σ_{54}-Dependent regulator DVU2956 switches *Desulfovibrio vulgaris* from biofilm formation to planktonic growth and regulates hydrogen sulfide production

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Summary

Microbiologically influenced corrosion causes $100 billion in damage per year, and biofilms formed by sulfate-reducing bacteria (SRB) are the major culprit. However, little is known about the regulation of SRB biofilm formation. Using *Desulfovibrio vulgaris* as a model SRB organism, we compared the transcriptomes of biofilm and planktonic cells and identified that the gene for σ_{54}-dependent regulator DVU2956 is repressed in biofilms. Utilizing a novel promoter that is primarily transcribed in biofilms (P_{dvu0304}), we found production of DVU2956 inhibits biofilm formation by 70%. Corroborating this result, deleting dvu2956 increased biofilm formation, and this biofilm phenotype could be complemented. By producing proteins in biofilms from genes controlled by DVU2956 (dvu2960 and dvu2962), biofilm formation was inhibited almost completely. A second round of RNA-seq for the production of DVU2956 revealed DVU2956 influences electron transport via an Hmc complex (high-molecular-weight cytochrome c encoded by dvu0531–dvu0536) and the Fe-only hydrogenase (encoded by dvu1769, hydA and dvu1770, hydB) to control H_{2}S production. Corroborating these results, producing DVU2956 in biofilms decreased H_{2}S production by half, deleting dvu2956 increased H_{2}S production by 131 ± 5%, and producing DVU2956 in the dvu2956 strain reduced H_{2}S production. Therefore, DVU2956 maintains SRB in the planktonic state and reduces H_{2}S formation.

Introduction

Sulfate-reducing bacteria (SRB) are the major cause of biocorrosion, and biocorrosion from all sources results in $100 billion in damage per year (Beech and Sunner, 2007; Koch et al., 2016). Hence, controlling SRB biofilm formation; i.e. preventing its formation and promoting its dispersal, is important.

The SRB biofilm extracellular matrix consists of protein (Clark et al., 2007) and polymers of mannose, N-acetylgalactosamine (GalNAc) and fucose (Poosarla et al., 2017). Hence, SRB biofilms may be dispersed by protease (Clark et al., 2007) and by its own glycoside hydrolase (Zhu et al., 2018).

In regard to the regulation of SRB biofilm formation, gene expression in *D. vulgaris* after 20 days of biofilm formation on a steel surface was studied using microarrays, and some chromosomal distributed EPS biosynthesis genes (not including those for mannos, fuose and GalNAc) were found to be induced (Zhang et al., 2007). In addition, gene and protein expression in 70-h SRB biofilms were examined by microarrays and iTRAQ, and some unknown extracellular proteins were identified as important for biofilm formation (Clark et al., 2012). Another report focused on eight genes differentially expressed in biofilm cells (84- and 144-h) and planktonic cells (18- and 36-h) at the single cell level; they found EPS biosynthesis gene dvu0281 as well as dvu1340 and dvu1397 involved in ferric iron uptake and storage were induced while another other five genes, including those involved in energy metabolism (dvu0434 and dvu0588), stress response (dvu2410), a response regulator (dvu3062), and iron transportation (dvu2571), were repressed in biofilms. Therefore, little has been discerned about early SRB biofilm formation.

For gene regulation in SRB, two component systems and their target binding sites have been studied by both computational and experimental techniques. The SRB
model bacterium *D. vulgaris* Hildenborough has at least 91 response regulators (Jenal and Galperin, 2009; Galperin, 2010; Kazakov et al., 2015), which are involved in metabolism, cell motility, biofilm formation, and the stress response (Rajeev et al., 2011). Different from other bacteria, in which $\sigma_{54}$-dependent regulators are less than 10% of the total response regulators (RRs) (Galperin, 2010); *D. vulgaris* Hildenborough has 37 $\sigma_{54}$-dependent regulators out of a total 91 RRs (41%; Kazakov et al., 2015). $\sigma_{54}$-dependent regulators (also called enhancer-binding proteins) specifically bind a conserved upstream activating sequence (UAS) located upstream from the $\sigma_{54}$-dependent promoter and interact with the $\sigma_{54}$-polymerase complex and the DNA looping between the UAS and promoter to turn on the regulated genes (Bush and Dixon, 2012). In *D. vulgaris*, the 37 regulons that are controlled by $\sigma_{54}$-dependent regulators include those for nitrogen, carbon and energy metabolism, transmembrane transport and various extracellular functions (Kazakov et al., 2015). In *Escherichia coli*, the products of $\sigma_{54}$-dependent operons are involved in nitrogen metabolism, formate, propionate and acetolactate metabolism, zinc tolerance, phage shock response and other functions (Reitzer and Schneider, 2001).

Given the importance of biofilms, it is likely some of these $\sigma_{54}$-dependent regulators are involved in biofilm formation.

In this work, RNA-seq was used to identify important proteins involved in *D. vulgaris* biofilm formation. Specifically, we identified that the $\sigma_{54}$-dependent regulator DVU2956 is repressed in biofilms. By utilizing a promoter that is mainly transcribed in biofilms (from gene dvu0304), we found that biofilm-phase production of DVU2956 significantly inhibited biofilm formation. DVU2956 binds upstream of its target operon dvu2957–dvu2964 (Kazakov et al., 2015) and includes genes of another $\sigma_{54}$-dependent regulator DVU2960, a putative two-component system that lacks a DNA binding domain (sensor histidine kinase DVU2962 and response regulator receiver protein DVU2963), and a putative sulfite exporter DVU2958 (the other 4 are unknown proteins); however, the role of these eight genes in biofilm formation and cell physiology is unknown. To reveal the relationship between regulator DVU2956 and SRB biofilm formation, a second round of RNA-seq was performed to see the effect of production of DVU2956 in biofilms. We found that DVU2956 reduces H$_2$S production by influencing electron transport via the Fe-only hydrogenase (encoded by dvu1769, hydA and dvu1770, hydB) and the Hmc complex (high-molecular-weight cytochrome c encoded by dvu0531–dvu0536).

