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Original Article

# Construction and analysis of deletion and complementation strains of the *extMNOPQS* gene cluster in *Geobacter sulfurreducens* PCA

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## Summary

Elemental sulfur respiration plays a pivotal role in the microbial sulfur cycle; however, the molecular mechanisms underlying this process remain poorly understood in many microorganisms, including *Geobacter sulfurreducens* PCA. A previous RNA-seq analysis showed that the upstream region of the *ext* gene cluster (*extHIJKL*) is transcriptionally upregulated in response to sulfur, whereas the downstream region (*extMNOPQS*) did not exhibit clear induction. However, the specific role of *extMNOPQS* in elemental sulfur reduction remains unclear. In this study, we investigated the function of *extMNOPQS* using transcriptional analysis and mutational approaches. RT-qPCR revealed that the expression of *extM* was not significantly induced by elemental sulfur, in contrast to the sulfur-responsive *extHIJKL* gene cluster, suggesting distinct transcriptional regulation. To examine its physiological role, we constructed a deletion mutant (*ΔextMNOPQS*) and a plasmid-based complementation strain. Western blotting confirmed the absence of ExtM in the deletion mutant and its elevated expression in the complemented strain. Growth assays showed that deletion of *extMNOPQS* did not severely impair cell growth, although the complemented strain exhibited slightly reduced growth when compared with the wild-type. Functional analysis using halo assays demonstrated that the *ΔextMNOPQS* mutant was markedly impaired in sulfur reduction, and this phenotype was almost completely restored by complementation. These findings indicate that *extMNOPQS* is essential for elemental sulfur respiration in *G. sulfurreducens* and may serve as a critical electron transfer module within the *ext* gene cluster.

## Introduction

Elemental sulfur ($S^0$) is widely distributed in sediments[1, 2)]. Anaerobic respiration using elemental sulfur as a terminal electron acceptor represents a key dissimilatory metabolic process in sulfur-reducing bacteria and certain archaea and simultaneously constitutes an essential component of the global biogeochemical sulfur cycle[3, 4)]. Sulfur-reducing microorganisms inhabit a broad spectrum of natural environments, ranging from mesophilic freshwater sediments to acidic volcanic hot springs and deep-sea hydrothermal vents[4, 5)].

The molecular mechanisms of sulfur reduction in bacteria have been elucidated only for a limited number of organisms, such as *Wolinella succinogenes*[6–11)] and the hyperthermophilic archaeon *Pyrococcus furiosus*[12–15)]. Reported systems include the molybdenum cofactor- and iron–sulfur cluster-containing polysulfide reductase PsrABC, which couples to hydrogenase or formate dehydrogenase[9, 16)], membrane-bound sulfur reductase MBS, respiratory complex I–like enzyme[17, 18)], and FAD/NADH-dependent sulfur reductase NSR[19–21)]. Compared with the extensively studied processes of sulfate, sulfite, and thiosulfate reduction, the molecular basis of elemental sulfur reduction remains poorly characterized[22)].

The gram-negative strict anaerobe *Geobacter sulfurreducens* PCA was originally isolated and identified as a dissimilatory metal- and sulfur-reducing bacterium capable of reducing insoluble metal compounds such as ferric iron, fumarate, and elemental sulfur, as part of its energy metabolism[23)]. Members of the genus *Geobacter* play key roles in the biogeochemical cycling of metals in soils and aquatic sediments. *G. sulfurreducens* PCA also belongs to the phylum Desulfobacterota and, as indicated by its spe-

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cies name, is a sulfur-reducing bacterium that lacks the ability to reduce sulfate[23]. Notably, despite extensive interest in the physiology and ecology of *G. sulfurreducens* PCA, no reports describing the proteins or genes responsible for sulfur respiration in this organism have been published for nearly three decades since its discovery.

Genome sequencing revealed that *G. sulfurreducens* encodes 111 *c*-type cytochromes, most of which contain heme cofactors and are thought to play essential roles in electron transfer to extracellular electron acceptors[24–29]. These *c*-type cytochromes may also contribute to elemental sulfur reduction, although their specific functions remain unclear. In this context, particular attention has been paid to the *ext* gene cluster (*extHIJKLMNOPQS*)[30, 31], which is predicted to encode multiple cytochromes and associated proteins potentially linked to sulfur respiration (Fig. 1)[32]. Recent transcriptomic studies have shown that genes in the upstream region (*extHIJKL*) are upregulated in response to sulfur, suggesting their role in sulfur reduction[32]. This gene cluster encodes several predicted proteins, such as a rhodanese-like sulfurtransferase (ExtH), porin-like protein (ExtI), protein of unknown function (ExtJ), multiheme cytochrome (ExtKL), and a set of putative redox proteins (ExtMNOPQS).

