Reduction of alternative electron acceptors drives biofilm formation in *Shewanella algae*

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Shewanella spp. possess a broad respiratory versatility, which contributes to the occupation of hypoxic and anoxic environmental or host-associated niches. Here, we observe a strain-specific induction of biofilm formation in response to supplementation with the anaerobic electron acceptors dimethyl sulfoxide (DMSO) and nitrate in a panel of *Shewanella algae* isolates. The respiration-driven biofilm response is not observed in DMSO and nitrate reductase deletion mutants of the type strain *S. algae* CECT 5071, and can be restored upon complementation with the corresponding reductase operon(s) but not by an operon containing a catalytically inactive nitrate reductase. The distinct transcriptional changes, proportional to the effect of these compounds on biofilm formation, include cyclic di-GMP (c-di-GMP) turnover genes. In support, ectopic expression of the c-di-GMP phosphodiesterase YhjH of *Salmonella* Typhimurium but not its catalytically inactive variant decreased biofilm formation. The respiration-dependent biofilm response of *S. algae* may permit differential colonization of environmental or host niches.

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INTRODUCTION

A hallmark of species of the genus *Shewanella* is their remarkable respiratory versatility since they can respire on an array of organic and inorganic compounds comprising virtually any electron acceptor more electronegative than sulfate. The respiratory capacity of *Shewanella* is reinforced by mediator-directed electron transfer mechanisms enabling the efficient reduction of insoluble substrates such as metal oxides. This respiratory flexibility results in an extraordinary physiological versatility that contributes to the environmental abundance of the chemoorganotroph *shewanellae* as versatile colonizers of oxic, hypoxic, and anoxic marine and freshwater habitats. Some species, primarily *Shewanella algae* and close relatives such as *Shewanella chilensis*, have been associated with human disease.

Biofilm formation promotes the colonization of biotic and abiotic substrata in *Shewanella* spp. and many other bacterial species. This predominantly sedentary lifestyle is a developmental process initiated by an attachment of single cells that subsequently form microcolonies prior to the establishment of mature biofilms. The intracellular second messenger cyclic di-γ-phosphate (c-di-GMP) is known to play a key role in the lifestyle switch from planktonic to sessile and vice versa in a plethora of Gram-negative species. Integration of extra- and intracellular stimuli modulates different features of this multicellular microbial lifestyle in response to changes in nutrient availability, temperature, oxygen tension, quorum sensing, and various chemical signals. For example, reduced oxygen tension differentially regulates biofilm formation in *Shewanella oneidensis* MR-1 and *Shewanella putrefaciens* CN32. For *Shewanella* spp., life within a biofilm provides structural and functional advantages, spanning from maintenance of redox balance to enhanced extracellular electron transfer. *Shewanella* spp. thrive in hypoxic and anoxic environments including sediments, hypoxic water bodies, and the intestinal tract of fish and invertebrates, where these non-fermentative species use some of the most abundant inorganic and organic alternative electron acceptors (AEAs). While biofilm formation on insoluble metal oxides has been well documented, knowledge on the behavioral and physiological biofilm responses upon use of the broad array of AEAs is limited in *Shewanella* species, as is the repertoire of terminal reductases enabling the respiration of AEAs.

Here we show that supplementation of a seawater-mimicking medium with organic or inorganic AEAs induces differential, strain-specific biofilm formation in *S. algae*. Assessment of the respiratory capacity of the strains combined with mutant analyses of the type strain *S. algae* CECT 5071 demonstrated that respiration is required, but not sufficient per se to increase biofilm production upon AEA supplementation. Transcriptome analyses showed that the number of differentially regulated genes positively correlated with the amount of biofilm formed by *S. algae* CECT 5071 upon addition of the electron acceptors DMSO and nitrate. Several of the differentially regulated genes encoded proteins that participate in c-di-GMP turnover. Altogether, our study suggests that differential biofilm formation in *S. algae* strains is governed beyond respiration by the induction of distinct biofilm developmental programs.

RESULTS

Alternative electron acceptors promote strain-specific biofilm formation in *S. algae*

While screening chemical libraries for biofilm inhibitors, we noticed that an addition of 35 mM DMSO enhanced biofilm formation of *S. algae* CECT 5071 static cultures ~2-fold compared to the non-supplemented control. Since a similar phenomenon had not been observed with other DMSO-respiring bacteria, we reasoned that this phenotype could be specific to *S. algae*.

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We therefore analyzed DMSO-mediated biofilm formation in a selection of 20 S. algae strains from various environmental and clinical sources (Supplementary Table 1). Biofilm formation in plain Marine Broth (MB) varied substantially between isolates, from poor biofilm formers to proficient biofilm producers (Fig. 1a and Supplementary Fig. 1). Biofilm formation values in MB were taken as baseline levels for comparisons upon AEA supplementation. Thus, for 9 of the 20 strains we observed a more than 1.5-fold induction of biofilm formation in response to DMSO (Fig. 1a and Supplementary Fig. 2).

Since the respiratory repertoire of Shewanella spp. is broad, we wondered whether other AEAs provoked a similar strain-specific biofilm response. To that end, we analyzed the biofilm response of the type strain S. algae CECT 5071 and the other 20 S. algae isolates towards 35 mM nitrate, another representative AEA during anaerobic respiration (Fig. 1c and Supplementary Fig. 3). With an increase of more than 4-fold, S. algae CECT 5071 showed the most pronounced increase in biofilm formation as determined by CV staining. Further 9 strains, 2 of which are different to those reported above for DMSO, responded with increased biofilm formation to nitrate supplementation.