### Results

**Biofilm-specific gene expression**

To identify the genes involved in SRB biofilm formation, RNA-seq was performed to compare the transcription of *D. vulgaris* biofilm cells versus planktonic cells. We chose to harvest the biofilm cells from glass wool when the planktonic cells were in mid-exponential growth (at 24 h and turbidity of ~0.2, Supporting Information - Table S1) so that we identified proteins responsible for early biofilm formation. The total RNA samples were isolated from triplicates of biofilm/planktonic cell samples. Comparative transcriptomic analysis between the *D. vulgaris* biofilm and planktonic cells was based on normalized gene transcript sequencing results by using Transcripts Per Kilobase Million (TPM).

With the normalized data, compared with planktonic cells, the number of induced genes in biofilms was 365 and the number of repressed genes was 2081 (greater than two-fold differential transcription excluding tRNA and ribosome protein genes, Supporting Information Table S2 and S3). The highly repressed genes in biofilms include those encoding the dispersal enzyme DisH (a glycoside hydrolase; Zhu et al., 2018) and chemotaxis proteins (e.g., MotA/MotB; Blair and Berg, 1990; Table S3).

**qRT-PCR confirmation of RNA-seq**

To confirm the RNA-seq results, qRT-PCR was performed with independent RNA samples of *D. vulgaris* biofilm and planktonic cells, following the same RNA isolation procedure. The qRT-PCR results show that dvu2956 and regulator gene dvu2960, which is regulated by DVU2956 (Kazakov et al., 2015), are highly repressed, since we found a reduction of $184 \pm 17$-fold and $27 \pm 10$-fold in biofilm cells, respectively. Genes disH and motA were also confirmed to be repressed since qRT-PCR showed a $22 \pm 3$-fold and $2.84 \pm 0.03$-fold reduction in biofilms by qRT-PCR, respectively. In addition, dvu0304 was confirmed to be induced 4 $\pm 1$-fold in biofilms (the promoter of this gene was used for biofilm gene expression as indicated below). Hence, all these qRT-PCR results serve to corroborate the RNA-seq results (Table 1).

**Bioinformatics analysis of DVU2956**

Among the most repressed genes in biofilms (25-fold), the gene encoding $\sigma_{54}$-dependent regulator DVU2956 was found transcribed primarily in planktonic cells (Table 1). Hence, because of the large change in transcription and its possible role in controlling other proteins, we focused on DVU2956 and hypothesized that it may regulate biofilm formation by controlling several other proteins.

DVU2956 has 345 amino acids and 45% amino acid identity with acetooacetate metabolism regulatory protein AtoC in *E. coli* (Matta et al., 2007). Domain analysis, via the NCBI Conserved Domain Search Service (Fig. 1A), shows that DVU2956 contains a $\sigma_{54}$ interaction/ATPase domain (NCBI accession no. PF00158, core motif for...
Table 1. Gene expression in *D. vulgaris* biofilm cells relative to planktonic cells for genes related to regulator DVU2956 as determined by RNA sequencing.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Protein size (aa)</th>
<th>Description</th>
<th>Fold change</th>
<th>Average TPM&lt;sup&gt;a&lt;/sup&gt; in biofilm cells</th>
<th>Average TPM&lt;sup&gt;a&lt;/sup&gt; in planktonic cells</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fold change</th>
<th>Fold change value&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>DVU2956</td>
<td>435</td>
<td>σ&lt;sub&gt;54&lt;/sub&gt; dependent transcriptional regulator</td>
<td>−25 ± 9</td>
<td>9 ± 3</td>
<td>223 ± 6</td>
<td>0.003</td>
<td>−1.64 ± 0.04</td>
<td>25 ± 9</td>
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<td>DVU2957</td>
<td>46</td>
<td>Hypothetical protein</td>
<td>−7 ± 3</td>
<td>26 ± 7</td>
<td>190 ± 65</td>
<td>0.006</td>
<td>−0.001</td>
<td>26 ± 7</td>
</tr>
<tr>
<td>DVU2958</td>
<td>432</td>
<td>Hypothetical protein</td>
<td>−1.3 ± 0.1</td>
<td>195 ± 3</td>
<td>250 ± 11</td>
<td>0.42</td>
<td>−1.3 ± 0.1</td>
<td>195 ± 3</td>
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<td>DVU2959</td>
<td>138</td>
<td>Hypothetical protein</td>
<td>+1.2 ± 0.1</td>
<td>491 ± 27</td>
<td>427 ± 11</td>
<td>0.02</td>
<td>+1.2 ± 0.1</td>
<td>491 ± 27</td>
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<tr>
<td>DVU2960</td>
<td>577</td>
<td>Sensor histidine kinase</td>
<td>−3.7 ± 0.1</td>
<td>129 ± 4</td>
<td>344 ± 12</td>
<td>0.004</td>
<td>−3.7 ± 0.1</td>
<td>129 ± 4</td>
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<td>Hypothetical protein</td>
<td>−3.6 ± 0.9</td>
<td>85 ± 13</td>
<td>306 ± 55</td>
<td>0.003</td>
<td>−3.6 ± 0.9</td>
<td>85 ± 13</td>
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<td>DVU2962</td>
<td>128</td>
<td>Response regulator</td>
<td>−1.9 ± 0.2</td>
<td>123 ± 9</td>
<td>232 ± 18</td>
<td>0.03</td>
<td>−1.9 ± 0.2</td>
<td>123 ± 9</td>
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<tr>
<td>DVU2963</td>
<td>577</td>
<td>Sensor histidine kinase</td>
<td>−3.6 ± 0.9</td>
<td>85 ± 13</td>
<td>306 ± 55</td>
<td>0.003</td>
<td>−3.6 ± 0.9</td>
<td>85 ± 13</td>
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<td>Hypothetical protein</td>
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<td>194 ± 4</td>
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<td>−1.64 ± 0.04</td>
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<td>DVU0512</td>
<td>260</td>
<td>Flagellar basal body rod protein</td>
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<td>195 ± 14</td>
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<td>M24/M37 family peptidase</td>
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<td>229 ± 16</td>
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<td>97 ± 13</td>
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<td>Hypothetical protein</td>
<td>−5.2 ± 0.3</td>
<td>24.7 ± 0.6</td>
<td>128 ± 7</td>
<td>0.02</td>
<td>−5.2 ± 0.3</td>
<td>24.7 ± 0.6</td>
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<td>DVU2733</td>
<td>249</td>
<td>Adenine specific dna methyltransferase</td>
<td>−14 ± 6</td>
<td>21 ± 9</td>
<td>306 ± 14</td>
<td>0.003</td>
<td>−14 ± 6</td>
<td>21 ± 9</td>
</tr>
<tr>
<td>DVU2768</td>
<td>298</td>
<td>ComN family protein, amidophosphoribosyltransferases, purine synthesis</td>
<td>−∞</td>
<td>0</td>
<td>80 ± 12</td>
<td>0.02</td>
<td>−∞</td>
<td>0</td>
</tr>
</tbody>
</table>

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<sup>a</sup> TPM is transcripts per kilobase million and was used to directly compare the proportion of reads that map to a gene in each sample.

