environmental microbiology

Environmental Microbiology (2019) **00**(00), 00–00 doi:10.1111/1462-2920.14679

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

Lei Zhu,[†,‡] Ting Gong,[‡] Thammajun L. Wood, Ryota Yamasaki and Thomas K. Wood[*]
Department of Chemical Engineering, Pennsylvania State University, University Park, State College, PA, 16802, USA.

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_2S production. Corroborating these results, producing DVU2956 in biofilms decreased H_2S production by half, deleting *dvu2956* increased H_2S production by 131 $\pm$ 5%, and producing DVU2956 in the *dvu2956* strain reduced H_2S production. Therefore, DVU2956 maintains SRB in the planktonic state and reduces H_2S 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*-acetyl-β-D-galactosamine (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 mannose, 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

Received 2 March, 2019; revised Month, 2019; accepted 11 May, 2019. *For correspondence. Tel. 814-863-4811; Fax 814-865-7846. E-mail tuw14@psu.edu [†]Present address: National Biopesticide Engineering Technology Research Center, Hubei Biopesticide Engineering Research Center, Hubei Academy of Agricultural Sciences, Biopesticide Branch of Hubei Innovation Centre of Agricultural Science and Technology, Wuhan, 430064, P. R. China [‡]These authors contributed equally to this work.

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 σ_{54}-dependent regulators are less than 10% of the total response regulators (RRs) (Galperin, 2010); *D. vulgaris* Hildenborough has 37 σ_{54}-dependent regulators out of a total 91 RRs (41%; Kazakov *et al.*, 2015). σ_{54}-dependent regulators (also called enhancer-binding proteins) specifically bind a conserved upstream activating sequence (UAS) located upstream from the σ_{54}-dependent promoter and interact with the σ_{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 σ_{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 σ_{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 σ_{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 σ_{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 σ_{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_2S 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 cells 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 σ_{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 acetoacetate 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 σ_{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.

Gene ID	Protein size (aa)	Description	Fold change	Average TPM[a] in biofilm cells	Average TPM[a] in planktonic cells	*P* value[b]	Fold change by qRT-PCR
DVU2956	345	σ_{54} dependent transcriptional regulator	−25 ± 9	9 ± 3	223 ± 6	0.003	−184 ± 17
DVU2957[c]	46	Hypothetical protein	−7 ± 3	26 ± 7	190 ± 65	0.006	–
DVU2958[c]	332	Hypothetical protein	−1.3 ± 0.1	195 ± 3	250 ± 11	0.04	–
DVU2959[c]	138	Hypothetical protein	+1.2 ± 0.1	491 ± 27	427 ± 11	0.2	–
DVU2960[c]	474	Σ_{54} dependent transcriptional regulator	−2.7 ± 0.1	129 ± 4	344 ± 12	0.004	−27 ± 10
DVU2961[c]	115	Hypothetical protein	−3.6 ± 0.9	85 ± 13	306 ± 55	0.003	–
DVU2962[c]	577	Sensor histidine kinase	−1.9 ± 0.2	123 ± 9	232 ± 18	0.03	–
DVU2963[c]	128	Response regulator	−1.5 ± 0.1	200.1 ± 0.1	304 ± 12	0.01	–
DVU2964[c]	219	Hypothetical protein	−1.64 ± 0.04	194 ± 4	318 ± 2	0.001	–
DVU0512	260	Flagellar basal body rod protein	−2.0 ± 0.1	195 ± 14	385 ± 6	0.006	–
DVU0517	610	M24/M37 family peptidase	−2.4 ± 0.4	97 ± 13	229 ± 16	0.02	–
DVU2732	66	Hypothetical protein	−5.2 ± 0.3	24.7 ± 0.6	128 ± 7	0.02	–
DVU2733	249	Adenine specific dna methyltransferase	−14 ± 6	21 ± 9	306 ± 14	0.003	–
DVU2768	298	Comf family protein, amidophosphoribosyltransferases, purine synthesis	−∞	0	80 ± 12	0.02	–
DVU2239	481	*N*-acetyl-β-*d*-hexosaminidase	−4.0 ± 0.2	59 ± 3	231.9 ± 0.2	0.0003	−22 ± 3
DVU0048	246	Chemotaxis protein motb	−5.8 ± 0.9	32.3 ± 0.4	187 ± 30	0.04	–
DVU0050	252	Chemotaxis protein mota	−23 ± 4	8 ± 2	186 ± 3	0.0004	−2.84 ± 0.03
DVU0304	93	Hypothetical protein	+8.5 ± 0.2	6519 ± 275	766 ± 189	0.003	+4 ± 1

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. The *P* value was calculated by one-way ANOVA test.
c. Indicates putative genes directly controlled by DVU2956.

interaction with σ_{54} 'GAFTGA') and a helix–turn–helix_8 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_{54}-dependent transcriptional regulator DVU2960, a putative NtrC family two-component system (DVU2962 and DVU2963) and some hypothetical proteins.