To ensure that the observed biofilm induction was not due to pH changes upon utilization of the different AEAs, experiments were validated by testing the type strain S. algae CECT 5071 in the absence or presence of AEAs in MB buffered by 30 mM HEPES pH = 7.5, which minimized pH oscillations during 24-h static incubation (Supplementary Fig. 4a). The corresponding results were similar to those recorded in unbuffered medium (Supplementary Fig. 4b) showing that AEA-induced changes in biofilm formation are not caused by pH changes. Taken together, these results indicate that DMSO and nitrate-induced biofilm formation is a strain-specific phenotype independent of pH.

**DMSO respiration is not conserved in S. algae**

We hypothesized that respiration of the supplemented compounds triggered the biofilm formation response. However, we wondered whether the respiratory activity differed for strains that responded with enhanced biofilm formation. To answer this question, we qualitatively assessed nitrate reductase activity in S. algae strains by measuring the accumulation of nitrite in static cultures supplemented with 35 mM nitrate. A straightforward colorimetric assay with endpoint determination of nitrate reductase activity in live bacteria showed similar overall nitrate reductase activity in all strains (Fig. 2a). This indicates that the biofilm formation response is not related to an intrinsic capacity of a given strain to reduce the supplemented AEA.

The genomes of these and other S. algae strains have been sequenced as part of ongoing comparative genomics studies (Martín-Rodríguez et al., unpublished). In S. algae, nitrate reduction occurs in the periplasm and relies in a duo-NAP system, comprised of NapA-α (encoded by the napEDAB operon) and NapA-β (encoded by the napDAGHB operon), being NapA-α and NapA-β the major catalytic subunits, respectively. To investigate genomic determinants for the observed biofilm phenotypes, BLASTp searches were conducted to identify CECT 5071 NapA-α and NapA-β orthologs in the S. algae genomes used. Both components of the NAP system were present in all S. algae genomes analyzed, with amino acid sequence identities between 97.1–99.9% for NapA-α and 98.1–99.1% for NapA-β with respect to the corresponding protein in the type strain (data not shown), thus indicating that the two nitrate reductases are conserved across S. algae isolates.

The terminal DMSO reductase DmsEFABGH is involved in extracellular DMSO respiration and uses several sulfoxides and

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**Fig. 1 Biofilm formation response of S. algae strains in the absence or presence of supplemented alternative electron acceptors.** a Biofilm formation for 21 S. algae strains in MB medium in the absence of added electron acceptors. b Heatmap representing the fold-change in biofilm formation with respect to the corresponding non-supplemented control upon addition of 35 mM DMSO. c Heatmap representing the fold-change in biofilm formation with respect to the corresponding non-supplemented control upon addition of 35 mM sodium nitrate. Data represent the average of two biological replicates with six technical replicates each.
N-oxides as substrates\textsuperscript{21,22}, with DmsA being the major catalytic subunit. We next performed BLASTp searches of \textit{S. algae} CECT 5071 DmsA orthologs to identify the presence of DMSO reductases in our sequenced \textit{S. algae} genomes. We could retrieve hits for all \textit{S. algae} strains except for A41, A57, and 97087. Genes predicted to encode the \textit{dmsEFABGH} operon are located between \textit{accB} and \textit{rpmE} on the \textit{S. algae} CECT 5071 chromosome. Inspection of the WGS of strains \textit{S. algae} A41, \textit{S. algae} A57, and \textit{S. algae} 97087 revealed absence of this operon (data not shown). Besides, DmsA orthologs with a significantly lower sequence identity were found in \textit{S. algae} A56 and \textit{S. algae} CCUG 58400 (not used in this study, originally described as \textit{Shewanella upenei}\textsuperscript{23} but demonstrated to be a later heterotypic synonym of \textit{S. algae}\textsuperscript{24}) that shared only modest sequence identity (55.8% both) with the reference protein. Interestingly, their sequence was almost identical to the DmsA homolog of \textit{S. chilikensis} (GenBank accession: WP\_165565699.1). A maximum-likelihood reconstruction of the evolutionary relationships of \textit{S. algae} DmsA in relation with other \textit{Shewanella} spp. DmsA orthologs is presented in Fig. 2b. DmsA orthologs from \textit{S. algae} A56 and \textit{S. algae} CCUG 58400 are within a well-supported separate clade, that is only distantly related to the housekeeping \textit{S. algae} DmsA. Altogether, these findings suggest DMSO respiration is a variable, potentially adaptive feature in \textit{S. algae}, with evidence of selective loss or acquisition from other \textit{Shewanella} spp.

**Surface-associated electron acceptors enhance \textit{S. algae} substrate colonization**

Since \textit{S. algae} encounters diverse AEs in its native ecosystems, we hypothesized that biofilm formation could be a selective response promoting bacterial colonization of distinct substrata that are abundant in AEs, a process that could lead to differential ecological niche occupation by distinct isolates. To test this hypothesis, we selected DMSO and nitrate as two representative AEs and two \textit{S. algae} strains, the DMSO and nitrate responder \textit{S. algae} CECT 5071 and the non-responder \textit{S. algae} A291. To mimic an environmentally relevant situation, the AEs were incorporated into an agarose pad (Fig. 3a), and biofilm formation of each strain was examined using non-supplemented agarose as a control. The release of immobilized AEs from the solid agarose pad could be visualized using the colored cobalt (II) nitrate as illustrated in Fig. 3b. \textit{S. algae} CECT 5071 exhibited substantially increased biofilm formation on the agarose surface upon incorporation of DMSO or nitrate (Fig. 3c), as observed with supplemented medium. Consistently, while a monolayer of cells was observed in non-supplemented agarose, elaborated biofilm
structures were observed primarily in the presence of nitrate and to a lesser degree with DMSO. Conversely, non-responsive *S. algae* A291 showed a similar low biofilm formation compared to the non-supplemented agarose pad irrespective of whether DMSO or nitrate had been added to the agarose (Fig. 3c). Thus, induction of biofilm formation is preserved upon incorporation of soluble AEAs into an agarose pad and their gradual release from a surface. This experimental set-up mimics environmentally relevant situations *S. algae* may find, for example, when bacteria encounter decaying organic matter in the aquatic milieu or on the surface of organisms such as fish or algae.