Previous studies from our laboratory suggested that ExtI interacts with ExtH and may facilitate the uptake of selenite[31, 33]. Bioinformatics analyses have suggested that ExtKL contains five heme-binding motifs (CXXCH) and one selenocysteine residue[34]. The proteins encoded by ExtMNOPQS are believed to be involved in electron transfer, potentially in cooperation with other components of the *ext* gene cluster. The *extMNOPQS* region encodes three putative multiheme *c*-type cytochromes, ExtM (12 CXXCH motifs), ExtN (10 motifs), and ExtS (4 motifs), as well as a membrane-associated *b*-type cytochrome-like protein (ExtP), Rieske Fe–S protein (ExtO), and a predicted membrane protein (ExtQ). These proteins have been proposed to form a quinol-oxidizing complex analogous to multiheme cytochromes or cytochrome *bc* complexes, transferring electrons from the quinone/quinol pool. Despite these predictions, the direct involvement of ExtMNOPQS in elemental sulfur reduction has not yet been elucidated.

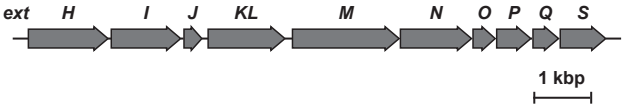


**Fig. 1.** The *ext* gene cluster (*extHIJKLMNOPQS*). The cluster encodes ExtH, a rhodanese-like sulfurtransferase; ExtI, a porin-like protein; ExtJ, a protein of unknown function; ExtKL, a multiheme cytochrome; ExtM, a multiheme *c*-type cytochrome with 12 CXXCH motifs; ExtN, a multiheme *c*-type cytochrome with 10 CXXCH motifs; ExtO, a Rieske Fe–S protein; ExtP, a membrane-associated *b*-type cytochrome-like protein; ExtQ, a predicted membrane protein; and ExtS, a multiheme *c*-type cytochrome with 4 CXXCH motifs.

In this study, we constructed deletion and complementation strains of the *extMNOPQS* gene cluster in *G. sulfurreducens* PCA to investigate its role in elemental sulfur respiration. By characterizing these strains, we aimed to clarify the contribution of ExtMNOPQS to the reduction of elemental sulfur and advance our understanding of sulfur-dependent respiratory pathways in this environmentally and biotechnologically important microorganism.

## Materials and Methods

### Bacterial strains and cultivation

*Geobacter sulfurreducens* PCA was obtained from the Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). Culture medium was inoculated under anaerobic conditions in an anaerobic chamber (Glovebox Japan Inc., Japan). Cells were routinely cultured at 35℃ in modified NBAFYE medium[35], supplemented with 20 mM acetate, 40 mM fumarate, 0.1% yeast extract, and 1 mM cysteine. A sulfur-supplemented liquid medium was prepared by adding 0.1% (w/v) sulfur powder, sublimed ( > 99% and ~100 mesh powder) (Strem Chemicals, USA) to the modified NBAFYE medium followed by autoclaving at 105℃ for 30 min. *E. coli* DH5*α* (TOYOBO, Japan) was used for general DNA manipulations. *E. coli* strains were grown aerobically 37℃ in lysogeny broth or tryptic soy broth (Becton Dickinson GmbH, Germany).

### Quantitative reverse transcription PCR (RT-qPCR)

*G. sulfurreducens* PCA was grown anaerobically in modified NBAFYE medium with or without 0.1% (w/v) sulfur powder for 48 h. The cells were harvested, and total RNA was extracted from three biological replicates using TRIzol Max Bacterial RNA Isolation Kit (Thermo Fisher Scientific, USA). Residual genomic DNA was removed using TURBO DNase (Thermo Fisher Scientific), and RNA was purified using Agencourt RNAClean XP beads (Beckman Coulter, USA). RNA quality was verified by agarose gel electrophoresis and quantified by UV spectrophotometry. Single-stranded cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix (TOYOBO).

RT-qPCR was performed using Applied Biosystems StepOne System (Thermo Fisher Scientific) with TB Green Fast qPCR Mix (Takara Bio, Japan). Each 10-μL

reaction contained 1× TB Green Fast qPCR Mix, 1× ROX Reference Dye, 0.4 μM of each primer, and cDNA. Primers for *extM* were 5′-AATGCTACGGCTGTCATACGAAATA-3′ and 5′-TTTCCCCTTGAAGGTAGAGACGTAG-3′; those for 16 S rRNA were 5′-TGAGACACGGTCCAGACTCCTAC-3′ and 5′-TCATTTCTTCCCTCCCGACA-3′. Cycling conditions were 95℃ for 30 s, followed by 40 cycles at 95℃ for 5 s and 60℃ for 10 s. Relative expression was determined from *Δ*Ct values normalized to 16 S rRNA.

