

Cation-driven envelope dynamics modulate outer membrane vesiculation and extracellular electron transfer in *Geobacter*

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Abstract

Geobacter bacteria use conductive pili and redox-active outer membrane vesicles to mediate metal transformations critical to the effectiveness of bioremediation and energy technologies. Mechanistic knowledge into these processes primarily comes from studies with *Geobacter sulfurreducens* grown in media closely formulated to mirror the mineral chemistry of contaminated sites. Although subtle differences in the media's cationic strength did not measurably change permeability, they reprogrammed outer membrane-peptidoglycan crosslinks modulating vesiculation and envelope functions impacting growth efficiency and mineralization. Cations that strongly bind and neutralize peptidoglycan carboxylates to prevent cell wall distortions, such as sodium and uranyl ions, ultimately determined the extent of envelope remodeling and cell bias toward pili-driven mineralization or membrane adsorption and release in vesicles. These findings identify cation chemistry as a key regulator of outer membrane vesiculation and the reprogramming of envelope functions ultimately determining the reproducibility of laboratory studies and effectiveness of bioremediation and energy-harvesting applications.

Introduction

The biomineralization of iron and other metals by electrically active (electric) microorganisms represents one of the oldest and most influential geobiological processes on Earth^{1–3}. These microbes, and the conductive minerals they generate, establish natural electric grids that metabolically couple microbial communities and shape the geochemistry of entire ecosystems^{4–7}. Among them, members of the *Geobacterales* provide foundational models for understanding how microbial electrical networks drive metal¹ and, indirectly, carbon⁸ sequestration into stable mineral sinks and how their metabolisms can be harnessed to mine and reclaim metals critical to energy storage technologies⁹. These electrical activities are primarily driven by a trans-envelope respiratory chain of c-cytochromes¹⁰ as well as retractable, conductive pili assembled on one side of the cell to extend the redox-active surface beyond outer membrane c-cytochromes.^{11,12} The conductive pili shared many of the conserved properties of bacterial Type IVa pili, yet are assembled with a uniquely short,¹¹ aromatically dense,¹³ and intrinsically conductive¹⁴ pilin peptide. The ubiquity of similar, aromatically dense pilin sequences in metal-rich environments¹⁵ suggests that pili-mediated electron flow is a widespread mechanism for metal cycling across ecosystems.

Pilus nanowires also drive the reductive mineralization of soluble toxic metals and radionuclides such as the uranyl oxycation (UO_2^{2+}).¹⁶ Surface motifs of anionic ligands promote the binding of cationic metals to the pilus fibers to facilitate their reductive mineralization,¹⁶ in a process that also conserves energy for growth.¹⁷ Pilus nanowires also permeate the exopolysaccharide (EPS) matrix of biofilms, establishing vertical redox gradients with EPS-anchored c-cytochromes for optimal electron flow¹⁸ and enhancing uranium mineralization.¹⁹ Field amendments with electron donors such as acetate stimulate these activities and promote the in situ immobilization of uranium and other toxic metals in contaminated

environments.^{20–24} Guiding these and other applications are laboratory studies with representative strains, primarily *Geobacter sulfurreducens*, grown in bicarbonate-based mineral media formulated with bicarbonate and mineral salts that replicate the geochemistry of contaminated sites.^{25,26} These studies identified a complementary pathway for the detoxification of uranyl cations via their adsorption to the rough (no O-antigen) lipopolysaccharide (LPS) that densely coats *Geobacter* cells and the disposal of LPS-bound metal in outer membrane vesicles (OMVs)²⁷ (**Fig. S1**). Vesiculation also provides a mechanism for the detoxification of any uranium which may have diffused and mineralized in the periplasm¹⁶ due to the high-affinity and low-potential of periplasmic c-cytochromes²⁸ (**Fig. S1**). The presence of c-cytochromes in the OMVs also suggests roles in extracellular electron transfer to minerals and other cells in biofilms.^{29,30} Furthermore, OMVs readily attach to solid surfaces and lower interfacial resistance.²⁹ Thus, the vesicles might complement or amplify mineralization reactions driven by the pili.