**Respiration and biofilm formation are intimately related in *S. algae***

To investigate the physiological basis of AEA-induced biofilm formation in *S. algae* CECT 5071, we next questioned whether respiration of the supplemented AEA is required for biofilm formation. To provide experimental evidence of an association between biofilm formation and reduction of AEAs, we first deleted the *dmsB* gene encoding the DMSO reductase subunit B of the DmsEFABGH reductase (Fig. 4a). To assess DMSO reduction by the WT strain and its Δ*dmsB* mutant derivative, we quantified the generation of dimethylsulfide (DMS), the product of DMSO...
Disruption of terminal reductase activity abrogates the biofilm response. 

**A** Schematic of the DMSO reductase encoding *dmsEFABGH* operon of *Shewanella algae*. 

**B** DMSO reduction of the WT strain and *dmsB* in-frame deletion mutant (**P < 0.0001, two-tailed unpaired t test**) as evidenced by measuring DMS production of three independent cultures supplemented with 35 mM DMSO. 

**C** Biofilm formation of *S. algae* CECT 5071 WT and Δ*dmsB* in the absence or presence of DMSO (**P < 0.0001, two-tailed unpaired t test**). Data represent the average and SD of three biological replicates with seven technical replicates each. 

**D** Schematic of the periplasmic nitrate reductase NAP-α encoding operon *napEDABC* and NAP-β encoding operon *napDAGHB* of *S. algae*. 

**E** Nitrate reduction by the single mutants Δ*napABC* (α−) and Δ*napA* (β−), and the double mutant Δ*napABC* Δ*napA* (αβ−) as determined by nitrite accumulation with respect to the WT strain upon supplementation with 35 mM nitrate (**P < 0.0001; one-way ANOVA followed by Dunnett’s post-hoc test; ND no nitrite production detected**). Data represent the average and SD of three biological replicates with four technical replicates per sample. 

**F** Biofilm formation of *S. algae* CECT 5071 WT and nitrate reduction mutants in the absence or presence of sodium nitrate (**P < 0.0001, one-way ANOVA followed by Dunnett’s post-hoc test**).
respiration, in static cultures supplemented with 35 mM DMSO. Deletion of \textit{dmsB} impaired the capacity to reduce DMSO by >75% (Fig. 4b) as determined by GC-MS.

We next studied biofilm formation in static cultures of the WT and mutant strain in MB in the absence or presence of 35 mM DMSO. In the absence of DMSO, both the WT strain and its \textit{ΔdmsB} mutant exhibited similar biofilm formation and growth patterns; the latter characterized by preferential growth as a pellicle at the air–liquid interface (Fig. 4c and Supplementary Fig. 5a). In the presence of DMSO, WT growth was stimulated as compared to the non-supplemented control and occurred at the air–liquid interface as well as in the lower layers of the well. In contrast, growth of the \textit{ΔdmsB} mutant in the presence of DMSO was similar to that in non-supplemented MB (Supplementary Fig. 5a). Biofilm formation in the WT was increased ~2-fold with respect to non-supplemented cultures as previously observed (Fig. 4c), an increase caused by higher biofilm formation on the bottom of the well (Supplementary Fig. 5a), whereas the \textit{ΔdmsB} mutant generated a similar amount of biofilm compared to the control without DMSO (Fig. 4c and Supplementary Fig. 5a), indicating that DMSO respiration is required for the increase in relative biofilm formation upon supplementation with DMSO.

In \textit{S. algae} CECT 5071 supplementation of MB with nitrate caused a more pronounced increase in biofilm formation than supplementation with DMSO (Fig. 3c). To extend the hypothesis of respiration-driven biofilm formation in \textit{S. algae}, we deleted the nitrate reductase subunits A–C (\textit{napABC}) from \textit{NapA}, and the nitrate reductase subunit \textit{napA} from \textit{NapA–β} (Fig. 4d) in the CECT 5071 genetic background that resulted in the single in-frame deletion mutants \textit{napA}ΔABC (hereafter referred to as \textit{napA}Δ) and \textit{napA}Δ (hereafter referred to as \textit{napA}Δβ), and the double deletion mutant \textit{napABC}ΔnapA (hereafter referred to as \textit{napA}ΔβΔ). To assess the capacity of the WT versus the mutant strains to respire nitrate, we measured the production of nitrite, the product of nitrate reduction, in static cultures supplemented with 35 mM nitrate. Deletion of \textit{NapA} genes reduced nitrite accumulation by almost 80%, whereas deletion of \textit{NapA–β} genes did not significantly affect nitrate reduction in comparison to the WT (Fig. 4e). Thus, the catalytic activity of \textit{NapA} represents the major fraction of the nitrate reduction capacity compared to \textit{NapA–β} under the assay conditions used for \textit{S. algae} CECT 5071. Deletion of \textit{napABC} and \textit{napA} rendered \textit{S. algae} unable to produce nitrite upon addition of nitrate (Fig. 4e).

