DVU1296	nrfA	2.51
DVU1297	nrfB	1.42
DVU1298	apbE	2.94

The putative *por* genes encode a large (1215 aa), multi-domain protein whereas the *oor* genes encode for putative subunits of a multi-meric protein; however, both are predicted to catalyze the TPP-mediated decarboxylation of pyruvate to acetyl-CoA. The altered expression may result from different regulatory features of the different PFOs under different conditions of carbon flow.

The *coo* genes encode for putative subunits of a NADH dehydrogenase: oxidoreductase system that function as a complex I during electron transport from NADH to the quinone pool. The *nrf* genes encode for a putative NADH oxidoreductase complex, and members of this protein family are known to transfer electrons to nitroreductase in *R. capsulatus* or redox sensors in *E. coli*. The respective up- and down expression of these electron transfer systems indicated that electron flow is altered in biofilm cells compared to both exponential- and stationary-phase cells.

Tree based upon predicted peptide sequences of putative *D. vulgaris* flagellin genes and closest orthologs in other sequenced genomes. Results show *flaB1*, *flaD*, *flaG*, and *flaB3* are closely related to genes in *D. desulfuricans* G20. *flaG* is unique to DVH1, G20, and *N. europaea*.

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