Cultivation conditions strongly bias whether cells assemble pili or vesiculate, thus undermining the reproducibility and predictive power of laboratory studies. For examples, pilus nanowires are required for iron or manganese oxides respiratory growth but not for the respiration of soluble electron acceptors such as fumarate.¹¹ Yet, non-piliated cells can grow optimally in fumarate cultures under uranium stress by hypervesiculating.²⁷ Furthermore, vesiculation is attenuated in the fumarate-grown cultures at temperatures (25°C) that slow down growth as in cultures with metal oxides and induce pili assembly.^{11,16} These reciprocal responses suggest that pili-wired and OMV-based detoxification pathways are co-regulated. Evidence further implicates outer membrane-peptidoglycan crosslink rearrangements in this coordination. Genetic disruption of one of several outer membrane OmpA-like anchors in *G. sulfurreducens* increases both vesiculation and current harvesting from electrode-associated biofilms.³⁰ Moreover, the hypervesiculating cells release OMVs enriched in OmcZ,³⁰ an outer membrane c-cytochrome that accumulates closer to the electrode surface to lower interfacial resistance.³¹ Yet as the biofilm cells grow away from the electrode, the wiring capacity of the conductive pili becomes increasingly more important¹⁸, enhancing current harvesting¹⁸ and uranium detoxification.¹⁹ These observations point to a coordinated regulation of pili- and OMV-based electron transfer pathways via yet unresolved envelope mechanisms.

Motivated by this gap in knowledge, we investigated how *G. sulfurreducens* modulates vesiculation when grown in two mineral media (NB and DB) indistinctively used for *Geobacter* cultivation. NB, a nutrient broth originally formulated for genetic studies,³² remains a popular medium for metal reduction and OMV research.^{27,29,30} DB is an NB-derived mineral formulation originally used for electrochemical studies of electrode-immobilized cells³³ used, once supplemented with the NB vitamin mix, for electrochemical³⁴ and metal detoxification³⁵ growth studies. Both media use bicarbonate buffering to recreate the complexation of cationic metals in the contaminated subsurface.^{25,26} Yet, as we show, previously overlooked differences in the media cationic strength, particularly from sodium (Na⁺), influence outer membrane-peptidoglycan crosslinks and reciprocally regulate piliation and vesiculation. These findings refine current models of OMV control in Gram-negative bacteria and reveal

physicochemical drivers of envelope remodeling that ultimately determine the reproducibility of laboratory phenotypes and metal transformations driven by *Geobacter* bacteria. We further discuss the implications of these findings for translating laboratory observations into field-scale and energy-harvesting applications with *Geobacter*.

Results

Control of outer membrane vesiculation by Na^+ ionic strength.

In order to standardize culture conditions for the study of vesiculation in *G. sulfurreducens*, we quantified OMVs in mid-exponential acetate-fumarate cultures using two popular mineral media (NBAF³² and DBAF,³⁴ respectively, but abbreviated as NB and DB herein). Incubation was at 35°C to support optimal planktonic growth and prevent piliation.^{11,16} OMV detection leveraged the high adsorption capacity of highly-oriented pyrolytic graphite (HOPG) for extracellular vesicles and the imaging power of an atomic force microscope (AFM)³⁶ to track vesiculation levels in culture samples, as previously described.²⁷ The method also avoided the artefacts introduced with protocols requiring OMV extraction, purification, and single-particle tracking analysis,³⁷ previously applied to characterize *G. sulfurreducens* OMVs.^{29,30} Although the NB and DB formulations contained comparable levels of sodium bicarbonate (21.4 and 23.8 mM NaHCO_3 , respectively) and were identically buffered (pH of 6.8) and amended with vitamins, their salt content differed subtly (**Table S1**). Most notably, the DB medium lacked two weakly complexed Na^+ salts (6 mM NaCl and 3.5 mM Na_2CO_3) from the NB formulation, decreasing the molar concentration of the alkali cation in DB from 89.5 to 79.3 mM. This difference markedly influenced the vesiculation response, stimulating it in the DB cultures (Fig. 1) despite measurably impacts on outer membrane permeability (**Fig. S2A**). However, addition of one or two of the NB salts to the DB cultures chemically rescued the hypervesiculation phenotype (Fig. 1A). Overall, average OMV yields per cell linearly scaled with the molar Na^+ concentration in the cultures ($R^2 = 0.999$).