We next analyzed biofilm formation of the mutants in static cultures with and without 35 mM nitrate. In the absence of nitrate, the WT and all mutants formed a similar amount of biofilm (Fig. 4f), and the patterns of growth and biofilm formation on the walls and bottom of the wells were also similar (Supplementary Fig. 5b). In the presence of nitrate, growth in the anoxic layers of the wells was observed for the WT and mutants lacking a functional \textit{NapA} or \textit{NapA–β}, but not in the nitrate reduction null mutant \textit{napA}ΔβΔ (Supplementary Fig. 5b). While biofilm formation in the \textit{NapA–β} mutant was not significantly different from that of the WT strain, the \textit{NapA} mutant formed significantly more biofilm than the WT (Fig. 4f), an increase that was due to higher biofilm formation on the bottom of the well as visually determined by CV staining (Supplementary Fig. 5b). In contrast, the double mutant \textit{napA}ΔβΔ, unable to reduce nitrate, did not respond to nitrate supplementation with increased biofilm formation with respect to non-supplemented cultures (Fig. 4f and Supplementary Fig. 5b). This indicates that, in analogy to DMSO, nitrate respiration drives biofilm formation in \textit{S. algae} CECT 5071, and suggests that each nitrate reductase plays a distinct role on biofilm formation upon nitrate addition.

\textbf{Reductase activity is required to elicit biofilm formation}

So far, our genetic analyses demonstrate that respiration of AEAs can selectively act as driver for biofilm formation. In WT \textit{S. algae} CECT 5071, AEA-induced biofilm formation is dose-dependent, as shown by dose-response measurements up to a DMSO or nitrate concentration of 70 mM (Supplementary Fig. 6). These experiments also showed that the onset of AEA mediated stimulation of biofilm formation was at ~4 mM for nitrate and 8 mM for DMSO. The \textit{ΔdmsB} mutant did not respond with enhanced biofilm formation upon addition of DMSO. In contrast, single mutants of nitrate reductases \textit{napA–α} (\textit{ΔnapABC}) and \textit{napA–β} (\textit{ΔnapA}) showed a dose-dependent response. As expected, the \textit{αβ}− mutant did not respond to nitrate.

To demonstrate that the mutant phenotypes were specific for the deletion of reductase subunits and not a consequence of secondary mutations, we complemented the \textit{ΔdmsB} mutant with the \textit{dmsEFABGH} operon cloned under the \textit{lac} promoter in the pSRK-Km vector. In the complemented \textit{ΔdmsB} mutant, DMS production was duplicated with respect to WT levels (Fig. 5a and Supplementary Fig. 7), compatible with overexpression of the reductase upon \textit{β−1-thiogalactopyranoside (IPTG)} induction. The introduction of the empty pSRK-Km plasmid in the WT or \textit{ΔdmsB} strains did not significantly change DMS production with respect to the corresponding parental strains (data not shown). Introduction of the empty vector pSRK-Km per se increased biofilm formation, leading to a higher baseline level of biofilm formation (Fig. 5b and Supplementary Fig. 8) that masked the effect of DMSO addition. While no significant differences were observed between biofilm levels of the WT and \textit{ΔdmsB} harboring the empty plasmid, the complemented mutant though showed increased biofilm formation (Fig. 5b). This could also be qualitatively assessed visually as the WT and \textit{ΔdmsB pSRK-Km:ΔdmsEFABGH} strains tended to form more biofilm on the bottom of the wells in the presence of DMSO, although the effect of the electron acceptor was negligible compared to the high level of biofilm formation at the air–liquid interface caused by plasmid introduction (Fig. 5c).

To complement the nitrate reductase mutants \textit{α−}, \textit{β−}, and \textit{αβ−}, individual \textit{napEDABC} and \textit{napDAGHB} operons and both operons in tandem were cloned under the \textit{lac} promoter into the pSRK-Km vector. Nitrate reductase activity was partially restored to ~40% by ectopic expression of \textit{napEDABC} in the \textit{α−} mutant with low levels of nitrate reductase (Fig. 5d). Nitrate reductase activity of the \textit{β−} mutant, which presented a nitrate reduction capacity similar to the WT, was significantly increased upon expression of \textit{napDAGHB}. Complementation of the \textit{αβ−} strain with \textit{napDAGHB} only marginally restored nitrate reductase activity, whereas expression of \textit{napEDABC}, or both \textit{napOAGHB} and \textit{napEDABC}, restored nitrate reductase activity to ~40% and 60% of the WT levels, respectively. As the induction of biofilm formation caused by nitrate is higher than that caused by DMSO, the effect of nitrate supplementation was not abrogated by plasmid introduction. Thus, complemented \textit{α−} and \textit{β−} mutants showed similar biofilm formation phenotypes than the WT strain upon nitrate supplementation (Fig. 5e).

Partial complementation of the WT biofilm phenotype upon nitrate addition was recorded for the \textit{αβ− pSRK-Km:ΔnapA} strain, whereas levels comparable to the WT were recorded for the \textit{αβ−} pSRK-Km:\textit{napA–α} and \textit{αβ−} pSRK-Km:\textit{napA–β} strains (Fig. 5e).

To demonstrate the specificity of our results, we engineered a \textit{napEDABC operon} containing a catalytically inactive \textit{NapA} reductase by replacing three of the four cysteines, Cys48, Cys51, and Cys55 responsible for coordination of the 4Fe–4S cluster of \textit{NapA}, with serine. Overexpression of the resulting mutant \textit{NapA–α} in the pSRK-Km plasmid in the \textit{αβ−} background did not restore nitrite production (Fig. 5d), and biofilm formation remained at the \textit{αβ−} mutant levels upon supplementation with nitrate (Fig. 5e).