### Construction of gene deletion mutants

An *extMNOPQS* gene deletion mutant (*ΔextMNOPQS*) was generated by homologous recombination with linear DNA fragments. Approximately 500-bp fragments upstream of *extM* and downstream of *extS* were amplified from genomic DNA of *G. sulfurreducens* PCA by using PCR with primers 5′-CACGAGCGCTTCGATATCCTCTTCCCCACC-3′ (P1) and 5′-AACATGGTCCGGTGGGCGTCCTCTTTGCTC-3′, and 5′-CGCGCGAAGGAAGACAACCTGTTCGTCACC-3′ and 5′-CACGGCTACCCCGACTATCTGGAGCACAAG-3′ (P4), respectively. A kanamycin-resistance gene cassette was amplified from the plasmid pJRD215[36] by using PCR with primers 5′-GAGCAAAGAGGACGCCCACCGGACCATGTTGGATGAATGTCAGCTAC-3′ and 5′-TCCACTTCCAGATCGTGGAGAAGGCGGCGGTGGAATCG-3′. The three PCR fragments were fused via overlap extension PCR, and the resulting DNA constructs were introduced into *G. sulfurreducens* PCA by electroporation (GTE-10; Shimadzu, Japan). Transformants were selected on NBAFYE agar plates containing 400 μg/mL kanamycin.

To confirm the gene deletion, PCR was performed using primers P1 and P4, together with two additional primers: P2, derived from the recombination region within *extMNOPQS* (5′-GGGCCAGACGAGCGACCAATTC-3′), and P3, derived from the kanamycin resistance gene (5′-TCAGAAGAACTCGTCAAGAAGGCGAT-3′) (Fig. 3).

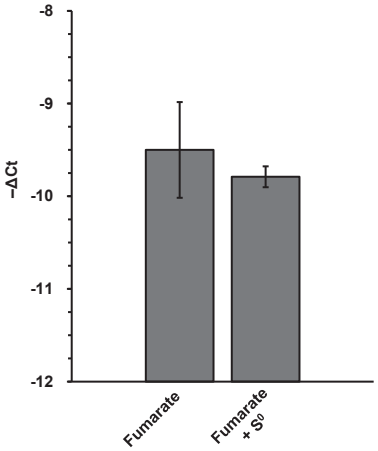


**Fig. 2.** Quantification of *extM* mRNA levels via RT-qPCR. Total RNA was extracted from cells grown with fumarate alone (Fumarate) or with fumarate plus 0.1% $S^0$ (Fumarate + $S^0$). Transcript abundance was expressed as the $-\Delta$Ct value of *extM* relative to 16 S rRNA. Data represent the mean of three biological replicates, and error bars indicate standard deviations. Statistical analysis by Welch's *t*-test indicated no significant difference between the two conditions ($p = 0.592$).

### Plasmid construction for complementation

For complementation of the *ΔextMNOPQS* strain, the broad-host-range vector pMMB206[37] was used. A synthetic DNA cassette containing a ribosome-binding site and restriction sites (NdeI, XhoI, and XbaI) was inserted into the EcoRI site of pMMB206 to generate the vector pMcas. The *extMNOPQS* fragment was amplified with PCR by using primers 5′-CTGACTGAGAAGGAGA-

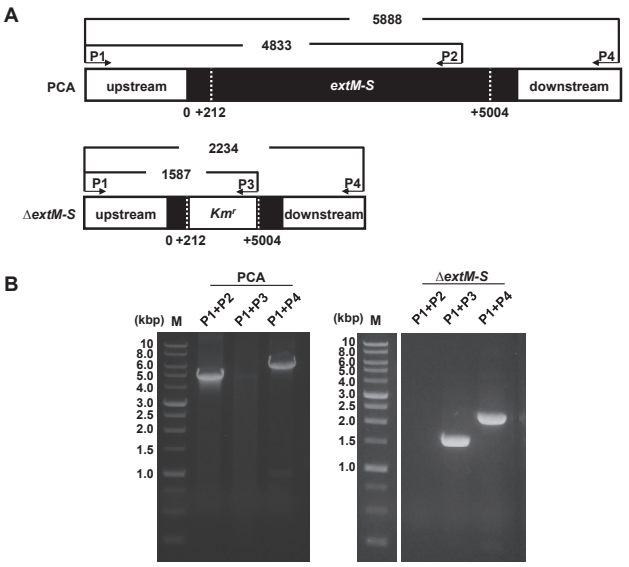


**Fig. 3.** Construction of the *extMNOPQS*-disrupted strain. (A) Schematic representation of the *extMNOPQS* locus and PCR strategy. *Top*, genomic context of the *extMNOPQS* gene in the *G. sulfurreducens* PCA parent strain, showing the annealing sites of primers deletion-check primers forward and reverse (P1/P4), the *extMNOPQS* internal primer within the deleted segment (P2), and the expected sizes of the PCR products. The region replaced by the kanamycin resistance gene ($Km^r$) spans nucleotides +212 to +5004 from the translational start site. *Bottom*, disrupted *extMNOPQS* locus in the Δ*extMNOPQS* strain, in which $Km^r$ replaces nucleotides +212 to +5004. Primer positions, deletion-check primers forward and reverse (P1/P4) and the primer targeting the 3′ end of the pJRD215-derived kanamycin resistance gene (P3) and expected PCR product sizes are indicated. (B) PCR verification of the *extMNOPQS* disruption. PCR was performed using genomic DNA from the PCA or *ΔextMNOPQS* strain as a template and the indicated primer pairs (P1/P2, P1/P3, P1/P4). Products were analyzed by agarose gel electrophoresis alongside a DNA size marker.