Further supporting the direct role of Na^+ in vesiculation control, OMV formation in the NB cultures was further attenuated by adding yeast extract (**Fig. S3**), a concentrate of the soluble fraction of osmotically autolyzed yeast that carries high molar concentrations of Na^+ salts³⁸ and is commonly added to *G. sulfurreducens* cultures.³⁹ By contrast, NB supplementation with cysteine, a reducing agent commonly added to ensure anoxic conditions,^{32,40} had no effect on OMV release (**Fig. S3**). These results suggested that Na^+ ionic strength is a key modulator of outer membrane vesiculation in *G. sulfurreducens*. Further supporting this, increasing the concentration of sodium acetate as an electron donor did not significantly impact vesiculation until the weakly complexed NB salts were added (**Fig. S2**), consistent with the stronger binding of Na^+ to acetate (CH_3COO^-) than to chloride (Cl^-) or carbonate (CO_3^{2-}) anions predicted from the Hofmeister series.⁴¹ By contrast, the type of alkaline ion mattered and higher concentrations of K^+ than Na^+ chloride and carbonate salts were needed to attenuate the hypervesiculation response of DB-grown cells to NB levels (**Fig. S2**). This is consistent with a

mechanism for vesiculation control mediated by electrostatic interactions that sterically favored the smaller Na⁺ cation.

Na⁺ ionic strength remodels outer membrane-peptidoglycan crosslinks and respiratory chains.

What type of electrostatic interactions with Na⁺ could modulate OMV formation? This alkali monovalent cation, more so than K⁺, diffuses freely through the outer membrane of Gram-negative cells⁴² and strongly binds the peptidoglycan's carboxylate groups,⁴³ minimizing repulsion forces that would otherwise cause cell wall expansions.⁴⁴ Consistent with this, cryo-EM imaging (**Fig. S4**) revealed enlarged periplasms (Fig. 1C) and, accordingly, larger cell surface area (measured by AFM, Fig. 1D) in DB-grown cells compared to those from NB or salt-amended DB cultures (Fig. 1D). Cell size also scaled linearly with OMV formation across all culture conditions (**Fig. S5**), strengthening the link between Na⁺-driven cell wall dynamics and vesiculation control.

We gained further insights into envelope adaptations to Na⁺ by comparing the proteomes (**Data S1**) and transcriptomes (**Data S2**) of DB-grown cells to NB controls. Though relatively modest (~ 10 mM), differences in Na⁺ molar concentrations in the two media drove broad changes in envelope-associated proteins (**Fig. S6-7**), including lipoproteins tethering the outer membrane to the underlying peptidoglycan (Fig. 2). The most significant change was the upregulation in the DB-grown cells of Lpp, the abundant Braun lipoprotein that covalently crosslinks the outer membrane to the peptidoglycan to preserve its integrity⁴⁵ and mechanically stabilize the cell envelope.⁴⁶ The Lpp of *G. sulfurreducens* is also longer than canonical Lpp proteins and well suited to accommodate periplasmic enlargements,⁴⁷ such as those observed in the DB-grown cells (Fig. 1). Pba, one of several OmpA-like peptidoglycan-binding protein anchors implicated in vesiculation control in *G. sulfurreducens*³⁰ and other Gram-negative bacteria,⁴⁸ was significantly downregulated in the transcriptomic dataset and, to a lesser extent, in the proteome (significance after imputation of a missing value was $p = 0.12$) (Fig. 2A).