This demonstrates that the reductase activity, and not the mere expression of the reductase per se in the periplasm, triggers the biofilm formation response.
Fig. 5  Ectopic expression of terminal reductases restores catalytic activity and biofilm formation.  

a Restoration of DMSO reductase activity as determined by DMS production in the ΔdmsB mutant upon overexpression of the dmsEFAGHB operon (average ± SD, n = 3) in comparison to the WT and ΔdmsB strains harboring the empty expression plasmid pSRK-Km (****P < 0.0001; one-way ANOVA followed by Tukey’s post-hoc test).  

b Biofilm formation of the complemented ΔdmsB mutant and the corresponding WT and ΔdmsB empty pSRK-Km vector controls. Data represent the average and SD of three biological replicates with seven technical replicates per sample. Statistical significance of the differences was determined by one-way ANOVA followed by Tukey’s post-hoc test (***P < 0.001, ns not significant).  

c Biofilm formation patterns on the walls and bottom of the wells of S. algae WT pSRK-Km, S. algae ΔdmsB pSRK-Km, and complemented S. algae ΔdmsB pSRK-Km-dmsEFABGH as visually assessed by CV staining. Note that biofilm formation differences on the bottom of the wells by the WT harboring the empty plasmid pSRK-Km and complemented mutant with respect to the ΔdmsB mutant harboring the empty expression vector are not apparent from the quantitative determinations shown on b because of the vector effect.  

d Nitrate reductase activity in complemented α−, β−, and αβ− mutants as determined by nitrite production, as well as in a catalytic napEDABC mutant in which three of the four cysteines of the 4Fe-4S cluster of NapA had been replaced by serine. Data represent the average and SD of three biological replicates with four technical replicates per sample. Statistical significance of the differences was determined by one-way ANOVA followed by Tukey’s post-hoc test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ND no nitrite production detected).  

e Biofilm formation of complemented nitrate reduction mutants and the corresponding WT and nitrate reduction null strains harboring the empty pSRK-Km plasmid, and the catalytic mutant. Data represent the average and SD of three biological replicates with seven technical replicates each. Statistical significance of the differences was determined by one-way ANOVA followed by Tukey’s post-hoc test.
Global transcriptional changes induced by alternative electron acceptors

To gain a deeper understanding of the physiological responses induced by different AEAs, transcriptional profiles were obtained from *S. algae* CECT 5071 grown in static culture in the absence or presence of 35 mM DMSO or nitrate (Supplementary Data 1). Statistically significant changes in transcript levels (P-adj ≤ 0.05) of a total of 3553 genes were noted in *S. algae* CECT 5071 exposed to DMSO or nitrate (Fig. 6a). There was a tendency between the number of transcripts changed in the presence of a given AEA and the corresponding effect on biofilm formation, since more genes had altered transcript levels in the presence of nitrate as compared to DMSO. Thus, there were 1573 genes with altered transcript levels in the presence of DMSO (Fig. 6b–d; 798 upregulated and 775 downregulated) and 3254 in the presence of nitrate (Fig. 6b, c, e; 1594 upregulated and 1660 downregulated). There was a substantial overlap in the transcriptional response to both AEAs, with 1323 genes having altered transcript levels (either upregulated or downregulated) in either condition (Fig. 6a).

Next, genes were classified according to functionality in clusters of orthologous genes (COGs). In the DMSO response, the highest number of upregulated and downregulated transcripts (132 genes, 65 upregulated, and 67 downregulated) belonged to the COG category signal transduction mechanisms [T] (Fig. 6f). This was also the case for nitrate, that modulated transcript levels of 273 genes belonging to this category (136 upregulated and 137 downregulated, Fig. 6g). These data suggest that respiration of AEAs involves downstream intracellular signaling events resulting in increased biofilm formation, consistent with the transcriptional landscape described above. An enrichment analysis using PANTHER<sup>®</sup> showed a significant enrichment (P < 0.05) for the ‘sulfate assimilation’ and ‘de novo purine biosynthesis’ categories for both treatments, although the high number of transcripts showing altered expression levels in the presence of DMSO and nitrate suggest that both electron acceptors elicit global physiological changes. We also classified upregulated and downregulated transcripts for each condition into gene ontology (GO) categories. GO terms of the categories “biological processes” and “molecular function” are presented for DMSO (Supplementary Fig. 9a, b) and nitrate (Supplementary Fig. 9c, d). In both treatments, the most populated GO terms under “biological processes” were metabolic processes (GO:0008152) and cellular processes (GO:0009987), irrespective of the directionality of the expression change, indicative of a major physiological reprogramming imposed by AEA supplementation. Likewise, “catalytic activity” (GO:0008324) was the most represented GO term of the “molecular function” category for both DMSO and nitrate supplementation, which is also consistent with the notion that both compounds induce a physiological re-adaptation.

A putative role of c-di-GMP signaling

As noted above, signal transduction mechanisms were highly affected by DMSO or nitrate supplementation. A hallmark of the genus *Shewanella* is the abundance of proteins that catalyze the synthesis or breakdown of c-di-GMP, suggesting a central role of c-di-GMP-mediated signaling in *Shewanella* biology. We identified 63 putative c-di-GMP turnover proteins in the genome of *S. algae* CECT 5071 as part of ongoing genomic studies (Martín-Rodríguez et al., in preparation). In the absence of specific gene nomenclature, we refer to the genes encoding these proteins with provisional generic names, but the corresponding GenBank locus tags are given in parentheses to unambiguously identify the genes. To investigate their transcriptional changes induced by DMSO or nitrate supplementation, we plotted the transcriptome of the 63 genes encoding putative diguanylate cyclases (DGCs) and c-di-GMP phosphodiesterases (PDEs) containing GGDEF/EAL/HD-GYP domains in a heatmap (Fig. 7a). This analysis revealed discrete changes caused by DMSO addition, with gene WT_00831 (E1N14_04100) encoding a PAS-GGDEF domain containing protein being the most differentially upregulated by 2.4-fold with respect to the control without electron acceptor supplementation. Nitrate supplementation caused more extensive changes in the c-di-GMP turnover transcriptome, with the putative DGCs WT_00655 (E1N14_03230) and WT_00831 (E1N14_04100) being the most differentially upregulated by 8.5- and 6.8-fold, respectively, with respect to control cultures. Another gene encoding a GGDEF-domain protein, WT_00826 (E1N14_04075) was the most downregulated, 0.14-fold, with respect to the control. Altogether, these data support the notion of an involvement of c-di-GMP signaling in respiration-mediated regulation of biofilm formation in *S. algae* CECT 5071.