TATACAATGAACAGGATTTCCGACGTCG-3′ and 5′ -CTTGGCTGCAGGTCGACTCAGTTTTCGAGATTGACG-3′ . The PCR product was digested with NdeI and SalI and cloned into the corresponding sites of pMcas, yielding pMcasExtMNOPQS. Construct integrity was verified with DNA sequencing by using the ABI Genetic Analyzer 3130 (Thermo Fisher Scientific).

### Western blotting

Proteins were separated with SDS–PAGE and transferred onto Immobilon-P membranes (0.45 μm; MilliporeSigma, USA) by using a Trans-Blot SD semi-dry transfer apparatus (Bio-Rad, USA). Anti-ExtM antibody was generated in rabbits against a synthetic peptide (DDPIYRKELDYRAL) derived from the ExtM amino acid sequence (Operon Biotechnologies, Japan). Immunoreactive proteins were detected with HRP-conjugated anti-rabbit IgG (H+L) secondary antibody (Promega, USA) and Chemi-Lumi One Super (Nacalai Tesque, Japan) and visualized using an Amersham Imager 600 (Cytiva, USA).

### Elemental sulfur reduction assay

Sulfur-containing NBAFYE agar plates were prepared by adding 2 mM polysulfide solution, which was generated by the addition of 0.25 M $Na_2S_4O_6$ in 0.5 M Tris-HCl (pH 8.5) to 1.0 M $Na_2S$ in 0.5 M Tris-HCl (pH 8.5) at 1:1 ratio under anaerobic conditions[8], to NBAFYE agar medium, followed by autoclaving and dispensing into petri dishes. The plates were exposed to air to oxidize polysulfide and remove excess hydrogen sulfide. Prior to use, the plates were rendered anaerobic for at least 16 h with an AnaeroPack-Kenki system (Mitsubishi Gas Chemical, Japan). One microliter of cell suspension ($OD_{600}$ = 1.0) was spotted onto $S^0$-containing NBAFYE agar and incubated at 35 ℃ for 6 days. The formation of clearance halos around the colonies was monitored and quantified by measuring the halo-to-colony area ratio with ImageJ software (NIH, USA).

## Results and Discussion

### RT-qPCR analysis of *extM*

A previous RNA-seq analysis demonstrated that the upstream region of the *ext* gene cluster (*extHIJKL*) is transcriptionally upregulated in response to sulfur, whereas the downstream region (*extMNOPQS*) shows little change[32]. To further verify this observation, we examined the expression of *extM* by using RT-qPCR. *G. sulfurreducens* PCA was cultured with and without elemental sulfur, and mRNA extracted from the cells was analyzed. The transcript levels of *extM* were not significantly altered by sulfur supplementation (Fig. 2), suggesting that *extMNOPQS* is not under the same transcriptional control as *extHIJKL*[32]. This difference implies that the *ext* cluster may be regulated in a modular manner, with *extHIJKL* responding specifically to sulfur availability, whereas *extMNOPQS* functions more constitutively as a part of the electron transfer chain. This modular regulation is consistent with previous observations that many *Geobacter c*-type cytochromes that mediate extracellular electron transfer are expressed under a range of conditions and can function as a constitutive electron-transfer backbone[25–29].

### Construction of deletion and complementation strains

To investigate the physiological role of *extMNOPQS* in sulfur reduction, we generated a deletion mutant in which the entire *extMNOPQS* region was replaced with a kanamycin-resistance cassette via homologous recombination. PCR analysis confirmed successful disruption of the *extMNOPQS* locus (Fig. 3). Growth assays in NBAFYE medium showed that the *ΔextMNOPQS* strain exhibited only slightly reduced growth when compared with the wild type (Fig. 4), indicating that this gene cluster is not essential for general metabolism or growth under fumarate-respiring conditions. Similar phenotypic patterns, *i.e.*, minor effects on growth, but pronounced defects in respiratory electron transfer, have been reported for other multi-heme cytochrome systems in *Geobacter*, particularly those involved in extracellular electron transfer to insoluble acceptors[25, 26].