σ_{54}-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_{54} interaction motif (GAFPGA). Therefore, the function of DVU2960 is unclear. However, besides a σ_{54} interaction/ATPase domain and a DNA binding domain, DVU2960 also contains a Per-ARNT-Sim (PAS) domain, which binds small molecules and other proteins (Moglich *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, 'RREAEVRVILKAMRATGGNKGEAA RLLGVSPRTLR YKFAEY' (Kazakov *et al.*, 2015) 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 'GCGGNNNNNNNNNGNCNN' 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*

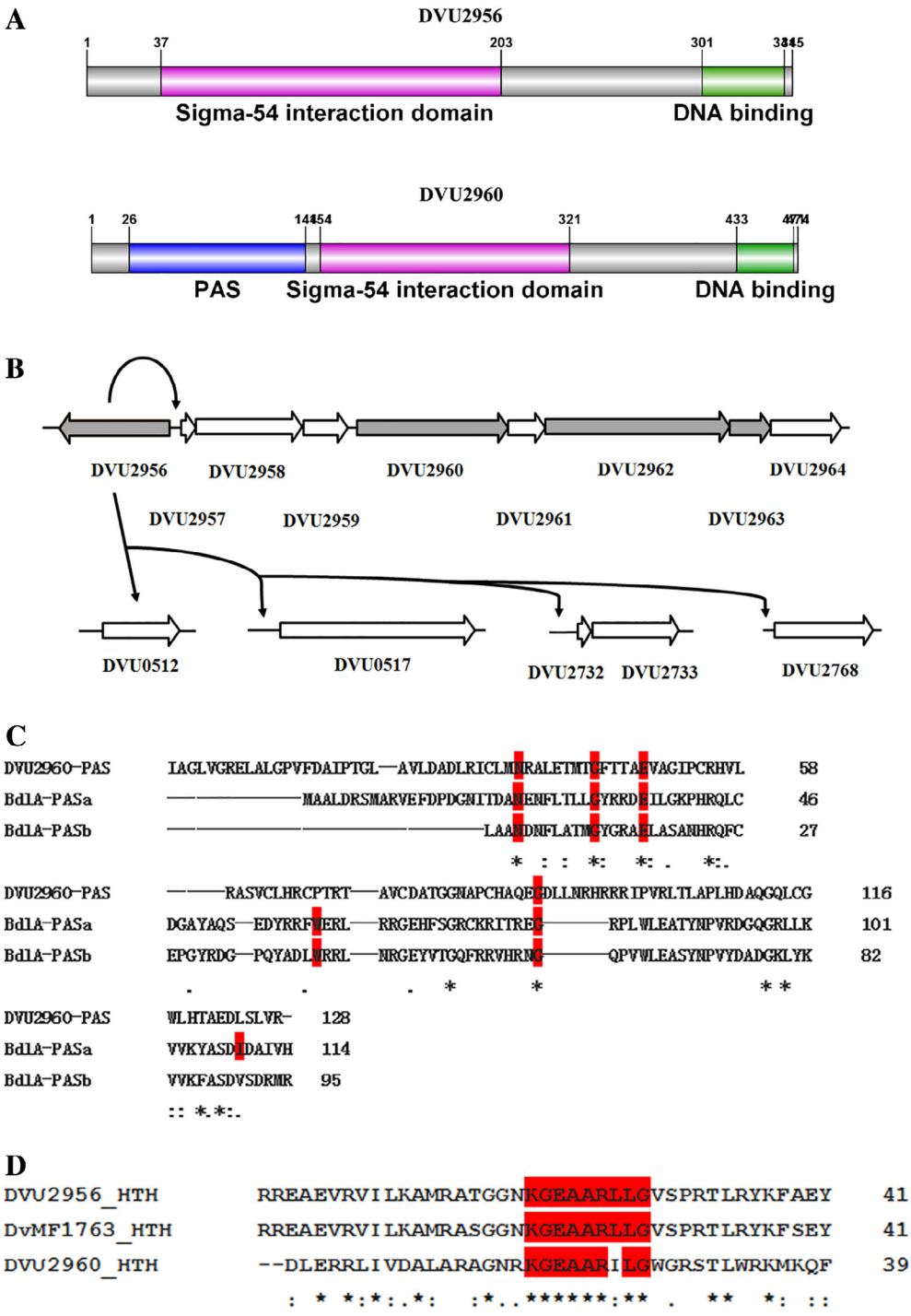

Fig. 1. (A) Domain organization of σ_{54}-dependent regulators DVU2956 and DVU2960. Pink regions indicate the σ54 factor interaction domains, green regions indicate the DNA binding domain, and blue indicates the PAS domain.(B) Gene regulation by DVU2956. Grey indicates regulatory proteins.(C) PAS domain alignment between σ_{54}-dependent regulator DVU2960 and biofilm dispersion protein BdlA. Protein BdlA 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 σ_{54}-dependent regulators DVU2956 and DVU2960 and DvMF_1763. The red colour indicates the conserved amino acid residues of the DNA binding motif.