Proteins of the Type IV pilus machinery (PilM) and the structural subunit of the conductive pili (PilA pilin)¹¹ were also upregulated in the DB proteomes (Fig. 2A). The trans-envelope pilus complex not only enhances the respiratory capacity of the cells,¹¹ it also reinforces outer membrane-peptidoglycan crosslinks^{49,50}. DB-grown cells also upregulated other proteins involved in extracellular electron transfer (**Table S2**), most notably the XapD ABC transporter and XapK glycosyltransferase required for the synthesis of the redox-active Xap EPS⁵¹ and the small PgcA c-cytochrome that facilitates interfacial electron transfer with minerals⁵² (Fig. 2A). Transcriptional profiling (**Fig. S7**) revealed additional reprogramming of membrane-bound proteins not captured within the significant thresholds set for the proteomic data (Fig. 2B and **Table S3**). These proteins included outer membrane c-cytochromes involved in iron oxide reduction such as OmcS⁵³ and those (OmcG and OmcH) involved in their regulation.¹⁰ Many of these c-cytochromes are enriched in OMVs^{29,30} and could have been underrepresented in the cells harvested for quantitative proteomics (Fig. 2A) and heme-staining detection (**Fig. S7**). Heme-staining is

further compounded by the similar electrophoretic mobility of many envelope c-cytochromes,^{54,55} which can mask expression changes. Similarly, transcriptomics but not proteomics analysis revealed the upregulation in DB-grown cells of the ATP-binding subunit of an ABC exporter (YbhF) with predicted roles in $\text{Na}^+:\text{H}^+$ antiport⁵⁶ and the extracytoplasmic function (ECF) sigma factor RpoE (**Fig. S7**), an alternative sigma factor that modulates the expression of pilus nanowires and c-cytochromes in *G. sulfurreducens*.⁵⁷ The functional significance of these transcriptional shifts in cation-driven envelope remodeling remains to be determined. Notably, RpoE regulates OMV production and other envelope responses in numerous Gram-negative bacteria,⁵⁸ yet its role in *G. sulfurreducens* is largely unexplored. This highlights the need for further studies to determine how this bacterium reprograms envelope responses to cation-driven structural adaptations.

Envelope adaptations to the uranyl oxyanion reveal dynamic trade-offs between vesiculation and piliation.

Like Na^+ , the uranyl oxyanion also strongly binds and effectively neutralizes the peptidoglycan carboxylates.⁴³ Consistent with this, culture supplementation with uranyl acetate ($\text{DB}^{+\text{U}}$ and $\text{NB}^{+\text{U}}$) abolished differences in the periplasmic width and surface area of the DB- versus NB-grown cells (**Fig. 3A**). The average periplasmic width of uranium-treated cells in both media was among the largest measured ($\sim 30\text{--}32\text{ nm}$), as expected from the complexation of the peptidoglycan carboxylates with the much larger uranyl oxyanion. However, the average size of uranium-treated cells was smaller than in untreated cultures (**Fig. S8A**), suggesting that peptidoglycan complexation with the rigid, linear uranyl oxyanion ($\text{O} = \text{U} = \text{O}^{2+}$) imposed constraints to overall cell expansions.

Uranium supplementation also triggered piliation in both cultures, albeit more strongly in the $\text{DB}^{+\text{U}}$ than in the $\text{NB}^{+\text{U}}$ cultures (14.4% of the cells had extensive mineralization compared to 8.5% in $\text{NB}^{+\text{U}}$) (**Fig. 3B-C**). AFM scans showed the characteristic monolateral mineralization of uranium by pilated cells¹⁶ in the cultures and identified, at closer magnification, uranium nanoparticles permeated by pilus fibers with the characteristic AFM height (2.5 nm) of uranium-reducing pili^{13,59} (**Fig. S9**). Furthermore, uranium-treated cells agglutinated (**Fig. S8B**), an aggregative phenotype associated with pili production.⁶⁰ Thus, uranyl cations not only counteracted the structural effects of Na^+ ionic strength but also shifted the cellular response toward enhanced piliation and mineralization. AFM scans of uranium-treated cultures revealed OMVs with a similar AFM diameter ($\sim 10\text{ nm}$) as in the untreated cultures (**Fig. S9**). However, vesiculation yields were reversed in uranium-treated cultures, decreasing in $\text{DB}^{+\text{U}}$ and increasing in $\text{NB}^{+\text{U}}$ (**Fig. 3B**). Furthermore, OMV release per cell inversely correlated with the levels of pilated cells in the uranium-treated cultures (**Fig. 3C**). These findings suggest dynamic trade-offs between pili and OMV pathways during uranium detoxification, where ionic conditions favoring piliation attenuate vesiculation and vice versa. Yet both mechanisms are needed to protect the cell from uranyl toxicity. This is because pilus nanowires confine mineralization to a single side of the cell,^{11,16} leaving the opposite side reliant on uranyl binding to its rough LPS layer and detoxification via OMV release.²⁷