Next, a complementation strain was constructed by in-

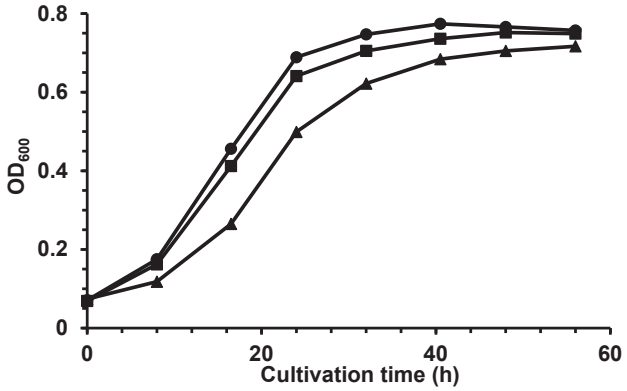


**Fig. 4.** Growth of the wild-type, *extMNOPQS*-disrupted, and *extMNOPQS*-complemented strains. Cells were cultured at 35 ℃ in NBAFYE medium supplemented with 20 mM acetate, 40 mM fumarate, 0.1% yeast extract, and 1 mM cysteine. The wild-type strain is shown as circles, the *ΔextMNOPQS* strain as squares, and the complemented strain as triangles.

troducing a plasmid carrying the *extMNOPQS* gene cluster into the deletion background. Because ExtM is predicted to be a membrane-associated lipoprotein, SDS-PAGE was performed using pellet fractions obtained after sonication and centrifugation. Under these conditions, the wild-type, *extMNOPQS*-disrupted, and *extMNOPQS*-complemented strains showed only minor differences in Coomassie Blue-stained protein profiles (Fig. 5 A). In contrast, western blotting confirmed the expression of ExtM in the complemented strain, with protein levels exceeding those observed in the wild-type and increasing over time during cultivation (Fig. 5B). Under these experimental conditions, the ExtM band was not visible in the wild-type strain, likely because of its low expression. These results suggest that the complementation system drives relatively strong expression, possibly because of the plasmid copy number or promoter activity. Interestingly, the complemented strain showed slightly slower growth than both the wild-type and deletion mutant (Fig. 4). This phenotype may reflect the metabolic burden associated with plasmid maintenance as well as potential imbalances in protein expression. Overexpression of cytochromes and membrane-spanning complexes is thought to accelerate the consumption of reducing equivalents such as NADH and reduced quinones, thereby competing with other metabolic pathways and imposing stress on central metabolism. Moreover, excessive production of membrane proteins has been reported to disrupt intracellular redox balance and to induce resource depletion and oxidative stress[38]. These factors are likely to act in concert and contribute to the reduced growth rate observed in the ExtMNOPQS-overexpressing strain.

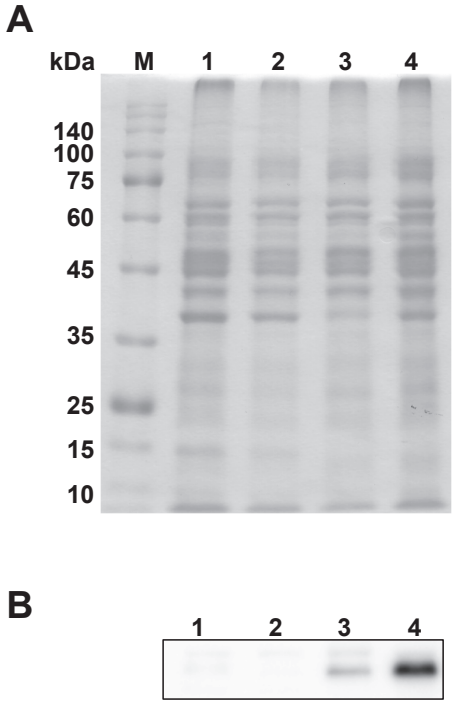


**Fig. 5.** SDS-PAGE (A) and western blot (B) analyses of pellet fractions obtained after sonication and centrifugation of cells from the wild-type, *extMNOPQS*-disrupted, and *extMNOPQS*-complemented strains. Samples were prepared after cultivation for 24 h (lane 3) or 48 h (lanes 1, 2, and 4). Lane 1, wild-type strain; lane 2, *ΔextMNOPQS* strain; lane 3, complemented strain (24 h); lane 4, complemented strain (48 h). Western blotting was performed using an anti-ExtM antibody.

### Role of *extMNOPQS* in sulfur reduction

The ability of these strains to reduce elemental sulfur was examined using the halo assay on sulfur-supplemented agar plates. Wild-type colonies produced clear halos around the colony, consistent with active sulfur respiration (Fig. 6)[16, 39]. In contrast, the *ΔextMNOPQS* strain displayed a pronounced defect in halo formation, indicating severely impaired sulfur reduction. Complementation with plasmid-borne *extMNOPQS* restored halo formation, confirming that the loss of activity was specifically attributable to the deletion of this gene cluster. However, the halo size of the complemented strain was not significantly different