(Supporting Information Table S3). 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.

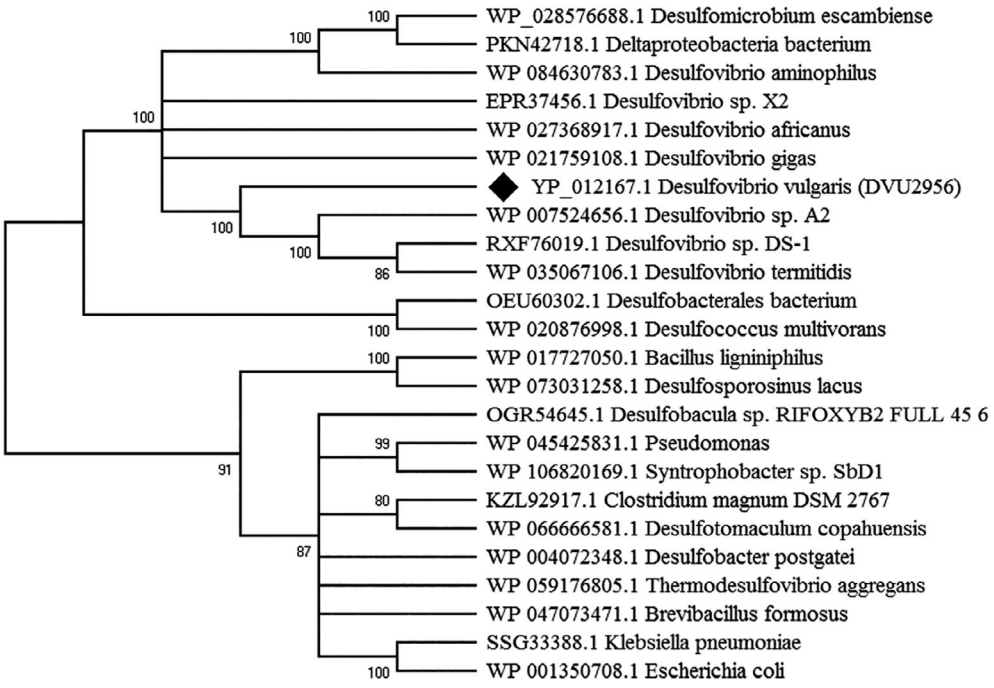

Fig. 2. Phylogenetic tree of DVU2956 homologous proteins. The phylogenetic tree was constructed by the maximum likelihood method which aligns homologues of DVU2956. The NCBI accession number and original strains of the homologous proteins are indicated on each branch. The Bootstrap support values were calculated from 1000 replicates. The black rhombus represents protein DVU2956 from *D. vulgaris* that was used in this study.

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 promotor 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′-*TCCGTGTTGAC ATTGATTTTCGTTTTCAATAAAAGGGTTCC*ATACAAACC ***AAGGAG***CCCGTC-3′ where the predicted promotor 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

Table 2. Transcription factor binding sites.

Gene ID	Strain	Target gene ID	Position	Sequence	Source
DVU2956	*D. vulgaris* Hildenborough	DVU2957	−131	GCGGTAATTTTTTGCCGC	(Kazakov *et al*., 2015)
DvMF_1763	*D. vulgaris* str. Miyazaki F	DvMF_1771	−39	GCGGCAACATTCTGCCGC	RegPrecise
		DvMF_1764	−271	GCGGAAAATTTCTGCCGT	
		DvMF_1764	−292	CCGGCAATTTGTTGCCGG	
Consensus sequence 'GCGGNNNNNNNNNGNCNN'					
DVU2956	*D. vulgaris* Hildenborough	DVU2957	−131	GCGGTAATTTTTTGCCGC	This work
		DVU0512	−108	GCGGCGAGAGGGGGCAT	
		DVU0517	−405	GCGGCAATCTCGTGCCGT	
		DVU2732	−386	GCGGCAAGCTTCTGCCGG	
		DVU2768	−72	GCGGCAACATCGTGCCGG	

(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-$P_{dvu2956}$-*dvu2956*; we found biofilm formation was inhibited in 24 h by 48 ± 11% compared with the empty plasmid control strain *D. vulgaris Δdvu2956*/pMQ70-$P_{dvu2956}$ (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 promotor *pBAD* fused to *dvu2956*, compared to the negative control *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 *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 Ddes_1305 (NCBI accession no. ACL49207, 379 aa) in *D. desulfuricans*, which also contains the σ_{54} interaction/ATPase domain and the helix–turn–helix_8 DNA binding domain. Using *D. desulfuricans* with pVLT33-$P_{dvu0304}$-*dvu2956* (DVU2956 produced using a biofilm-specific promoter) and negative control pVLT33-$P_{dvu0304}$, we found DVU2956 inhibited *D. desulfuricans* biofilm formation by 78 ± 9% after 24 h (Fig. 3). Hence, DVU2956 functions in multiple SRB strains.