Differential impact of Na⁺ and uranyl cations on growth and metal mineralization.

Rigorous control of media preparation and culture passage ensured phenotypic reproducibility and unmasked subtle, yet significant, differences in growth efficiency as a function of cation chemistry (Na⁺ and uranyl) (Fig. 4A). The DB-grown cells reproducibly underwent slightly longer (> 1 hour) lag phases yet, once acclimated, they doubled faster than in the NB medium. Moreover, growth yields measured by optical density were similar in both cultures but masked the lower viability of the DB cells upon entry into stationary phase. Mid-exponential DB cells also experienced more viability losses upon growth arrest in a bicarbonate-buffered solution (Fig. 4B). Additionally, the enzymatic removal of uranyl acetate per viable cell in the resting state was higher when using DB-grown cells compared to NB (Fig. 4B). The upregulation in the DB cells of envelope proteins for extracellular electron transfer such as pili and c-cytochromes (Fig. 2) could have accounted for these differences, as previously shown.¹⁶ Cell wall and periplasmic expansions in the DB-grown cells (Fig. 1) may have also made more peptidoglycan carboxylates available for binding the uranyl cations and prime them for more efficient uranium removal once in the resting state. Ultimately, these results caution about the importance of cultivation conditions in the metal-reducing phenotypes reported in resting cell assays, whose reproducibility depends on respiratory capacity⁶¹ and cellular integrity.⁶²

We also compared the growth efficiency of *G. sulfurreducens* in NB or DB cultures supplemented with 500 μ M uranyl acetate (NB^{+U} and DB^{+U} cultures) (Fig. 4A). The NB^{+U} and DB^{+U} cultures acclimated and grew at comparable rates in the presence of uranium, albeit less efficiently than in untreated controls (**Fig. S10**). However, growth yields (both by optical density and viable cell counts) were significantly lower in the DB^{+U} medium compared to NB^{+U} (Fig. 4A). The heightened susceptibility of growth-arrested DB cells to uranium likely reflects the compounded effects of uranium-induced envelope stress on cells already compromised by low Na⁺ ionic strength, which makes their envelope more vulnerable to uranium toxicity once growth is arrested and active mechanisms for detoxification and repair are no longer active.

Discussion

Findings from this work demonstrate that cation chemistry is a key and previously underappreciated regulator of outer-membrane vesiculation and envelope remodeling in Gram-negative bacteria. Subtle, yet physiologically relevant, differences in the medium's Na⁺ ionic strength remodeled outer membrane-peptidoglycan crosslinks, adjusted the periplasmic volume and cell size, and modulated vesiculation in *G. sulfurreducens* (Fig. 1). These adaptive responses to Na⁺ ionic strength reflect the free diffusion of the alkali cation through the outer membrane,⁴² its strong affinity for peptidoglycan carboxylates,⁴³ and its ability to neutralize carboxylate charges to prevent repulsion forces that would otherwise cause cell wall expansions.⁴⁴ Insufficient free Na⁺ in the DB medium led to cell wall expansions that widened the periplasm and destabilized non-covalent tethering via Pba, inducing curvature stress and vesiculation, even though the outer membrane-peptidoglycan lattice was covalently reinforced with Lpp anchors (Figs. 1 and 2). However, culture supplementation with weakly complexed Na⁺ salts alleviated these

constraints and chemically rescued the hypervesiculating phenotype of DB-grown cells (Fig. 1). K^+ similarly modulated OMV formation but less effectively than Na^+ due its larger ionic radius and lower ionization potential,⁶³ which makes local ion pairing with peptidoglycan carboxylate ligands more inefficient.⁶⁴ These results highlight how steric and electrostatic factors unique to each alkali metal ion contribute to envelope remodeling. Conversely, the inability of additional sodium acetate to suppress vesiculation in DB cultures (**Fig. S2**) underscores how anion pairing can strongly limit free Na^+ and cation-driven envelope adaptations.