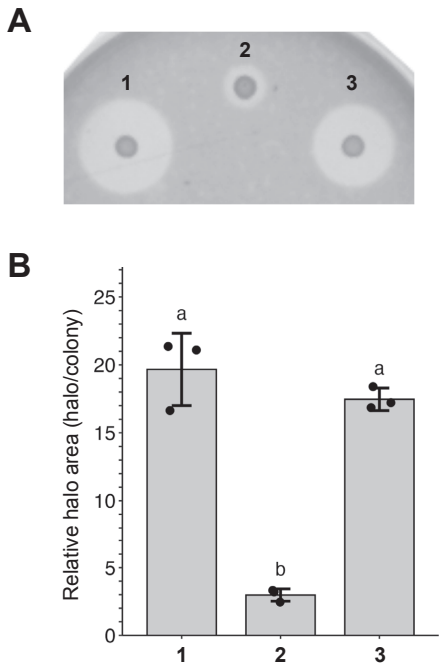


**Fig. 6.** Phenotype analysis of *extMNOPQS*-deficient and complemented strains with respect to sulfur reduction. *A*, Sulfur reduction halo assay. The parent strain *G. sulfurreducens* PCA (1), gene-disrupted mutant *ΔextMNOPQS* (2), and complemented strain carrying *extMNOPQS* on a plasmid (3) were spotted on an NBAFYE agar plate supplemented with $S^0$ and incubated anaerobically for 6 days. *B*, Comparison of normalized halo area (to colony size) shown in (*A*). Quantification of sulfur reduction activity based on the halo-to-colony area ratio shown in (A). Halo and colony areas were measured using ImageJ. Statistical significance was determined using one-way ANOVA followed by Tukey's honestly significant difference test. Bars labeled with different letters indicate significant differences ($p < 0.05$).

from that of the wild type (Fig. 6B), despite the substantially higher expression levels of ExtM (and likely ExtNOPQS) observed in western blot analysis (Fig. 5). The apparent discrepancy may reflect unbalanced overexpression of multiheme cytochromes, which can disrupt the stoichiometry and proper assembly of the functional complex. Moreover, excessive expression of membrane-associated proteins may perturb intracellular redox balance and impose metabolic stress[38], thereby constraining overall sulfur-reducing activity. These factors together likely account for the slightly smaller halo formation trend observed in the complemented strain, even though the difference was not statistically significant ($p$ = 0.296). Our genetic results implicate *extMNOPQS* in elemental sulfur respiration; however, it is unclear how electrons are routed to the sulfur-reducing step. Based on domain predictions and subunit annotations (multiheme *c*-type cytochromes ExtM/ExtN/ExtS, putative *b*-type cytochrome ExtP, Rieske 2Fe-2 S protein ExtO, and membrane protein ExtQ), we considered a working model in which ExtMNOPQS potentially acts as or contributes to, a quinone/quinol-oxidizing module that may deliver electrons to downstream sulfur-processing components. This hypothesis is consistent with the electron transfer systems observed elsewhere: the MBX complex of *P. furiosus* couples reduced ferredoxin to $S^0$ reduction[21,40], and *W. succinogenes* PsrABC uses a membrane *b*-type subunit (PsrC) to accept quinol and pass electrons to periplasmic catalysts[9]. In addition, ACIII systems demonstrate that noncanonical assemblies comprising multiheme *c*-type cytochromes, a Rieske Fe-S subunit, and *b*-type elements can execute a *bc*-like quinol oxidoreductase function[41]. These findings support the plausibility that *extMNOPQS* has a *bc*-type function in *G. sulfurreducens*; however, targeted biochemical assays need to be performed to test this model.

## Conclusion

In summary, our study provides the first genetic evidence that the *extMNOPQS* gene cluster is required for elemental sulfur reduction in *G. sulfurreducens* PCA. RT-qPCR analysis demonstrated that *extM* is not transcriptionally responsive to sulfur, distinguishing the cluster from the sulfur-inducible *extHIJKL* region[32]. Nevertheless, deletion of *extMNOPQS* abolished sulfur reduction activity and complementation restored it, clearly demonstrating its indispensable role in sulfur respiration. These findings suggest a model in which *extMNOPQS* functions as a constitutively expressed electron transfer module, delivering electrons from the quinone/quinol pool to downstream sulfur-reducing components. These results increase our understanding of sulfur-dependent respiratory pathways in *G. sulfurreducens* and provide a foundation for future biochemical and structural studies aimed at elucidating the mechanisms underlying this unique electron transfer system.

