FINAL REPORT

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Identification of Molecular and Cellular Responses of Desulfovibrio vulgaris Biofilms under Culture Conditions Relevant to Field Conditions for Bioreduction of Toxic Metals and Radionuclides
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Progress under funding period. During growth of *D. vulgaris* Hildenborough with lactate and sulfate, internal carbohydrate levels increased throughout exponential-phase, and peaked as the cells transitioned to stationary-phase. The carbohydrate to protein ratio (C:P) peaked at 0.05 ug/ug as the cells transitioned to stationary-phase, and then declined to 0.02 ug/ug during extended stationary-phase. In contrast, a strain of *D. vulgaris* that does not contain the 254 kb plasmid, referred to as the megaplasmid (MP), maintained higher internal carbohydrate levels and the C:P ratio peaked at 0.1 ug/ug (2-fold increase compared to wild-type). Under the tested growth conditions, we observed biofilm formation in wild-type cells, but the plasmid-less strain formed less biofilm (3-fold decrease). We hypothesized that carbohydrate was re-allocated outside the cell proper for biofilm formation. However, the biofilm contained relatively little carbohydrate (0.6 to 1.0 ug/ml) and had a C:P ratio similar to the wild-type cells in stationary-phase. Staining with calcafluor white also indicated the presence of little external carbohydrate in *D. vulgaris* biofilms. Less biofilm was formed in the presence of protinease K, trypsin, and chymotrypsin, however, the growth of planktonic cells was not affected. In addition, when *D. vulgaris* biofilm was treated with a protease, less biofilm was observed. Electron micrographs suggested the presence of filaments between the biofilm cells, and filaments appeared to be susceptible to protease treatment. Biofilm filtrates contained soluble protein, and SDS-PAGE analysis suggested different polypeptide profiles between a cell-free biofilm filtrate, whole-cell extract of planktonic cells, and a biofilm whole-cell sample.

Omic Characterization. Biofilm formation was observed on glass slides submerged in reactor that contained a defined medium and a dilution rate of approximately 0.09 h^{-1}. Biofilms cultivated in batch contained little carbohydrate (hexose, pentose, or uronic acids), nor did the biofilm contain significant amounts of extracellular DNA. Significant levels of carbohydrates (hexose, pentose, uronic acids) were not detected in biofilms grown in continuous mode, and less than 2 ug/cm^2 of carbohydrate was detected at any stage of biofilm growth. In addition, the carbohydrate to protein ratios were lower for biofilm cells compared to cells in different growth modes. 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 (including TEM) suggested that the extracellular filaments were flagella.

Transcriptomic analyses of the biofilm cells compared to planktonic 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. Transcript and protein expression profiles were unique, but sets of genes had similar trends in transcript and protein levels, namely genes associated with energy conservation. Genes annotated to encode Ech hydrogenase, formate dehydrogenase and pyruvate ferredoxin oxidoreductase had increased transcripts and the predicted proteins were also increased in the biofilm samples. Several other hydrogenases and formate

dehydrogenases also showed an increased protein level for biofilm cells and proteins for each step in the reduction of sulfate to sulfide were increased. Decreased transcript and protein levels were observed within the biofilm for putative Coo hydrogenases as well as a lactate permease and Hyp hydrogenases. Genes annotated for amino acid synthesis enzymes and nitrogen utilization proteins were also among the most dominant changers within the biofilm state. There was an increase in putative proteins for the synthesis of amino acids arginine, cysteine, methionine, and histidine. Alternatively, enzymes predicted to synthesize aromatic amino acids showed decreased protein levels in the biofilm samples. Both transcript and protein levels for ribosomal proteins were notably decreased within the biofilm cells. The results indicated that biofilm cells may have an alternate flux of carbon and energy which may influence metal-reducing and metal-interacting capacity. In addition, fluxes within amino acid production suggested nutrient limitations within biofilm cells.

With respect to flagella, *D. vulgaris* has six putative flagellin proteins, but only one gene encoding a novel putative flagellin was up-expressed in biofilm cells compared to planktonic cells. Biofilms were also analyzed for the presence of proteins within the biofilm matrix, and a 1-D gel revealed several polypeptides that could be enriched during fractionation and filtration. 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. These results contradict some studies with aerobic bacteria that suggest biofilms cells are in a physiological state similar to stationary-phase. In addition to initial attachment, flagella could be involved in biofilm maturation.