We reasoned that if c-di-GMP signaling mediates AEA-dependent biofilm formation, a decrease of intracellular c-di-GMP pools would revert the phenotype upon AEA supplementation. To artificially alter c-di-GMP levels, the heterologous c-di-GMP PDE *YhjH* from *S. typhimurium* and the catalytically inactive derivative *YhjH* E136A were cloned in plasmid pBRR1MCS-2, introduced in *S. algae* CECT 5071, and the biofilm phenotypes of *YhjH* and *YhjH* E136A expressing strains were compared with respect to that of the control strain harboring the empty vector in strain MB and MB supplemented with 35 mM DMSO or nitrate (Fig. 7b). Expression of *YhjH* significantly decreased biofilm formation in MB, whereas biofilm formation of the strain expressing *YhjH* E136A was not significantly different than that of the vector control. In the presence of DMSO there were no statistically significant differences between the vector control and the *YhjH* or *YhjH* E136A expressing strains, although a trend towards lower biofilm formation was noted in the strain expressing the active PDE. In the presence of nitrate, *YhjH* expressing cells formed significantly lower biofilm than the vector control whereas cells expressing *YhjH* E136A formed statistically significantly higher biofilm. Taken together, these results suggest that a decrease of intracellular [c-di-GMP] decreases biofilm formation in static culture and counteracts to some extent the effect of AEA supplementation.

Finally, to validate RNA-seq results and to test the reproducibility of our data, RNA was isolated from static *S. algae* CECT 5071 cultures as performed for transcriptomic analysis, and the expression of six relevant genes was tested by qRT-PCR and compared to transcript levels obtained by RNA-seq: *dmsB* (E1N14_09855); *napA α* (E1N14_17675); *napA β* (E1N14_03175); *WT_00826* (E1N14_04075); *WT_00831* (E1N14_04100); and *WT_00655* (E1N14_03230) (Supplementary Fig. 10). The differential expression of these genes was similar in the transcriptomic and qRT-PCR analyses, which validates the RNA-seq data.

**DISCUSSION**

In this study we demonstrate a specific differential induction of biofilm formation in response to respiration in *S. algae*. We show that the presence and subsequent utilization of soluble electron acceptors in a seawater-mimicking growth medium induces biofilm formation in some but not all *S. algae* strains tested. Disruption of the corresponding respiratory pathway as well as mutant complementation with a catalytically inactive variant abolished the capacity of DMSO or nitrate to stimulate biofilm formation.

In *S. algae*, biofilm formation is strain-specifically upregulated upon supplementation with various electron acceptors. Thereby, respiration and biofilm formation are directly coupled as mutants in the respective terminal reductase did not respond with altered biofilm formation. A physiological link between cellular respiration and regulation of biofilm formation has been observed in other bacteria. In *Pseudomonas aeruginosa*, nitrate respiration has been found to contribute to biofilm formation and development under static growth<sup>20</sup>, while in *Burkholderia pseudomallei*, nitrate...
Transcriptomic profiles of *S. algae* CECT 5071 static cultures supplemented with DMSO or nitrate. Venn diagrams of (a) differentially expressed genes (P-adj < 0.05) in both treatments, (b) genes with upregulated transcript levels and (c) genes with downregulated transcript levels. Volcano plots showing alterations in gene transcript levels in static cultures supplemented with 35 mM DMSO (d) and nitrate (e). In red are the genes that are significantly differentially regulated (P-adj < 0.05) with a fold-change value below 2 or above 0.5. In orange are the rest of differentially expressed genes. Black indicates genes not significantly differentially regulated. COG assignment of differentially expressed genes in *S. algae* CECT 5071 upon addition of 35 mM DMSO (f) or sodium nitrate (g) versus control cultures without electron acceptor addition. COG categories: [A] RNA processing and modification; [B] Chromatin structure and dynamics; [C] Energy production and conversion; [D] Cell cycle control, cell division, chromosome partitioning; [E] Amino acid transport and metabolism; [F] Nucleotide transport and metabolism; [G] Carbohydrate transport and metabolism; [H] Coenzyme transport and metabolism; [I] Lipid transport and metabolism; [J] Translation, ribosomal structure and biogenesis; [K] Transcription; [L] Replication, recombination and repair; [M] Cell wall/membrane/envelope biogenesis; [N] Cell motility; [O] Post-translational modification, protein turnover, and chaperones; [P] Inorganic ion transport and metabolism; [Q] Secondary metabolites biosynthesis, transport, and catabolism; [R] General function prediction only; [S] Function unknown; [T] Signal transduction mechanisms; [U] Intracellular trafficking, secretion, and vesicular transport; [V] Defense mechanisms; [W] Extracellular structures; [Y] Nuclear structure; and [Z] Cytoskeleton.
respiration was found to inhibit biofilm formation by decreasing global c-di-GMP cellular pools. Anaerobic respiration has been reported to induce biofilm formation in Vibrio cholerae and Staphylococcus epidermidis, thereby promoting host colonization. In Staphylococcus aureus, biofilm formation is promoted under anoxic conditions in response to the oxidation state of the membrane quinone pool. Opposite roles for oxygen have been reported for other Shewanella spp. where low oxygen promoted c-di-GMP-mediated biofilm formation in S. putrefaciens CN32 or induced biofilm detachment in S. oneidensis MR-1. Altogether, these findings suggest the existence of an extensive, but variable interplay between biofilm formation and the nature of the electron acceptor in respiration.