<sup>b</sup> The *P* value was calculated by one-way ANOVA test.

<sup>c</sup> Indicates putative genes directly controlled by DVU2956.

Interaction with σ<sub>54</sub> ‘GAFTGA’ and a helix–turn–helix<sub>8</sub> DNA binding domain (NCBI accession no. PF02954). DVU2956 belongs to the enhancer-binding protein NtrC family, which was originally identified as a nitrogen regulatory protein involved in the *E. coli* nitrogen limitation response and controls ~2% of the *E. coli* genome (Zimmer et al., 2000). DVU2956 is conserved since it is found in all SRB and in some other bacteria (Fig. 2).

DVU2956 binds to the gene operon dvu2957–dvu2964 (Kazakov et al., 2015) (Fig. 1B). In our study, the RNA-seq data analysis of *D. vulgaris* biofilm cells versus planktonic cells revealed that six of eight genes in this operon are repressed in *D. vulgaris* biofilm cells (greater than 1.5-fold change, Table 1). This operon contains the genes encoding a putative sulfoacetate transmembrane exporter (DVU2958), another σ<sub>54</sub>-dependent transcriptional regulator DVU2956, a putative NtrC family two-component system (DVU2962 and DVU2963) and some hypothetical proteins.

σ<sub>54</sub>-Dependent transcriptional regulator DVU2960 does not regulate itself since it is unable to bind to the upstream sequence of its operon like DVU2956 (Kazakov et al., 2015). In addition, DVU2960 has a substitution of threonine for proline (‘T’ to ‘P’) in the core motif of the σ<sub>54</sub> interaction motif (GAFTGA). Therefore, the function of DVU2960 is unclear. However, besides a σ<sub>54</sub> interaction/ATPase domain and a DNA binding domain, DVU2960 also contains a Per ARNT-Sim (PAS) domain, which binds small molecules and other proteins (Moghich et al., 2009). PAS domains are widely distributed, versatile, sensor and interaction modules in signal transduction proteins, and the signals recognized by PAS domains include light, oxygen and redox potential (Taylor and Zhulin, 1999). Critically, the biofilm dispersion protein BdlA of *Pseudomonas aeruginosa* harbours 70 μmol of heme per mg of purified protein through its PAS domain and uses heme as an environmental signal to induce biofilm dispersion (Petrova and Sauer, 2012). DVU2960 contains many of the amino acid residues essential for chemotaxis (4/6) (red highlight in Fig. 1C). This suggests that regulator DVU2960 may sense oxygen or heme. However, an aerotaxis assay with the *D. vulgaris* wildtype and the dvu2956 knockout shows that there is no significant difference in chemotaxis (Supporting Information Fig. S1), which suggests that regulator DVU2960, controlled by DVU2956, does not sense oxygen.

Furthermore, the DNA binding site of regulator DVU2956, ‘RREAERVLKAMRATGGNKGEAARLLGVSPRTLR’, is similar with homologues in *D. vulgaris* strain Miyazaki F’ (Fig. 1D), with known binding sites sequences listed in the RegPrecise database (Table 2), so by comparison, we derived the consensus sequence ‘GCGGNNNNNNNGCN’ based on the common binding sites of DVU2956 and DvMF_1763 (Table 2). Using this consensus sequence, putative DNA binding sites were identified in the whole genome of *D. vulgaris* (Table 2). Our analysis suggests that besides the promoter of dvu2957, DVU2956 might also bind to the upstream sites of genes dvu0512, dvu0517, dvu2732 and dvu2768 (Table 2). All of these genes were repressed in our SRB biofilms similar to dvu2956.

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Furthermore, both DVU0512 and DVU0517 are related to flagellar assembly (Supporting Information Table S3). Together, these bioinformatic results suggest that DVU2956 activates some flagellar assembly genes to maintain SRB in a motile planktonic state.

Fig. 1. (A) Domain organization of $\sigma^{54}$-dependent regulators DVU2956 and DVU2960. Pink regions indicate the $\sigma^{54}$ factor interaction domains, green regions indicate the DNA binding domain, and blue indicates the PAS domain. (B) Gene regulation by DVU2956. Grey indicates regulatory proteins. (C) PAS domain alignment between $\sigma^{54}$-dependent regulator DVU2960 and biofilm dispersion protein BdIA. Protein BdIA contains two Pas domain, PASa and PASb. The red colour indicates the conserved amino acid residues essential for chemotaxis. (D) DNA binding motif (HTH_8) alignment between $\sigma^{54}$-dependent regulators DVU2956 and DVU2960 and DvMF_1763. The red colour indicates the conserved amino acid residues of the DNA binding motif.
**DVU2956 inhibits and disperses SRB biofilms**

Since the transcription of *dvu2956* gene was significantly repressed in biofilms, we hypothesized that DVU2956 represses biofilm formation; hence, we desired to produce DVU2956 in biofilms and assay its effect. To test this hypothesis, we required a promoter that is induced in biofilms but not induced in planktonic cells, since *dvu2956* is not normally produced in biofilms. Using the RNA-seq data for expression in biofilms, we chose the promoter of *dvu0304* since it was induced ninefold in biofilms (Supporting Information Table S2) and is found in an operon (via the prediction database http://www.microbesonline.org/operons/gnc882.html). The Neural Network Promoter Prediction tool (http://www.fruitfly.org/seq_tools/promoter.html) was used to predict the promoter as 5'-TCCGTGTGGAC ATGATTTTCTGTTTTCAATAAAAGGGTTCATACAAAACCAAGGAGCCCGTC-3' where the predicted promoter sequence is italicized, and the ribosome binding site is indicated in bold. The promoter region of P*_{dvu0304}* and ribosome binding site were fused to *dvu2956* and cloned into broad host vector pVLT33 (de Lorenzo *et al.*, 1993).