## Acknowledgments

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## References

1) Jørgensen, BB, Findlay, AJ, Pellerin, A (2019) The biogeochemical sulfur cycle of marine sediments. Front Microbiol 10: 849.
2) Findlay, AJ, Kamyshny, A (2017) Turnover rates of intermediate sulfur species ($S_x^{2-}$, $S^0$, $S_2O_3^{2-}$, $S_4O_6^{2-}$, $SO_3^{2-}$) in anoxic freshwater and sediments. Front Microbiol 8: 2551.
3) Bonch-Osmolovskaya, EA (1994) Bacterial sulfur reduction in hot vents. FEMS Microbiol Rev 15: 65–77.
4) Wasmund, K, Mussmann, M, Loy, A (2017) The life sulfuric: microbial ecology of sulfur cycling in marine sediments. Environ Microbiol Rep 9: 323–344.
5) Pjevac, P, Kamyshny, A, Jr., Dyksma, S, Mussmann, M (2014) Microbial consumption of zero-valence sulfur in marine benthic habitats. Environ Microbiol 16: 3416–3430.
6) Macy, JM, Schroder, I, Thauer, RK, Kroger, A (1986) Growth the *Wolinella succinogenes* on $H_2S$ plus fumarate and on formate plus sulfur as energy-sources. Arch Microbiol 144: 147–150.
7) Schröder, I, Kröger, A, Macy, JM (1988) Isolation of the sulphur reductase and reconstitution of the sulphur respiration of *Wolinella succinogenes*. Arch Microbiol 149: 572–579.
8) Klimmek, O, Kröger, A, Steudel, R, Holdt, G (1991) Growth of *Wolinella succinogenes* with polysulphide as terminal acceptor of phosphorylative electron transport. Arch Microbiol 155: 177–182.
9) Krafft, T, Gross, R, Kröger, A (1995) The function of *Wolinella succinogenes psr* genes in electron trans-

port with polysulphide as the terminal electron acceptor. Eur J Biochem 230: 601–606.
10) Ringel, M, Gross, R, Krafft, T, Kröger, A, Schauder, R (1996) Growth of *Wolinella succinogenes* with elemental sulfur in the absence of polysulfide. Arch Microbiol 165: 62–64.
11) Klimmek, O, Kreis, V, Klein, C, Simon, J, Wittershagen, A, Kröger, A (1998) The function of the periplasmic Sud protein in polysulfide respiration of *Wolinella succinogenes*. Eur J Biochem 253: 263–269.
12) Blumentals, II, Itoh, M, Olson, GJ, Kelly, RM (1990) Role of polysulfides in reduction of elemental sulfur by the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl Environ Microbiol 56: 1255–1262.
13) Ma, K, Schicho, RN, Kelly, RM, Adams, MWW (1993) Hydrogenase of the hyperthermophile *Pyrococcus furiosus* is an elemental sulfur reductase or sulfhydrogenase: Evidence for a sulfur-reducing hydrogenase ancestor. Proc Natl Acad Sci USA 90: 5341–5344.
14) Ma, K, Weiss, R, Adams, MWW. (2000) Characterization of hydrogenase II from the hyperthermophilic archaeon *Pyrococcus furiosus* and assessment of its role in sulfur reduction. J Bacteriol 182: 1864–1871.
15) Wu, CH, Schut, GJ, Poole, FL, Haja, DK, Adams, MWW (2019) Characterization of membrane-bound sulfane reductase: A missing link in the evolution of modern day respiratory complexes. J Biol Chem 293: 16687–16696.
16) Hedderich, R, Klimmek, O, Kröger, A, Dirmeier, R, Keller, M, Stetter, KO (1998) Anaerobic respiration with elemental sulfur and with disulfides. FEMS Microbiol Rev 22: 353–381.
17) Yu, H, Haja, DK, Schut, GJ, Wu, CH, Meng, X, Zhao, G, Li, H, Adams, MWW (2020) Structure of the respiratory MBS complex reveals iron-sulfur cluster catalyzed sulfane sulfur reduction in ancient life. Nature Commun 11: 1–13.
18) Yu, H, Schut, GJ, Haja, DK, Adams, MWW, Li, H (2021) Evolution of complex I–like respiratory complexes. J Biol Chem 296: 100740–100740.
19) Jelen, B, Giovannelli, D, Falkowski, PG, Vetriani, C (2018) Elemental sulfur reduction in the deep-sea vent thermophile, *Thermovibrio ammonificans*. Environ Microbiol 20: 2301–2316.
20) Liang, J, Huang, H, Wang, Y, Li, L, Yi, J, Wang, S (2022) A cytoplasmic NAD(P)H-dependent polysulfide reductase with thiosulfate reductase activity from the hyperthermophilic bacterium *Thermotoga maritima*. Microbiol Spectr 10: e0043622.
21) Schut, GJ, Bridger, SL, Adams, MWW (2007) Insights into the metabolism of elemental sulfur by the hyperthermophilic archaeon *Pyrococcus furiosus*: Characterization of a coenzyme A-dependent NAD(P)H sulfur oxidoreductase. J Bacteriol 189: 4431–4441.
22) Grein, F, Ramos, AR, Venceslau, SS, Pereira, IAC (2013) Unifying concepts in anaerobic respiration: Insights from dissimilatory sulfur metabolism. Biochim. Biophys. Acta Bioenergetics 1827: 145–160.
23) Caccavo, F, Lonergan, DJ, Lovley, DR, Davis, M, Stolz, JF, McInerneyl, MJ (1994) *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate- oxidizing dissimilatory metal-reducing microorganism. Appl Environ Microbiol 60: 3752–3759.
24) Methé, BA, Nelson, KE, Eisen, JA, Paulsen, IT, Nelson, W, Heidelberg, JF, Wu, D, Wu, M, Ward, N, Beanan, MJ, Dodson, RJ, Madupu, R, Brinkac, LM, Daugherty, SC, DeBoy, RT, Durkin, AS, Gwinn, M, Kolonay, JF, Sullivan, SA, Haft, DH, Selengut, J, Davidsen, TM, Zafar, N, White, O, Tran, B, Romero, C, Forberger, HA, Weidman, J, Khouri, H, Khouri, H, Feldblyum, TV, Utterback, TR, Van Aken, SE, Lovley, DR, Fraser, CM (2003) Genome of *Geobacter sulfurreducens*: metal reduction in subsurface environments. Science 302: 1967–1969.
25) Reguera, G, McCarthy, KD, Mehta, T, Nicoll, JS, Tuominen, MT, Lovley, DR (2005) Extracellular electron transfer via microbial nanowires. Nature 435: 1098–1101.
26) Shi, L, Squier, TC, Zachara, JM, Fredrickson, JK. (2007) Respiration of metal (hydr)oxides by *Shewanella* and *Geobacter*: A key role for multihaem *c*-type cytochromes.
27) Butler, JE, Young, ND, Lovley, DR (2010) Evolution of electron transfer out of the cell: Comparative genomics of six *Geobacter* genomes. BMC Genomics 11: 1–12.
28) Ueki, T (2021) Cytochromes in extracellular electron transfer in *Geobacter*. Appl Environ Microbiol 87:
29) Paquete, CM, Morgado, L, Salgueiro, CA, Louro, RO (2022) Molecular mechanisms of microbial extracellular electron transfer: The importance of multiheme cytochromes. Front Biosci (Landmark Ed) 27: 174.
30) Jiménez Otero, F, Chan, CH, Bond, DR (2018) Identification of different putative outer membrane electron conduits necessary for Fe(III) citrate, Fe(III) oxide, Mn(IV) oxide, or electrode reduction by *Geobacter sulfurreducens*. J Bacteriol 200: e00347–18.
31) Jahan, MI, Tobe, R, Mihara, H (2018) Characterization