In our study we used two representative electron acceptors, nitrate and DMSO. Our results implicate that the two periplasmic nitrate reductases of S. algae play different, non-redundant roles under nitrate-rich conditions. Thus, a mutant lacking a functional NAP-β does not show alterations in biofilm formation and global nitrate reductase activity in comparison to the WT as determined by nitrite assessment in culture supernatants, but a mutant lacking a functional NAP-α exhibits a strong impairment in nitrate reduction capacity and altered biofilm formation patterns. The greater contribution of NAP-α to nitrate reduction under static growth conditions is also reflected by a higher expression of this reductase than the other isoform, NAP-β. The nitrate reductase is a complex multidomain protein consisting of the core subunits NapA and NapB, the chaperone NapD and variable accessory subunits. NapA is the catalytic subunit containing a [4Fe-4S] cluster, whereas NapB shuttles electrons from the membrane electron transport protein CymA to NapA. Replacement of NapA-α Cys48, Cys51, and Cys55 involved in the coordination of the [4Fe-4S] cluster with serine was shown to prevent nitrate reduction and we show that a nitrate reduction-null mutant expressing such a catalytically deficient NapA-α is unable to induce biofilm formation.
formation upon nitrate supplementation. To the best of our knowledge, our work is the first to functionally analyze nitrate reductase activity (and their contribution to biofilm formation) in a Shewanella species harboring both NAP isoforms, as is the case for most shewanellaceae as shown by whole-genome sequencing, thus establishing a functional link between respiration and biofilm formation, two prima facie independent processes.

Like nitrate, DMSO also elicited strain-specific biofilm formation. Remarkably, genome sequence analysis of S. algae strains showed the DMSO reductase DmsEFABGH not to be conserved across the sequenced isolates, and in some cases, not present at all. In some S. algae isolates, high sequence identity of the catalytic subunit DmsA with orthologs from other Shewanella spp. was noted and demonstrated by phylogenetic analysis. The diversity of gene clusters encoding DMSO reductases in different Shewanella species has been shown. However, the intra-species variability of the DMSO reductase operon indicates that DMSO respiration is under strong evolutionary pressure in S. algae. Loss or lateral acquisition of respiratory features by S. algae from other Shewanella spp. points to currently unexplored ecological and physiological advantages.

The ability to reduce a given electron acceptor was not necessarily associated with increased biofilm formation. As illustrated for nitrate, while all S. algae isolates reduced nitrate to nitrite, not all of them responded with enhanced biofilm formation indicating that AEA sensing with subsequent upregulation of biofilm formation can be functionally uncoupled from AEA respiration. Sensory domains in signal transduction systems have been reported to detect nitrate and directly couple it to enhanced respiration. Sensory domains in signal transduction systems have been shown to functionally analyze nitrate supplementation. To the best of our knowledge, our work is the first to functionally analyze nitrate reductase activity (and their contribution to biofilm formation) in a Shewanella species harboring both NAP isoforms, as is the case for most shewanellaceae as shown by whole-genome sequencing, thus establishing a functional link between respiration and biofilm formation, two prima facie independent processes.

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presumably as a consequence of artificially altered intracellular c-di-GMP pools.

In summary, in this work we have provided evidence that reductase activity is required for AEA-induced biofilm formation in *S. algae*. Deletion mutants in the different reductases characterized the physiological role of respiration in terms of activity and biofilm induction. Given the complexity of the electron transport systems and signal transduction pathways in *S. algae*, and the strain-specific nature of this phenomenon, this work provides an entry point for follow-up studies of comparative transcriptomic analyses of responsive and non-responsive *S. algae* isolates as well as extensive mutational analyses to identify and characterize specific regulatory pathways linking respiration with biofilm formation. Furthermore, we report that biofilm formation is closely intertwined with the use of different electron acceptors by *S. algae*. We show that the two NAP isofoms of *S. algae* play substantially different roles on cellular nitrate reduction and nitrate reduction-mediated biofilm formation. We also demonstrate DMSO reduction to be a variable feature in *S. algae* with evidence of loss or horizontal acquisition in certain isolates. Respiration-driven biofilm formation may therefore constitute a mechanism of niche colonization by specialized *S. algae* strains, not only in anoxic environments where certain AEAs are abundant, but also in moderately oxygenated environments by taking advantage of their broad respiratory repertoire and reducing thereby the consequences of microbial competition for oxygen. Apart from deciphering the underlying molecular mechanisms, this work provides a sound basis for future research aimed at establishing the phylogenetic spread of the phenomenon studied here.

**METHODS**

**Strains and growth conditions**

The *Shewanella* strains used in this study (Supplementary Table 1) were routinely grown in MB or on marine agar (MA) (Difco). *Escherichia coli* strains (Supplementary Table 1) were grown on Luria broth (LB) or on Luria agar (LA). When necessary, the medium was supplemented with kanamycin (50 µg ml⁻¹), streptomycin (200 µg ml⁻¹), IPTG (0.5 mM), and/or diaminopimelic acid (0.3 mM).