Compared with the negative control harbouring the empty plasmid, after 48 h incubation, the biofilm of *D. vulgaris/pVLT33-dvu2956* decreased by 72 ± 11% (−4 ± 2-fold; Fig. 3), and consistently, after 72 h incubation, DVU2956 inhibited the biofilm formation by 70 ± 11% (−4 ± 1-fold, Supporting Information Fig. S2). Furthermore, there was no significant difference in growth from producing DVU2956...
(Supporting Information Table S4). In addition, an adhesion assay showed that production of DVU2956 does not change the number of cells that attach to glass vs. the empty plasmid control (Supporting Information Fig. S3). Hence, DVU2956 reduces biofilm formation either directly or indirectly without affecting growth or initial cell attachment.

Since production of DVU2956 in D. vulgaris inhibited biofilm formation, we investigated further the role of DVU2956 in biofilm formation by replacing dvu2956 with the kanamycin gene. As expected, compared with the wild-type strain, knocking out dvu2956 increased biofilm formation by 30.1 ± 0.6% (Fig. 3) in a consistent manner (Supporting Information Fig. S2) but did not alter growth (Supporting Information Table S4). Furthermore, this biofilm phenotype could be complemented in dvu2956 by producing DVU2956 from its own promoter via pMQ70-P\textit{dvu2956}Δ\textit{dvu2956}; we found biofilm formation was inhibited in 24 h by 48 ± 11% compared with the empty plasmid control strain \textit{D. vulgaris} \textit{Δ}dvu2956/pMQ70-P\textit{dvu2956} (Fig. 3).

Since DVU2956 inhibits biofilm formation when produced at the beginning of biofilm formation, we tested whether DVU2956 is able to disperse mature SRB biofilms. Using broad host range vector pMQ70 with the inducible promoter pBAD fused to \textit{dvu2956}, compared to the negative control \textit{D. vulgaris}/pMQ70, DVU2956 disperses SRB biofilms by 42 ± 4% when it is produced 24 h after initial biofilm formation.

In addition, since DVU2956 inhibited biofilm formation in \textit{D. vulgaris}, we also tested whether DVU2956 is able to reduce biofilm formation in other SRB species. DVU2956 has 52% amino acid identity with the protein encoded by \textit{Ddes}_1305 (NCBI accession no. ACL49207, 379 aa) in \textit{D. desulfuricans}, which also contains the \textit{σ}_{34} interaction/ATPase domain and the helix-turn-helix_8 DNA binding domain. Using \textit{D. desulfuricans} with pVLT33-P\textit{dvu0304}\textit{dvu2956} (DVU2956 produced using a biofilm-specific promoter) and negative control pVLT33-P\textit{dvu0304}Δ\textit{dvu2956}, we found DVU2956 inhibited \textit{D. desulfuricans} biofilm formation by 78 ± 9% after 24 h (Fig. 3). Hence, DVU2956 functions in multiple SRB strains.


dvu2960, dvu2962 and dvu2964 inhibit SRB biofilm formation

Given that DVU2956 reduces biofilm formation and that DVU2956 regulates the \textit{dvu2957–dvu2964} operon, four genes of this operon were chosen to see the impact of their product on biofilm formation: \textit{σ}_{34}-dependent regulator DVU2960, sensor histidine kinase DVU2962, and two hypothetical proteins DVU2961 and DVU2964. As with \textit{dvu2956}, biofilm-specific promoter P\textit{dvu0304} and broad host vector pVLT33 were used. Compared with the negative control (empty plasmid), DVU2960, DVU2962 and DVU2964 inhibited biofilm formation by 95%, 90% and 45%, respectively, after 48 h (Fig. 3). In contrast, DVU2961 did not affect biofilm formation (Fig. 3). These results were consistent from 0 to 72 h (Supporting Information Fig. S2), and, as with DVU2956, growth was not changed by producing DVU2960, DVU2961, DVU2962 and DVU2964 (Supporting Information Table S4). These results indicate DVU2956 negatively controls SRB biofilm formation by regulating the expression of the \textit{dvu2957–dvu2964} operon.

RNA-seq of \textit{D. vulgaris} with biofilm phase expression dvu2956 gene

To identify additional targets of DVU2956 as a biofilm regulator, a whole-transcriptome analysis between
D. vulgaris/pVLT33-dvu2956 and D. vulgaris/pVLT33 (empty plasmid control) in biofilm cells was performed by utilizing RNA-seq. We used the same conditions as the original RNA-seq in which DVU2956 was identified as important for planktonic growth (turbidity of planktonic cells ~0.2), since we reasoned promotor P_dvu0306, which is used for DVU2956 production, would be induced. We found that DVU2956 induces the hmc operon dvu0529–dvu0536 (encodes the high-molecular-weight cytochrome c Hmc complex) by approximately sixfold and induces the Fe-only hydrogenase operon dvu1769–dvu1770 (hydA and hydB) by 8.9- and 5.3-fold (Table 3). Furthermore, we identified putative DVU2956 binding sites upstream of both operons; i.e. in dvu0536 and dvu1769 (‘CCGGATGCAGGTC GAAGA’ and ‘CCGGGCACATGCTGGGGG’, respectively). These results suggest that DVU2956 controls both electron transport and H₂S production.