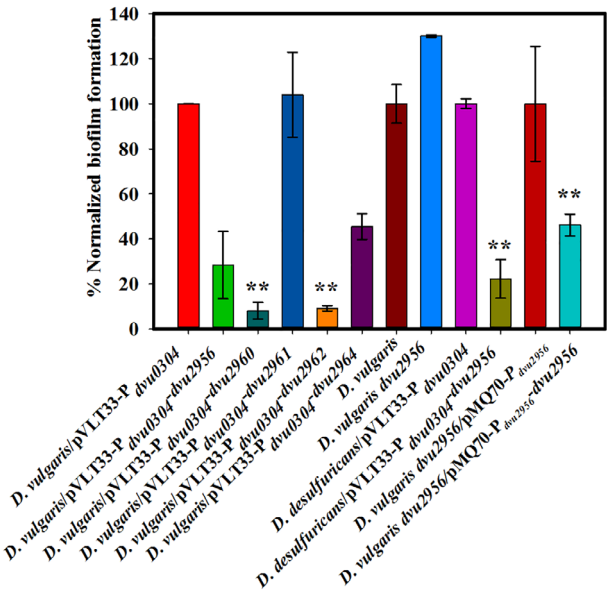

Fig. 3. Production of DVU2956, DVU2960, DVU2962, and DVU2964 inhibits *D. vulgaris* biofilm formation and DVU2956 inhibits *D. desulfuricans* biofilm formation. DVU2956, DVU2960, DVU2961, DVU2962, and DVU2964 were produced using pVLT33 in *D. vulgaris* using the biofilm-specific promoter $P_{dvu0304}$. Normalized biofilm formation percentage (based on $OD_{540\ nm}/OD_{620\ nm}$) is shown to compare to the empty plasmid control after 48 h of anaerobic growth at 30 °C in modified Baar's medium. Four independent biofilm assays were performed using *D. vulgaris* producing DVU2956 in the biofilm phase. pVLT33-$P_{dvu0304}$-*dvu2956* was also produced in *D. desulfuricans*, and biofilm formation was compared to the empty plasmid control after 24 h under the same growth conditions. For the *dvu2956* knockout, *D. vulgaris* wild-type was used for comparison, and normalized biofilm formation percentage (based on $OD_{540\ nm}/OD_{620\ nm}$) is shown after 24 h of anaerobic growth at 30 °C in modified Baar's medium. For complementing the *dvu2956* knockout, *D. vulgaris Δdvu2956* with empty plasmid pMQ70-$P_{dvu2956}$, was used for comparison, and normalized biofilm formation (based on $OD_{540\ nm}/OD_{620\ nm}$) is shown after 24 h of anaerobic growth at 30 °C in modified Baar's medium. The error bar indicates one standard deviation. The symbols ** ($P < 0.01$) indicate significant differences versus the empty plasmid control group via one-way ANOVA.

DVU2960, DVU2962 and DVU2964 inhibit SRB biofilm formation

Given that DVU2956 reduces biofilm formation and that DVU2956 regulates the *dvu2957–dvu2964* operon, four genes of this operon were chosen to see the impact of their product on biofilm formation: σ_{54}-dependent regulator DVU2960, sensor histidine kinase DVU2962, and two hypothetical proteins DVU2961 and DVU2964. As with *dvu2956*, biofilm-specific promoter $P_{dvu0304}$ 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 *dvu2957–dvu2964* operon.

RNA-seq of 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 promoter $P_{dvu0304}$, 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 'GCGGGCACATGCTGGGGC', respectively). These results suggest that DVU2956 controls both electron transport and H_2S production.