**Genetic manipulations**

The R6K-origin plasmid pKNG101 was used for allelic replacement in *S. algae* CECT 5071. For in-frame deletion of the target genes dmsB (minor subunit of the DMSO reductase DmsAB), napABC (periplasmic nitrate reductase NapA-C), napA (periplasmic nitrate reductase NapB, subunit A), the chromosomal regions immediately up- and downstream of the genes of interest were PCR-amplified and fused in plasmid pKNG101 using the primers and restriction sites indicated in Supplementary Table 2. To create the dmsB deletion, the DNA fragments comprising the 347-bp upstream and 355-bp downstream of the *dmsB* gene were initially cloned into pUC18Not using the restriction sites indicated in Supplementary Table 2, and then subcloned into pKNG101 at the NotI site. The resulting plasmids were propagated in *E. coli* DH5α and isolated, and electroporated into *E. coli* MFDPir from which they were mobilized into *S. algae* CECT 5071 by conjugation. Recombinants generated by single-crossover recombination were selected on LA plates containing 5µg ml⁻¹ Sm. Double crossover was induced upon re-streaking merodiploids on plates containing 10% (w/v) sucrose. In-frame gene deletion mutants were confirmed by PCR using primers flanking the recombination sites (Supplementary Table 2) followed by Sanger sequencing. To complement the mutations, the relevant operons *dmsEFABGH*, *napDAGBH* and *napEDABC* were cloned individually or in tandem under the control of the *lac* promoter in pSRK-Km using the primers listed in Supplementary Table 2. The cloning strains *E. coli* TOP10 and NEB-Sa were used for plasmid propagation. All plasmid constructs were confirmed by Sanger sequencing.

The wild-type yhjH gene from *Salmonella enterica* serovar Typhimurium ATCC 14028 (UMR1) and the catalytically inactive derivative yhjHE136Δ were cloned into pBBR1MCS-2 with primers yhjH-F-HindIII and yhjH-
spectrometer with an El ion source running in TIC Full Scan mode (scanning m/e 32–300). Experiments involved three biological replicates.

**Determination of nitrate reductase activity**

Nitrate reductase activity in static cultures of strains grown in MB supplemented with 35 mM nitrate was determined spectrophotometrically by measuring the accumulation of nitrite in supernatants upon addition of 0.02% (w/v) N-(1-naphthyl)ethylenediamine in 95% (v/v) ethanol and 1% sulfuramidole (w/v) in 1.5 N HCl as previously described63. Optical readings were taken at OD460 in 96-well plates. Experiments involved at least three biological replicates.

**RNA isolation and sequencing**

Total RNA was isolated from static overnight cultures of _S. algae_ CECT 5071 in non-supplemented MB or the same medium supplemented with 35 mM DMSO or 35 mM nitrate. Prior to RNA isolation, cultures (three biological replicates per test condition) were homogenized by inserting the tubes to ensure representative sampling of the entire bacterial population. RNA purification was performed with the Direct-Zol RNA Miniprep Plus kit (Zymo Research) following the instructions of the manufacturer. Residual genomic DNA was removed by DNase treatment with the Turbo DNA-free kit (Ambion). DNA contamination was assessed with a 40-cycle PCR using the DNase-treated RNA samples as templates. The RNA quality was examined on a 2100 Bioanalyzer system (Agilent). The relative integrity (RIN) values were 9.6–10.

Prior to sequencing, rRNA was depleted from 550 ng of total RNA using Illumina Ribozero RNA removal kit for Gram-negative bacteria. Sequencing libraries were then prepared using Illumina TruSeq stranded total RNA kit. The libraries were pooled and 1% of PhiX control library was added to the pool. The pool was sequenced on a HiSeq 2500 machine in High Output run mode using v4 chemistry. The read length was PE125.

Low quality sequence reads and adapters were removed with AfterQCC64 using default parameters and the remaining contaminating rRNA was removed with SortMeRNA65. Read counts per gene were obtained using the package Salmon66 with default parameters. To identify differentially expressed genes, the DESeq2 R/Bioconductor package was used69. RNA-seq reads were deposited at the NCBI database (accession number: GSE133627).

The annotation of _S. algae_ CECT 5071 genes was classified into functional families using the Cluster of Orthologous Groups (COG) by BLASTP with 60% minimum alignment coverage, 50 minimum bitscore, and 35% minimum percent identity.

Proteins were classified into families and subfamilies with an ontology term (GO), PANTHER protein class, and PANTHER pathway using its classification system (www.pantherdb.org70), Statistical enrichment analysis for differentially expressed genes was considered as significant when the FDR-adjusted P-value was inferior to 0.05. Analyses were assisted by Python scripts.

**qRT-PCR validation of RNA-seq data**

Quantitative real time PCR was used to validate differential gene transcript levels using RNA isolated from _S. algae_ CECT 5071 cultures under the same conditions as described above. The primers used in qRT-PCR reactions are listed in Supplementary Table 2. Complementary DNA (cDNA) was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen) using 1 µg of RNA. Quantitative PCR reactions were prepared with the Power SYBR Green PCR Master Mix (Applied Biosystems) and run on a LightCycler 480 instrument (Roche) using the pre-defined SYBR Green reaction protocol. Relative gene expression was calculated using the ΔΔCt method70.

**Reporting summary**

Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

**DATA AVAILABILITY**

RNA sequencing data that support the findings of this study have been deposited in the NCBI GenBank with the accession code GSE133627. The Whole Genome Shotgun project of _S. algae_ CECT 5071 is available at the NCBI GenBank under the accession code SMNR00000000. The nucleotide sequences of _dmsA_ orthologs used for phylogenetic analysis are available at the NCBI GenBank under accession codes MT953034–MT953072.

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AUTHOR CONTRIBUTIONS
Study - original idea: A.J.M.-R. Study - design and conceptualization: A.J.M.-R. and U.R. Experiments: A.J.M.-R., assisted by J.A.R.-D. and D.M.-M. Data analysis: A.J.M.-R., J.M.G., T.K., and U.R. Writing - original draft: A.J.M.-R. Writing - review and editing: A.J.M.-R., T.K., and U.R., with the input of all co-authors. All authors read and approved the final version before submission.

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The authors declare no competing interests.

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