**DVU2956 reduces D. vulgaris H₂S production**

Based on the RNA-seq results, we investigated whether regulator DVU2956 affects the production of H₂S. Our initial 96-well screen of H₂S production showed that compared with the empty plasmid control, biofilm-phase expression of dvu2956 decreased H₂S production by ~51 ± 2%. Corroborating these results, compared with the *D. vulgaris* wild-type strain, inactivation of the dvu2956 increased H₂S production by 131 ± 5%. In addition, biofilm production of DVU2960, DVU2962 and DVU2964, whose genes are directly regulated by DVU2956, inhibited H₂S production by ~62 ± 3%, ~82 ± 6% and ~38 ± 3%, respectively. In contrast, biofilm production of DVU2961 increased H₂S production by +134 ± 3% (Fig. 4). Corroborating these results using sealed vials, we found that production of DVU2956 in biofilms inhibited H₂S production by ~34.6 ± 0.6% and inactivating dvu2956 increased H₂S production by 136 ± 3%. This phenotype could be complemented by producing DVU2956 from its own promoter in that H₂S production in *D. vulgaris Δdvu2956/pMQ70-PΔdvu2956-dvu2956* was decreased by ~45 ± 13% compared with empty plasmid control *D. vulgaris Δdvu2956/ pMQ70-P_dvu2956* (Fig. 4). Therefore, these consistent results demonstrate regulator DVU2956 reduces H₂S production.

**DVU2956 is produced in *D. vulgaris***

To confirm that regulator DVU2956 was produced in *D. vulgaris* (rather than to determine physiological levels), we cloned *dvu2956* downstream of the *pBAD* promotor with a His-tag using broad host range vector pMQ70 to construct pMQ70-dvu2956-His and performed both SDS-PAGE and a western blot assay. Both the SDS-PAGE and the western (Supporting Information Fig. S4) indicate DVU2956 is produced in *D. vulgaris* albeit at far lower amounts than the positive control *E. coli* BL21 (DE3)/pET27-b-dvu2956. Hence, DVU2956 is produced in SRB.

**Discussion**

In this work, we determined that the σ₅₄-dependent regulator DVU2956 is inactive in biofilms; hence, we found that producing DVU2956 results in two phenotypes: a reduction in biofilm formation and a reduction in H₂S production. For the reduction in biofilm formation, we determined that it is primarily a result of the DVU2956-controlled production of a second σ₅₄-dependent regulator DVU2960 and production of sensor histidine kinase DVU2962 (Fig. 3). Hence, we have identified a novel and conserved biofilm pathway in SRB. The downstream steps resulting

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**Table 3.** Gene expression in *D. vulgaris* biofilm cells during production of regulator DVU2956 versus the empty plasmid as determined by RNA sequencing.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Protein size (aa)</th>
<th>Description</th>
<th>Fold change</th>
<th>Avg. TPM² + DVU2956</th>
<th>Avg. TPM² no <em>D. vulgaris</em></th>
<th>P value²</th>
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<tr>
<td>DVU2956</td>
<td>345</td>
<td>Sigma-54 dependent transcriptional regulator (positive control)</td>
<td>+26 ± 2</td>
<td>1965 ± 62</td>
<td>77 ± 6</td>
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<td>150</td>
<td>Rfr2 family transcriptional regulator</td>
<td>+7 ± 1</td>
<td>220 ± 25</td>
<td>33 ± 6</td>
<td>0.006</td>
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<td>DVU0530</td>
<td>138</td>
<td>Response regulator, rrf protein</td>
<td>+6 ± 3</td>
<td>203 ± 7</td>
<td>33 ± 16</td>
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<td>DVU0531</td>
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<td>Hmc operon protein 6</td>
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<td>449 ± 216</td>
<td>72 ± 17</td>
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<td>238 ± 18</td>
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<td>DVU0534</td>
<td>388</td>
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<td>Hmc operon protein 2</td>
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<td>1399 ± 77</td>
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</table>

a. TPM is transcripts per kilobase million and was used to directly compare the proportion of reads that map to a gene in each sample.

b. P values were calculated via a one-way ANOVA test.

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Therefore, it is unlikely that DVU2956 senses nitrogen.

Transcription of other nitrogen-related genes was also not affected significantly compared to the empty plasmid control group (D. vulgaris wild-type strain, pVLT33-Pdvu0304 for biofilm phase production of DVU2956, DVU2964; Table 4). Critically, in the wild-type strain (for the dvua0143 knockout), the RNA-seq data of the second set of RNA-seq data for the effect of production of DVU2956, DVU2960, DVU2961, DVU2962, DVU2964 and pMO70-P\textsubscript{dvua0143} for gene dvua0143 complementation, respectively) and to the wild-type strain (for the dvua2956 knockout). The symbols * (P < 0.05) and ** (P < 0.01) indicate significant differences versus the empty plasmid control group via one-way ANOVA.

In conclusion, we have identified a conserved biofilm and H\textsubscript{2}S regulator, DVU2956, that controls SRB biofilm formation and H\textsubscript{2}S production. Since biofilm formation and H\textsubscript{2}S production are relevant for corrosion, future studies should investigate the role of DVU2956 in corrosion. As neither phosphorylation nor cyclic diguanylate appears to influence DVU2956, it appears this regulator uses a novel mechanism to switch SRB from biofilm to planktonic growth.

**Experimental procedures**

**Bacterial strains, medium and growth conditions**

The strains and plasmids used in this study are listed in Table 4. *Desulfovibrio vulgaris* Hildenborough (ATCC 29579) was grown anaerobically at 30°C in 25 ml screwcap tubes containing 10 ml of modified Baar’s medium (ATCC medium no. 1249) with 0.025% sodium sul\textsubscript{2}de (as an oxygen scavenger). Initial cultures were grown from glycerol stocks stored at −80°C; all subcultures were grown from a 5% inoculum from the initial culture and were incubated without shaking; 400 μg/ml genetin (G418) was used to maintain plasmids. *Escherichia coli* strains were cultured at 37°C with shaking at 250 rpm using LB medium with 50 ng/μL kanamycin to maintain broad host vector plasmid pVLT33 carrying dvu2956, dvu2960, dvu2961, dvu2962 and dvu2964 fused with biofilm phase promoter P\textsubscript{dvu2964}. Growth rates were determined by monitoring the increase in turbidity at 620 nm of 10 ml cultures in sealed glass tubes via a spectrophotometer (Spectronic 20, Milton Roy Company).

**Plasmid construction**

The primers used are listed in Supporting Information Table S5, and *E. coli* TG1 was used for the constructions.