D. vulgaris ATCC 29579 (wild-type) and three mutants, Δ*fla*G, Δ*fli*A, and ΔMP (lacking the 200kb plasmid) were grown in batch mode in a defined medium with lactate and sulfate and biofilms were allowed to form on glass slides. Wild-type cells were motile and formed a continuous mono-layer of cells on the glass as observed via crystal violet staining and SEM. Initial results indicated that Δ*fla*G mutants were motile, while the ΔMP and Δ*fli*A mutants were less motile or not motile. Significant amounts of carbohydrate were not measured within wild-type biofilms (0.01 ug hexose sugar per ug protein), and biofilms stained with calcofluor white, Concanavalin A, and congo red revealed little external carbohydrate (e.g., EPS) within the wild-type *D. vulgaris* biofilm. TEM analysis of wild-type biofilms grown on SiO_2 grids also showed little EPS, but the presence of 'filaments' were observed in both TEM and SEM images. The filaments, possibly a form of modified flagella, were present within wild-type biofilms but fewer were seen in Δ*fla*G, and were almost completely lacking in the Δ*fli*A and ΔMP mutants. Crystal violet staining revealed that Δ*fla*G, Δ*fli*A, and ΔMP mutants produced 5-fold, 2-fold, and 3-fold less biofilm compared to the wild-type, respectively. As observed with wild-type biofilms, negligible amounts of carbohydrate were detected within the mutant biofilms. Filtrate samples of the wild-type biofilms were also analyzed and a 1D protein gel indicated that the biofilm matrix was enriched for 5 to 7 polypeptides. These results indicated that *D. vulgaris* flagella play an important role in not only initial formation of *D. vulgaris* biofilm but also in biofilm stability. The data also indicated that a novel flagellin played a crucial role in the ability of *D. vulgaris* to form and maintain biofilms.

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 and have been isolated from biofilm communities within mine drainage systems, activated-sludge basins, and oil pipelines. 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 biofilm morphology when grown on different surfaces (unpublished results, Clark and Fields), and this point highlights the plausibility that the biofilm growth state can vary depending on 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. 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.

The results indicated that *D. vulgaris* changes carbohydrate distributions in response to growth phase, biofilm formation can respond to changes in physiological conditions for the cells, the megaplasmid contains genes important for carbohydrate distribution and biofilm formation, and *D. vulgaris* biofilms contain extracellular, polypeptides that are important for biofilm formation. In addition, in contrast to typically studied biofilm systems that use carbohydrate-based attachment, the data suggested that *D. vulgaris* uses a different mechanism(s), possibly specialized filaments unique to biofilm cells. An understanding of cell attachment and biofilm formation will provide great insight into how SRBs may interact and persist with heavy metals under relevant growth conditions (i.e., biofouling and bioremediation).

Chromate Responses. In order to study heavy metal resistance [e.g., Cr(VI)] of *D. vulgaris* biofilms, we initiated studies to characterize Cr(VI) sensitivity in planktonic cells first so that a base-line could be determined for cellular susceptibility. Cultures grown in a defined medium had a lag period of approximately 30 h when exposed to 0.05 mM Cr(VI). Substrate analyses revealed that although Cr(VI) was reduced within the first 5 h, growth was not observed for an additional 20 h. The growth lag could be explained by a decline in cell viability; however, during this time small amounts of lactate were still utilized without sulfate reduction or acetate formation. Approximately 40 h after Cr exposure (0.05 mM), sulfate reduction occurred concurrently with the accumulation of acetate. Similar amounts of hydrogen were produced by Cr-exposed cells compared to control cells, and lactate was not converted to glycogen during non-growth conditions. *D. vulgaris* cells treated with a reducing agent and then exposed to Cr(VI) still experienced a growth lag, but the addition of ascorbate at the time of Cr(VI) addition prevented the lag period. In addition, cells grown on pyruvate displayed more tolerance to Cr(VI) compared to lactate grown cells. These results indicated that *D. vulgaris* utilized lactate during Cr(VI) exposure without the reduction of sulfate or production of acetate, and that ascorbate and pyruvate could protect *D. vulgaris* cells from Cr(VI)/Cr(III) toxicity.

In conjunction with the VIMSS group (Genomes:GTL), we have also complemented on-going work by the groups dealing with Cr(VI) exposure. Whole-genome expression profiles indicated that the following groups of genes were up-expressed in response to Cr(VI) exposure: those encoding reductases and transporters. Multiple heavy metal transporters were up-expressed and included putative permeases, drug efflux, and metal ATPases. These results suggest the importance of Cr(III) efflux after Cr(VI) has been reduced. Based upon the results a FMN-dependent nitroreductase might reduce Cr(VI) directly or reduce a Cr-complex. The FMN reductase could synthesize $FMNH_2$ and the NADP dehydrogenase might be used to regenerate $NADPH_2$. The nitroreductase, FMN reductase, and NADP dehydrogenase were all up-expressed based upon the microarray data. The *chr*AB genes on the megaplasmid most likely play a key role in Cr(III) efflux based upon microarray data and growth data. Additional toxicological effects could be occurring once the Cr(III) is produced via protein denaturation in the cytoplasm, periplasm, and outer cell proper. In the future, Cr(VI) exposure of *D. vulgaris* biofilms will be characterized and compared to

planktonic cells, and preliminary data indicate that biofilms maintain a greater degree of viability compared to planktonic cells.

Presentations funded by project

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Clark, M.E. et al. 2008. Transcriptomic and Proteomic Analysis of *Desulfovibrio vulgaris* ATCC 29579 Biofilms Under Conditions Conducive to Metal Reduction. 108th General Meeting of the American Society for Microbiology, Boston, MA

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