DVU2956 reduces D. vulgaris H_2S production

Based on the RNA-seq results, we investigated whether regulator DVU2956 affects the production of H_2S. Our initial 96-well screen of H_2S production showed that compared with the empty plasmid control, biofilm-phase expression of *dvu2956* decreased H_2S production by $-51 \pm 2\%$. Corroborating these results, compared with the *D. vulgaris* wild-type strain, inactivation of the *dvu2956* increased H_2S production by $131 \pm 5\%$. In addition, biofilm production of DVU2960, DVU2962 and DVU2964, whose genes are directly regulated by DVU2956, inhibited H_2S production by $-62 \pm 3\%$, $-82 \pm 6\%$ and $-38 \pm 3\%$, respectively. In contrast, biofilm production of DVU2961 increased H_2S production by $+134 \pm 3\%$ (Fig. 4). Corroborating these results using sealed vials, we found that production of DVU2956 in biofilms inhibited H_2S production by $-34.6 \pm 0.6\%$ and inactivating *dvu2956* increased H_2S production by $136 \pm 3\%$. This phenotype could be complemented by producing DVU2956 from its own promoter in that H_2S production in *D. vulgaris Δdvu2956*/pMQ70-$P_{dvu2956}$-*dvu2956* was decreased by $-45 \pm 13\%$ compared with empty plasmid control *D. vulgaris Δdvu2956*/pMQ70-$P_{dvu2956}$ (Fig. 4). Therefore, these consistent results demonstrate regulator DVU2956 reduces H_2S 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*i-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)/pET27b-*dvu2956.* Hence, DVU2956 is produced in SRB.

Discussion

In this work, we determined that the σ_{54}-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 H_2S production. For the reduction in biofilm formation, we determined that it is primarily a result of the DVU2956-controlled production of a second σ_{54}-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

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

Gene ID	Protein size (aa)	Description	Fold change	Avg. TPM[a] + DVU2956	Avg. TPM[a] no *D. vulgaris*	*P* value[b]
DVU2956	345	Sigma-54 dependent transcriptional regulator (positive control)	+26 ± 2	1965 ± 62	77 ± 6	0.0003
DVU0529	150	Rrf2 family transcriptional regulator	+7 ± 1	220 ± 25	33 ± 6	0.006
DVU0530	138	Response regulator, rrf1 protein	+6 ± 3	203 ± 7	33 ± 16	0.003
DVU0531	461	Hmc operon protein 6	+6 ± 3	449 ± 216	72 ± 17	0.01
DVU0532	226	Hmc operon protein 5	+6 ± 2	238 ± 38	38 ± 11	0.01
DVU0533	47	Hmc operon protein 4	+6 ± 3	245 ± 67	41 ± 17	0.05
DVU0534	388	Hmc operon protein 3	+6 ± 2	312 ± 71	54 ± 11	0.03
DVU0535	370	Hmc operon protein 2	+7 ± 3	331 ± 89	49 ± 15	0.03
DVU0536	560	Hmc periplasmic [Fe]	+5.1 ± 0.9	335 ± 51	66 ± 8	0.01
DVU1769	421	Hydrogenase, large subunit	+9 ± 2	851 ± 117	95 ± 20	0.006
DVU1770	123	Hydrogenase, small subunit	+5 ± 2	1399 ± 77	262 ± 92	0.003

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.

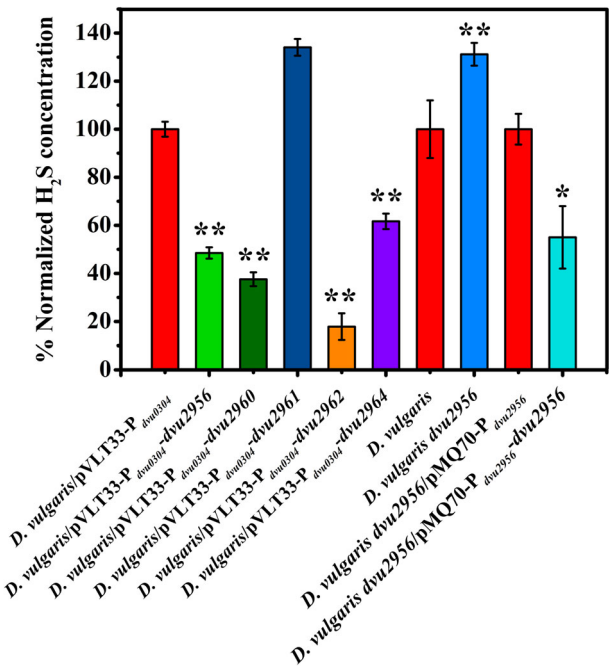

Fig. 4. DVU2956 inhibits H_2S formation. The percentage of normalized H_2S production in biofilms (ppm H_2S/turbidity at 620 nm) is shown compared to the empty plasmid controls (pVLT33-$P_{dvu0304}$ for biofilm phase production of DVU2956, DVU2960, DVU2961, DVU2962, DVU2964 and pMQ70-$P_{dvu2956}$ for gene *dvu2956* complementation, respectively) and to the wild-type strain (for the *dvu2956* knockout). The symbols * ($P < 0.05$) and ** ($P < 0.01$) indicate significant differences versus the empty plasmid control group via one-way ANOVA.

in reduced biofilm formation remain to be determined for these two regulators as well as the upstream trigger for DVU2956.