Fig. 4. DVU2956 inhibits H\textsubscript{2}S formation. The percentage of normalized H\textsubscript{2}S production in biofilms (ppm H\textsubscript{2}S/turbidity at 620 nm) is shown compared to the empty plasmid controls (pVLT33-P\textsubscript{dvu0304} for biofilm phase production of DVU2956, DVU2960, DVU2961, DVU2962, DVU2964 and pMO70-P\textsubscript{dvua0143} for gene dvua0143 complementation, respectively) and to the wild-type strain (for the dvua2956 knockout). The symbols * (P < 0.05) and ** (P < 0.01) indicate significant differences versus the empty plasmid control group via one-way ANOVA.

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To investigate whether the proteins identified from the differentially expressed genes in the RNA-seq data for biofilm versus planktonic cells affect biofilm formation (Supporting Information Table S2), we cloned genes dvu2956, dvu2960, dvu2961, dvu2962 and dvu2964, into broad-host-range plasmid pVLT33 (de Lorenzo et al., 1993) under control of the biofilm promoter P_{dvu2934} (Supporting Information Table S2) as predicted by the Neural Network Promoter Prediction tool (http://www.fruitfly.org/seq_tools/promoter.html) and with the ribosome binding site ‘AAGGAG’. dvu2956 was also cloned into pMQ70 and induced via its inducible promoter pBAD. In addition, dvu2956 was knocked out by using pBluescriptII (SK-) that does not replicate in SRB by cloning the 1008 bp upstream and 915 bp downstream sequence of dvu2956, and kanamycin resistance gene from vector pBSKan (Canada et al., 2002). In addition, to express and purify protein D. vulgaris dvu2956, the dvu2956 gene was amplified using forward primer sig54R-F-Ndel and reverse primer sig54R-R-HindIII, which introduce the Ndel and HindIII sites and the His-tag, and cloned into PET-27b(+). Plasmid PET27b-dvu2956 was then transferred into protein expression host E. coli BL21 (DE3). To demonstrate that DVU2956 can be produced in SRB, dvu2956 was also was amplified using forward primer dvu2956-IF and reverse primer sig54R-R-HindIII, which introduce EcoRI and HindIII sites and a His-tag, and cloned into broad host range vector pMQ70 with inducible promoter pBAD (Table 4). To complement D. vulgaris dvu2956 phenotypes, promoter of dvu2956 was cloned into broad-host-range plasmid pMQ70 using primers Pdvu2956-F-HindIII and Pdvu2956-R-SalI to construct plasmid pMQ70-P_{dvu2956}. Then the ribosome binding site ‘AAGGAG’ and gene dvu2956 were inserted downstream of P_{dvu2956} to make plasmid pMQ70-P_{dvu2956}-dvu2956 (Table 4) using primers dvu2956-F-Sal and dvu2956-R-EcoRI.

The plasmids were confirmed by sequencing with the primers pVLT33-SF and pVLT33-SB from the vector pVLT33 derivatives, T7 promoter/terminator primers for plasmid PET27b-dvu2956 and pMQ70-F/R primers for plasmid pMQ70-dvu2956i-His and pMQ70-F/R primers for plasmid pMQ70-P_{dvu2956}-dvu2956. Competent SRB cells (turbidity ~ 0.3) were prepared by washing twice anaerobically with pre-chilled, sterile 10% glycerol. Plasmid DNA (0.5–1 μg) was added to the competent cells (50 μl) by mixing gently, and the solution was transferred to a pre-chilled (0 °C), 1 mm electroporation cuvette in the anaerobic chamber. Electroporation (25 μF, 200 Ω, and 1.5 kV/cm) was performed aerobically, and the cuvette was moved back to the anaerobic chamber immediately, where modified Baar’s medium (1 ml) was added. The cells were mixed gently and transferred to a 1.5 ml Eppendorf tube where they recovered overnight at 30 °C, and 50 μl was inoculated into either 10 ml modified Baar’s medium with 0.2% yeast extract or 1% agar plates, both with G418 (400 μg/ml for D. vulgaris, and 800 μg/ml for D. desulfuricans) or 300 μg/ml carbenicillin for D. vulgaris. Genomic DNA from 1 to 2 ml of culture or the colony was isolated by UltraClean® Microbial DNA

Table 4. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
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</tr>
<tr>
<td>D. vulgaris Hildenborough</td>
<td>Wild-type, ATCC 29579</td>
<td>ATCC</td>
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<tr>
<td>D. desulfuricans</td>
<td>Wild-type, isolated from sulfidic mud</td>
<td>DSMZ (DSM-642)</td>
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<td>E. coli TG1</td>
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<td>J. Minshull</td>
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<td>E. coli BL21 (DE3)</td>
<td>F– ampR Tn5Δr(3–) gal dcm (DE3)</td>
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<td>Plasmids</td>
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<td>pVLT33</td>
<td>broad-host-range expression vector (IncQ, RSF1010 replicon), Km', Plac, lacIq tra’ mob'</td>
<td>V. de Lorenzo</td>
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<td>pBSKan</td>
<td>E. coli vector, pUC ori, f1 (-) ori, Ampq, Plac</td>
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<td>pET-27b(+)</td>
<td>P_{T7}, pBR322 ori, KmR</td>
<td>(Canada et al., 2002)</td>
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<td>pMQ70</td>
<td>pBAD, CarR, shuttle vector</td>
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<td>P_{dvu2960}, CarR</td>
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KmR, AmpR and CarR are kanamycin, ampicillin and carbenicillin resistance, respectively.

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isolation kit (MO BIO cat#12224) for PCR verification of the correct plasmids in SRB.

For PCR verification of the cloned genes, dvu2956, dvu2960, dvu2961, dvu2962 and dvu2964 in *D. vulgaris*, primers pVLT33-SF and dvu2956-HindIIIIR2, pVLT33-SB and dvu2960MR, pVLT33-SB and dvu2961DB, pVLT33-SB and dvu2962MR, and pVLT33-SB and dvu2964DB were used, respectively. For PCR verification of pVLT33-Pdvu0304*, dvu2956 and pVLT33-Pdvu0304 in *D. desulfuricans*, primers pVLT33-SF and pVLT33-SB were used. For the dvu2956 gene knockout in *D. vulgaris*, primers dvu2956front and km_P6, km_P5 and dvu2956rear were used for verification.