Control of vesiculation via cation-driven remodeling of envelope crosslinks complements genetic studies linking outer membrane-peptidoglycan tethers to OMV formation, particularly those mediated by non-covalent OmpA-like anchors such as Pba^{30,48} and the Braun lipoprotein Lpp.⁶⁵ Importantly, because vesiculation control in *G. sulfurreducens* only required subtle changes in cation chemistry, we circumvented the broad pleiotropic defects reported for anchor mutants⁴⁸ and instead examined envelope adaptations under physiological relevant conditions. As a mechanically tethered system,⁶⁶ the outer membrane-cell wall lattice synchronously responds to cation deficiencies via peptidoglycan expansions that widen the periplasm.^{67,68} *G. sulfurreducens* adapted to the periplasmic enlargements in DB cultures by making more Lpp (Fig. 2), a trimeric anchor that covalently reinforces outer membrane-peptidoglycan crosslinks to stabilize the envelope.^{69,70} By transitioning from tilted to stretched triple coiled-coil configurations, the flexible Lpp trimer stabilizes the envelope and preserves the integrity of the outer membrane during periplasmic enlargements.⁴⁷ This capacity is further enhanced in *Geobacter* due to its unusually long Lpp.⁴⁷ Studies also show that the tilted-to-stretched transition of the Lpp trimer during periplasmic enlargements quickly dislodges non-covalent Pba anchors,⁴⁷ leaving untethered areas more prone to vesiculation and producing undulating membrane features, the same phenotypes we observed in the DB-grown cells (Fig. 1). The reciprocal regulation of Pba and Lpp anchors likely arises as a compensatory response to the structural remodeling of cross-links triggered by low ionic strength. The possibility of fine-tuning these crosslinks and regulatory feedbacks via adjustments in the culture's Na^+ ionic strength makes *Geobacter* a powerful system to study the plasticity of the Gram-negative envelope to spatial constraints imposed by chemical cues.

Like Na^+ , the uranyl oxycation (UO_2^{2+}) exhibits a strong affinity for peptidoglycan carboxylate groups,⁴³ effectively neutralizing their negative charges and counteracting the differential effects of Na^+ ionic strength on periplasmic and cell surface area expansions (Fig. 3). Nevertheless, uranium imposes additional physicochemical constraints on the envelope due its larger size, rigid linear structure ($O = U = O^{2+}$), strong bidentate bonds, and ability to form polynuclear U(VI) complexes with carboxylates that yield a rigid and expanded uranium-peptidoglycan scaffold.⁴³ Consequently, uranium-treated cells had, on average, larger periplasms than untreated controls (Fig. 3). Yet their cell size was constrained by the rigidity of the cell wall (**Fig. S8**). These physicochemical properties also promoted robust assembly of the Type IV pilus machinery for the reductive mineralization of uranium (Fig. 3). The slightly enlarged periplasms of the uranium-treated cells likely alleviated spatial barriers to the orderly assembly and

alignment of pre-pilus intermediates across the *Geobacter* multi-layered envelope,⁷¹ whereas the more rigid peptidoglycan scaffold provided mechanical support to the peptidoglycan anchors of the pilus complex.^{49,50} As a result, *G. sulfurreducens* pili can more effectively counteract the destabilizing forces imposed by the antagonistic cycles of pilus protrusion and retraction that are key to effective uranium mineralization and respiratory gains.¹² However, DB⁺U cultivation, more so than NB⁺U, markedly increased piliation, consistent with compounding effects of peptidoglycan-binding cations on envelope remodeling. However, cation-driven changes in the envelope imposed a dynamic tradeoff between pili-mediated mineralization and OMV-mediated export, reciprocally regulating the two pathways (Fig. 3). This balance likely reflects the compounding effects of steric crowding and stronger tethering of the outer membrane in piliated cells, which spatially and structurally limits OMV biogenesis. Such constraints reciprocally coordinate the selection of pathways during metal detoxification, preventing envelope stress yet having opposing impacts on respiratory gains and metal transformations (pili-mediated mineralization versus reversible adsorption to vesicles).