Although nitrogen sensing has been linked to the σ_{54}-dependent regulators DVU1949, DVU3220 and DVUA0143 (Kazakov *et al.*, 2015), and could be a possible upstream trigger for DVU2956, in our work, the RNA-seq data of *D. vulgaris* biofilm cells vs. planktonic cells showed that the transcription of genes encoding the chromosomal-based nitrogen regulators *dvu1949* and *dvu3220* was not significantly changed (–1.3 ± 0.1-fold and – 1.68 ± 0.03-fold, respectively). Transcription of plasmid-based nitrogen regulator *dvua0143* was repressed –39 ± 9-fold in biofilms; however, transcription of many plasmid genes was repressed in biofilms (Supporting Information Table S3). Critically, in the second set of RNA-seq data for the effect of production of DVU2956 in *D. vulgaris* biofilms, the transcription of the nitrogen regulators encoded by *dvu1949*, *dvu3220* and *dvua0143* were not significantly affected by DVU2956 (1.4 ± 0.3-, –1.7 ± 0.2- and 2.3 ± 0.8-fold, respectively). The transcription of other nitrogen-related genes was also not affected (e.g. *dvu0665*, 1.1 ± 0.1-fold and *dvu1232*, –2.2 ± 0.4-fold). Therefore, it is unlikely that DVU2956 senses nitrogen.

For the reduction in H_2S, we found production of the σ_{54}-dependent regulator DVU2956 in biofilms induces gene transcription from one of the four *D. vulgaris* hydrogenase gene operons (Heidelberg *et al.*, 2004), the Fe-only hydrogenase (Voordouw, 2002), and one of the five transmembrane electron circuits (Heidelberg *et al.*, 2004), the Hmc complex (Dolla *et al.*, 2000). *D. vulgaris* derives energy from oxidative phosphorylation by coupling H_2S production from the reduction of sulfate (or other sulfur oxyanions like sulfite or thiosulfate) with the oxidation of hydrogen to H^+. This process includes periplasmic hydrogen oxidation and electron transfer from *c3*-type cytochromes within the periplasm to a transmembrane electron circuit of the inner membrane. In the cytoplasm, the electrons are used to reduce sulfate or thiosulfate to H_2S (Heidelberg *et al.*, 2004). Since H_2S production is reduced by producing DVU2956, DVU2956 reduces H_2S by causing dysregulation of this H_2S production circuit.

In conclusion, we have identified a conserved biofilm and H_2S regulator, DVU2956, that controls SRB biofilm formation and H_2S production. Since biofilm formation and H_2S 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 sulfide (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 geneticin (G418) was used to maintain plasmids. *E. 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 $P_{dvu0304}$. 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.

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 $P_{dvu0304}$ (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 promotor *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 DVU2956, the *dvu2956* gene was amplified using forward primer sig54R-F-*Nde*I and reverse primer sig54R-R-*Hin*dIII, which introduce the *Nde*I and *Hin*dIII 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-*Hin*dIII, which introduce *Eco*RI and *Hin*dIII sites and a His-tag, and cloned into broad host range vector pMQ70 with inducible promotor *pBAD* (Table 4). To complement *D. vulgaris dvu2956* phenotypes, promoter of *dvu2956* was cloned into broad-host-range plasmid pMQ70 using primers Pdvu2956-F-*Hin*dIII and Pdvu2956-R-*Sal*I 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*I and dvu2956-R-*Eco*RI.

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-*dvu2956*i-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.