For PCR verification of pMQ70-P* _dvu2956* and pMQ70-P* _dvu2956* in *D. vulgaris* *dvu2956*, primers pMQ70-F and pMQ70-R were used (Supporting Information Table S5).

**RNA isolation and sequencing**

For the RNA-seq of *D. vulgaris* biofilm and planktonic cells, 300 ml Baar’s modified medium was inoculated with *D. vulgaris* culture. The culture was anaerobically incubated for 3 days to a turbidity of greater than 0.3. Subsequently, 3 x 400 ml of Baar’s modified medium was inoculated with this culture (initial turbidity of 0.1). This medium was added to 3 x 1 l beakers along with 10 g of glass wool. All beakers with glass wool were autoclaved prior to start of the experiment. The cultures were grown anaerobically without shaking for 16–24 h until the turbidity reached ~0.2. Loose biofilm cells were removed from the glass wool by immersing sequentially in two chilled beakers containing 2 x 200 ml of RNase-free 0.85% NaCl solution in RNase-free beakers, and the biofilm cells were removed by immersing the glass wool in 200 ml of RNase-free 0.85% NaCl solution plus 2 ml RNALater (Invitrogen™, cat no. AM7024) and sonicating for 2 min (Fisher Scientific, model FS3). Simultaneously, the planktonic cells were rapidly decanted into pre-chilled RNase-free 250 ml centrifuge bottles containing 2 ml of chilled RNALater. Cells were centrifuged in a pre-chilled JA-14 rotor (Beckman Coulter, Avanti J-E) at -2 °C for 2 min at 10 000 g. The centrifuge bottles were rapidly removed and put in dry ice +95% ethanol for 5 s. The supernatant was then discarded, and the cells were resuspended in 4 ml of pre-chilled RNALater and transferred to pre-chilled 2 ml tubes with O-rings. The tubes were immersed in dry ice +95% ethanol bath for 5 s for rapid cooling. The tubes were centrifuged for 15 s at 15 000 g and 4 °C in a cold bench-top microcentrifuge. The supernatants were discarded, and the tubes were then immersed in a dry ice/ethanol bath for flash freezing. The tubes were stored at -80°C until RNA isolation.

Similarly, for the RNA-seq of *D. vulgaris* producing DVU2956 that formed less biofilm and empty plasmid control strain, the strains were inoculated and subcultured with 400 μg/ml G418. The biofilm cell samples were harvested following the procedures mentioned above. The RNA was isolated by Roche High Pure RNA isolation kit (Roche Cat # 11828665001).

Comparative transcriptomic analysis between *D. vulgaris* biofilm cells and planktonic cells, and the strains expressing DVU2956 and the empty plasmid control was based on normalizing the gene transcript sequencing results to transcripts per kilobase million (TPM). TPM was calculated by first dividing the read counts by the length of each gene in kilobases to yield reads per kilobase (RPK) for each gene. Then the total RPK values of all the genes in a sample were added together and divided by 1 000 000 to yield the ‘per million scaling factor’. Finally, the RPK value of each gene was divided by the ‘per million scaling factor’ to get the TPM value of each gene. Hence, the sum of all the TPMs in each sample is 1 000 000 so this method allows a direct comparison of the transcription of each gene in different data sets.

**qRT-PCR**

The individual RNA samples were prepared following the procedures mentioned above. The primers are listed in Supporting Information Table S5, and the 16S rDNA gene of *D. vulgaris* was used as the internal reference control. Prior to performing qRT-PCR, a regular PCR with *D. vulgaris* genomic DNA was used to ensure that only a single band is produced by the primers. The qRT-PCR thermocycling protocol was used (95 °C for 5 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min). For qRT-PCR, two replicate reactions were set up for each sample/primer pair. Components from the iTaq™ universal SYBR® Green One-Step kit (Bio-Rad, Hercules, CA) were used to set up qRT-PCRs. The annealing temperature was 60 °C for all primers.

**Protein domain analysis and sequence alignment**

Protein domain analysis was performed using the Conserved Domain Search Service of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). Sequence alignment was performed via Basic Local Alignment Search Tool at NCBI. Multiple protein sequence alignments were analysed using Clustal X2.0 (Larkin et al., 2007). The phylogenetic tree was constructed by the maximum likelihood method via MEGA X (Kumar et al., 2018). Bootstrap support values were calculated from 1000 replicates.

**Biofilm inhibition and dispersal assays**

Biofilms of *D. vulgaris* were developed in modified Baar’s medium (300 μl) in 96-well polystyrene plates

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(Fisher Scientific, cat no. 07-200-656) by incubating in an anaerobic glove box for 24–72 h without shaking. The planktonic cell turbidity was measured at 620 nm using a Sunrise microplate reader (Tecan, Austria Gesellschaft, Salzburg, Austria). After incubation, the cultures were discarded, the wells were washed three times with distilled water by dipping the plates into a 1 l of distilled water, and the plates were dried using a piece of paper towel. Crystal violet (0.1% in 300 μl) was added to each well (Fletcher, 1977), the plates were incubated for 20 min at room temperature, and the staining solution was discarded. The plates were washed three times with distilled water by dipping the plates into a 1 l solution of distilled water, 300 μl of 95% ethanol was added to each well, and the plates were soaked for 5 min to dissolve the crystal violet. Total biofilm was measured spectrophotometrically at 540 nm using the Sunrise microplate reader (TECAN, Switzerland) and was normalized the planktonic metrically at 540 nm using the Sunrise microplate reader (TECAN, Austria). All experiments were conducted for at least three biological independent replicates. Technical replicates were averaged to produce replicate means that were subsequently used for analysis. For comparisons, differences were evaluated by a one-way ANOVA test of mean replicate measurements, which were considered statistically significant if \( P < 0.05 \) and \( P < 0.01 \) (Sigma plot, version 12, Systat Software, Inc., San Jose, CA).