of a novel porin-like protein, ExtI, from *Geobacter sulfurreducens* and its implication in the reduction of selenite and tellurite. Int J Mol Sci 19: 809.

32) Inoue, M, Fujita, D, Izu, Y, Aono, R, Mihara, H (2025) Transcriptional response to elemental sulfur in *Geobacter sulfurreducens*. Biosci Biotechnol Biochem, zbaf106, https://doi.org/10.1093/bbb/zbaf106

33) Jahan, MI, Juengwiwattanakitti, P, Izu, Y, Tobe, R, Imai, T, Mihara, H (2019) Selenite uptake by outer membrane porin ExtI and its involvement in the subcellular localization of rhodanese-like lipoprotein ExtH in *Geobacter sulfurreducens*. Biochem Biophys Res Commun 516: 474–479.

34) Fujita, M, Mihara, H, Goto, S, Esaki, N, Kanehisa, M (2007) Mining prokaryotic genomes for unknown amino acids: A stop-codon-based approach. BMC Bioinformatics 8: 225–225.

35) Coppi, MV, Leang, C, Sandler, SJ, Lovley, DR (2001) Development of a genetic system for *Geobacter sulfurreducens*. Appl Environ Microbiol 67: 3180–3187.

36) Davison, J, Heusterspreute, M, Chevalier, N, Ha-Thi, V, Brunel, F (1987) Vectors with restriction site banks. V. pJRD215, a wide-host-range cosmid vector with multiple cloning sites. Gene 51: 275–280.

37) Morales, VM, Backman, A, Bagdasarian, M (1991) A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene 97: 39–47.

38) Wagner, S, Baars, L, Ytterberg, AJ, Klussmeier, A, Wagner, CS, Nord, O, Nygren, PA, van Wijk, KJ, de Gier, JW (2007) Consequences of membrane protein overexpression in *Escherichia coli*. Mol Cell Proteomics 6: 1527–1550.

39) Fauque, GD, Barton, LL (2012) Hemoproteins in dissimilatory sulfate- and sulfur-reducing prokaryotes. Adv Microb Physiol 60: 1–90.

40) Silva, PJ, van den Ban, EC, Wassink, H, Haaker, H, de Castro, B, Robb, FT, Hagen, WR (2000) Enzymes of hydrogen metabolism in *Pyrococcus furiosus*. Eur J Biochem 267: 6541–6551.

41) Refojo, PN, Teixeira, M, Pereira, MM (2012) The Alternative complex III: properties and possible mechanisms for electron transfer and energy conservation. Biochim Biophys Acta 1817: 1852–1859.