Strains and plasmids	Features	Source
Strains		
D. vulgaris Hildenborough	Wild-type, ATCC 29579	ATCC
D. desulfuricans	Wild-type, isolated from sulfidic mud	DSMZ (DSM-642)
E. coli TG1	K-12 *supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5*, $(r_K^- m_K^-)$	J. Minshull
E. coli BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	M. Nomura
Plasmids		
pVLT33	broad-host-range expression vector (IncQ, RSF1010 replicon), Km^r, *Ptac*, *lacIq tra*$^-$ *mob*$^+$	V. de Lorenzo
pBluescriptII (SK-)	*E. coli* vector, pUC ori, f1 (–) ori, Amp^r, *Plac*	Stratagene
pBSKan	*E. coli* vector, pUC ori, f1 (–) ori, Km^r, *Plac*	(Canada *et al*., 2002)
pET-27b(+)	P_{T7}, pBR322 ori, Km^R	Novagen®
pMQ70	*pBAD*, Car^R, shuttle vector	(Shanks *et al*., 2006)
pVLT33-$P_{dvu0304}$-*dvu2956*	$P_{dvu0304}$::*dvu2956*, RSF1010 replicon, Km^r, *Ptac*, *lacIq tra*$^-$ *mob*$^+$	This work
pVLT33-$P_{dvu0304}$-*dvu2960*	$P_{dvu0304}$::*dvu2960*, RSF1010 replicon, Km^r, *Ptac*, *lacIq tra*$^-$ *mob*$^+$	This work
pVLT33-$P_{dvu0304}$-*dvu2961*	$P_{dvu0304}$::*dvu2961*, RSF1010 replicon, Km^r, *Ptac*, *lacIq tra*$^-$ *mob*$^+$	This work
pVLT33-$P_{dvu0304}$-*dvu2962*	$P_{dvu0304}$::*dvu2962*, RSF1010 replicon, Km^r, *Ptac*, *lacIq tra*$^-$ *mob*$^+$	This work
pVLT33-$P_{dvu0304}$-*dvu2964*	$P_{dvu0304}$::*dvu2964*, RSF1010 replicon, Km^r, *Ptac*, *lacIq tra*$^-$ *mob*$^+$	This work
pVLT33-P_{tac}-*dvu2956*	P*tac*::*dvu2956*, RSF1010 replicon, Km^r, *lacIq tra*$^-$ *mob*$^+$	This work
pBluescriptII (SK-)-*Δdvu2956*	pBluescriptII (SK-)- *Δdvu2956* Ω Km^r	This work
pET27b-*dvu2956*	PT7::*dvu2956*, pBR322 ori, Km^R	This work
pMQ70-*dvu2956*i-His	*pBAD*:*dvu2956*, Car^R	This work
pMQ70-$P_{dvu2956}$	$P_{dvu2956}$, Car^R	This work
pMQ70-$P_{dvu2956}$–*dvu2956*	$P_{dvu2956}$:*dvu2956*, Car^R	This work

Km^R, Amp^R and Car^R are kanamycin, ampicillin and carbenicillin resistance, respectively.

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-*Hin*dIIIR2, 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-$P_{dvu0304}$-*dvu2956* and pVLT33-$P_{dvu0304}$ 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-$P_{dvu2956}$-*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× 400 ml of Baar's modified medium was inoculated with this culture (initial turbidity of 0.1). This medium was added to 3 × 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× 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

(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 cell turbidity at 620 nm.

For biofilm dispersal, after 24 h of biofilm formation, arabinose (10 mM) was added to 96-well plates to produce DVU2956. The biofilm was incubated anaerobically for another 24 h and quantified as for the biofilm inhibition experiments.

H_2S assay

H_2S 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 methylthioninium chloride (methylene blue) by reacting with H_2S dissolved in hydrochloride 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 $FeCl_3{\cdot}6H_2O$ (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 H_2S assay, sealed glass vials were used to prevent H_2S 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 H_2S 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 $FeCl_3$ were added and the mixture was shaken for 20 min at 300 rpm at room temperature. H_2O (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*i-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 β-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 sonicating 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 at room temperature for 15 min, and covered by aluminium-foil to avoid light. During the microscope observation, 485/500 nm was used for fluorescence excitation/emission. Two independent cultures were used for each strain.

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 have been submitted to the NCBI Sequence Read Archive (SRA) database (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?) 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

Beech, I.B., and Sunner, J.A. (2007) Sulphate-reducing bacteria and their role in corrosion of ferrous materials. In *Sulphate-Reducing Bacteria: Environmental and Engineered Systems*, Barton, L.L., and Hamilton, W.A. (eds). Cambridge, UK: Cambridge University Press, pp. 459–482.

Blair, D.F., and Berg, H.C. (1990) The MotA protein of *E. coli* is a proton-conducting component of the flagellar motor. *Cell* **60**: 439–449.

Bush, M., and Dixon, R. (2012) The role of bacterial enhancer binding proteins as specialized activators of sigma54-dependent transcription. *Microbiol Mol Biol Rev* **76**: 497–529.

Canada, K.A., Iwashita, S., Shim, H., and Wood, T.K. (2002) Directed evolution of toluene ortho-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. *J Bacteriol* **184**: 344–349.

Clark, M.E., Edelmann, R.E., Duley, M.L., Wall, J.D., and Fields, M.W. (2007) Biofilm formation in *Desulfovibrio vulgaris* Hildenborough is dependent upon protein filaments. *Environ Microbiol* **9**: 2844–2854.

Clark, M.E., He, Z., Redding, A.M., Joachimiak, M.P., Keasling, J.D., Zhou, J.Z., *et al.* (2012) Transcriptomic and proteomic analyses of *Desulfovibrio vulgaris* biofilms: carbon and energy flow contribute to the distinct biofilm growth state. *BMC Genomics* **13**: 138.