**H₂S assay**

H₂S was measured via a methylene blue spectrophotometric assay (Rabinowitz, 1978); in brief, \( N,N \)-dimethyl-p-phenylenediamine dihydrochloride (Sigma Aldrich, catalogue no. 536-46-9) is converted into methylenionium chloride (methylene blue) by reacting with H₂S dissolved in hydrochloric acid in the presence of ferric chloride. For the 96-well screening method, anaerobically grown \( D. \ vulgaris \) cultures adjusted to a turbidity of 0.05 at 620 nm in modified Baar’s medium were added anaerobically (150 μl) into each well, and the 96-well plate was incubated for 48 h for biofilm formation. Anaerobically, 25 μl of the biofilm cultures were transferred to another 96-well plate with 225 μl deoxygenated water per well to make a 10× dilution. 5% \( N, N \)-dimethyl-p-phenylenediamine dihydrochloride (24.5 μl, prepared in 5.5 N HCl) and 5 μl 23 mM FeCl₃ 6H₂O (prepared in 1.2 M HCl) was added into each well. After mixing gently by pipetting and incubating at room temperature for 3 min anaerobically, the 96-well plate was taken out of the anaerobic chamber and the absorbance at 670 nm was measured using a Sunrise microplate reader (TECAN, Switzerland).

For a more rigorous H₂S assay, sealed glass vials were used to prevent H₂S losses. SRB strains were grown anaerobically in 10 ml of modified Baar’s medium with 2.5 g sterilized glass wool to promote biofilm formation for 7–10 days. Supernatants were transferred to another sealed vial with 10 ml PBS buffer by syringe for dilution. Anaerobically, 1 ml diluted sample was transferred to another sealed bottle with 125 μl 12% sodium hydroxide and 3.25 ml 1% zinc acetate by syringe (to fix the sulfide from H₂S by forming ZnS precipitates). After mixing gently and incubating for 30 min at room temperature, 625 μl of 5% \( N, N \)-dimethyl-p-phenylenediamine dihydrochloride and 125 μl 0.023 M FeCl₃ were added and the mixture was shaken for 20 min at 300 rpm at room temperature. H₂O (2.125 ml) was added and mixed, and at least 1 ml of each reaction was taken to determine the absorbance at 670 nm.

**Statistical analysis**

All experiments were conducted for at least three biologically independent replicates. Technical replicates were averaged to produce replicate means that were subsequently used for analysis. For comparisons, differences were evaluated by a one-way ANOVA test of mean replicate measurements, which were considered statistically significant if \( P < 0.05 \) and \( P < 0.01 \) (Sigma plot, version 12, Systat Software, Inc., San Jose, CA).

**Western blot**

To produce DVU2956 with a His-tag proteins in \( D. \ vulgaris \), \( D. \ vulgaris/pMQ70-dvu2956-His \) and the empty plasmid control strains (\( D. \ vulgaris/pMQ70 \)) were induced by 10 mM arabinose at 30 °C for 24 h, anaerobically. \( E. \ coli \) BL21 (DE3)/pET27b-dvu2956 was used as the positive control, which was induced by final 1 mM isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG) at a turbidity of 620 nm of about 0.5, then incubated at 37 °C, 250 rpm for 6 h, aerobically. After measuring the turbidity, the cells were harvested at 10 000 g for 10 min at 4 °C and resuspended with PBS buffer (pH 7.4) to normalize the turbidity to 1, lysed by sonication at level 3, 20 s, three times on ice using a Sonic Dismembrator 60 (Fisher Scientific).

For SDS-PAGE, protein DVU2956 expressed from \( E. \ coli \) BL21 (DE3) was used as a positive control. Proteins from SDS-PAGE gel were transferred by a Mini Trans-Blot Cell (Bio-Rad) to polyvinylidene difluoride membranes (Immun-Blot PVDF Membrane, Bio-Rad cat#162–0177); a His-tag antibody was used to detect DVU2956 (6× His Tag Antibody, HRP conjugate (His. H8), Invitrogen, cat#MA1-21315-HRP). The antibody of the blot was detected using the SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific cat#34077) and CL-XPosure™ Film (Thermo Scientific cat#34090) in 1 min within an Autoradiography Cassette (Fisher Scientific cat#FBCS 57).
Aerotaxis assay

Anaerobically-prepared SRB cell suspensions (turbidity of 2.0 at 620 nm) in modified Baar’s medium were added to flat glass capillary tubes (100 mm length × 8 mm width × 0.8 mm height; Vitro Dynamics Inc., Rockaway, NJ) that were sealed with multiple layers of Parafilm M (Bemis, NA). An oxygen gas bubble (50 μl and 5 μl) of 95% pure oxygen was injected by a gastight syringe (#1705, Hamilton, Reno, NV) into the capillary tubes, and the tubes were incubated for 24 h without movement in the anaerobic chamber.

Adhesion assay

When SRB cells reached the stationary phase (turbidity greater than 0.3 at 620 nm), cell culture (1 ml) was placed on a sterilized glass slide and incubated anaerobically for 30 min. Then the slides were gently dipped into PBS buffer twice, stained with SYTO9 (L7012, Invitrogen) anaerobically 30 min. Then the slides were gently dipped into PBS buffer on a sterilized glass slide and incubated anaerobically for 24 h without movement in the anaerobic chamber.

Acknowledgements

This work was supported by the Dow Chemical Company and funds derived from the Biotechnology Endowed Professorship at the Pennsylvania State University. The authors have no conflicts of interest. We appreciate our discussions with Dr Bei Yin.

Data availability

The raw RNA sequencing reads for the D. vulgaris biofilm cell replicates 1 and 2 and D. vulgaris planktonic cell replicates 1 and 2 of D. vulgaris/pVLT33-P_dvu0304-dvu2956 and D. vulgaris/pVLT33-P_dvu0304 have been submitted to the NCBI SRA database under accession SRP156817. In addition, the raw RNA sequencing reads for biofilm cell replicates 1 and 2 of D. vulgaris/pVLT33-P_dvu0304-dvu2956 and D. vulgaris/pVLT33-P_dvu0304 have been submitted to the NCBI SRA database under accession SRP158123.

References


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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Appendix S1. Supporting Information

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