Dolla, A., Pohorelic, B.K., Voordouw, J.K., and Voordouw, G. (2000) Deletion of the *hmc* operon of *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough hampers hydrogen metabolism and low-redox-potential niche establishment. *Arch Microbiol* **174**: 143–151.

Fletcher, M. (1977) The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. *Can J Microbiol* **23**: 1–6.

Galperin, M.Y. (2010) Diversity of structure and function of response regulator output domains. *Curr Opin Microbiol* **13**: 150–159.

Heidelberg, J.F., Seshadri, R., Haveman, S.A., Hemme, C. L., Paulsen, I.T., Kolonay, J.F., *et al.* (2004) The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Nat Biotechnol* **22**: 554–559.

Jenal, U., and Galperin, M.Y. (2009) Single domain response regulators: molecular switches with emerging roles in cell organization and dynamics. *Curr Opin Microbiol* **12**: 152–160.

Kazakov, A.E., Rajeev, L., Chen, A., Luning, E.G., Dubchak, I., Mukhopadhyay, A., and Novichkov, P.S. (2015) sigma54-dependent regulome in *Desulfovibrio vulgaris* Hildenborough. *BMC Genomics* **16**: 919.

Koch, G., Varney, J., Thompson, N., Moghissi, O., Gould, M., and Payer, J. (2016) *International Measures of Prevention, Application, and Economics of Corrosion Technologies Study*. Houstan, TX: NACE International.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* **35**: 1547–1549.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., *et al.* (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948.

de Lorenzo, V., Eltis, L., Kessler, B., and Timmis, K.N. (1993) Analysis of *Pseudomonas* gene products using lacIq/Ptrp-lac plasmids and transposons that confer conditional phenotypes. *Gene* **123**: 17–24.

Matta, M.K., Lioliou, E.E., Panagiotidis, C.H., Kyriakidis, D. A., and Panagiotidis, C.A. (2007) Interactions of the antizyme AtoC with regulatory elements of the *Escherichia coli* atoDAEB operon. *J Bacteriol* **189**: 6324–6332.

Moglich, A., Ayers, R.A., and Moffat, K. (2009) Structure and signaling mechanism of per-ARNT-Sim domains. *Structure* **17**: 1282–1294.

Petrova, O.E., and Sauer, K. (2012) PAS domain residues and prosthetic group involved in BdlA-dependent dispersion response by *Pseudomonas aeruginosa* biofilms. *J Bacteriol* **194**: 5817–5828.

Poosarla, V.G., Wood, T.L., Zhu, L., Miller, D.S., Yin, B., and Wood, T.K. (2017) Dispersal and inhibitory roles of mannose, 2-deoxy-*D*-glucose, and *N*-acetylgalactosaminidase on the biofilm of *Desulfovibrio vulgaris*. *Environ Microbiol Rep* **9**: 779–787.

Rabinowitz, J.C. (1978) Analysis of acid-labile sulfide and sulfhydryl groups. *Methods Enzymol* **53**: 275–277.

Rajeev, L., Luning, E.G., Dehal, P.S., Price, M.N., Arkin, A. P., and Mukhopadhyay, A. (2011) Systematic mapping of two component response regulators to gene targets in a model sulfate reducing bacterium. *Genome Biol* **12**: R99.

Reitzer, L., and Schneider, B.L. (2001) Metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. *Microbiol Mol Biol Rev* **65**: 422–444.

Shanks, R.M., Caiazza, N.C., Hinsa, S.M., Toutain, C.M., and O'Toole, G.A. (2006) *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from gram-negative bacteria. *Appl Environ Microbiol* **72**: 5027–5036.

Taylor, B.L., and Zhulin, I.B. (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* **63**: 479–506.

Voordouw, G. (2002) Carbon monoxide cycling by *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol* **184**: 5903–5911.

Zhang, W., Culley, D.E., Nie, L., and Scholten, J.C. (2007) Comparative transcriptome analysis of *Desulfovibrio vulgaris* grown in planktonic culture and mature biofilm on a steel surface. *Appl Microbiol Biotechnol* **76**: 447–457.

Zhu, L., Poosarla, V.G., Song, S., Wood, T.L., Miller, D.S., Yin, B., and Wood, T.K. (2018) Glycoside hydrolase DisH from *Desulfovibrio vulgaris* degrades the *N*-acetylgalactosamine component of diverse biofilms. *Environ Microbiol* **20**: 2026–2037. https://doi.org/10.1111/1462-2920.14064

Zimmer, D.P., Soupene, E., Lee, H.L., Wendisch, V.F., Khodursky, A.B., Peter, B.J., *et al.* (2000) Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. *Proc Natl Acad Sci U S A* **97**: 14674–14679.

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