Taken together, our results demonstrate that cation chemistry is an unappreciated experimental variable that must be rigorously controlled to ensure reproducibility in studies of extracellular electron transfer and metal transformations. Many discrepancies in the *Geobacter* literature, and likely in other Gram-negative systems, might be traced to unstandardized cations (e.g., Na⁺, K⁺) and associated counterions in media formulations and, potentially, other cultivation variables modulating piliation such as temperature.¹ Genetic analyses in *Geobacter* further confound experimental results across labs due to the pleiotropic nature of most disruptions of envelope proteins, particularly pili and outer-membrane c-cytochromes.^{16,18} In contrast, chemical modulation of envelope mechanics provides a powerful and physiologically relevant tool to dissect these pathways without exacerbating compensatory responses. The compounding effects of cation-driven envelope adaptations on bioremediation outcomes and the performance of energy-harvesting technologies driven by *Geobacter* are yet to be addressed. Moreover, the diverse and poorly understood repertoire of OmpA-like anchors in *G. sulfurreducens*³⁰ represents an opportunity to dissect how chemical cues selectively bias envelope crosslinks toward piliation or vesiculation. Given the roles of OMVs in transporting c-type cytochromes and modulating interfacial electron transfer reactions in *Geobacter*^{29,30} and their ability to form conductive nanotubes in other bacteria⁷² accounting for local ionic conditions will prove essential for producing reproducible datasets that can meaningfully guide bioremediation and energy systems driven by *Geobacter*.

Materials and Methods

Bacterial strains and culture conditions. *G. sulfurreducens* PCA⁷³ and $\Delta omcS$ ⁵³ and $\Delta omcB$ mutants⁷⁴ from our culture collection were routinely grown at 35°C in NBAFYE³², a nutrient broth (NB) medium with acetate (15 mM), fumarate (40 mM), cysteine (1 mM) and yeast extract (0.1%, w/v). After three transfers (starting OD₆₀₀ of 0.01–0.03) in mid-exponential phase (0.4–0.6 OD₆₀₀), the cells were inoculated in test media: NBAF (no cysteine or yeast extract) or a modified media formulation DBAF.³⁴ When indicated,

cultures were amended with various salts (NaCl, Na₂CO₃, KCl, and/or potassium phosphate salts) or with 500 µM uranyl acetate. Growth parameters (length of lag phase, generation time, and maximum growth yields) were calculated using the growthcurver package (v0.3.1) in R. Pili-mediated agglutination in the cultures was measured as the OD₆₀₀ difference before and after gentle tube inversion.⁶⁰

Microscopy. Mid-exponential cultures were routinely imaged in tapping mode at 0.3 Hz with an Asylum Research Cypher S Atomic Force Microscope (AFM) system equipped with an AC240TS tip to count OMVs²⁷ and uranium-mineralizing cells expressing pili (AFM amplitude images analyzed with the multi-point tool in ImageJ). OMV and pilus filament diameters were measured as AFM height with the Igor Pro (6.38B01) and Asylum AFM software (v16.31.232). The area of the cells in AFM Zsensor images was measured with the “analyze particles” tool of ImageJ, as described in the Supplementary materials.

Cryo-EM imaging used a Thermo Scientific™ Talos™ Arctica™ instrument outfitted with a Falcon 3 Direct Detection Device and a Ceta CCD camera and operated at 200 kV. Mid-exponential cell suspensions concentrated via centrifugation (5000xg for 10 min) were deposited (3–5 µl) on 300 copper mesh grids coated with a Quantifoil™ R 3.5/1.0 carbon film (45 sec of negative glow discharge with a Ted Pella Pelco easiGlow), blotted (2.5 and 10 sec blot and wait times, respectively; blot force, -2; humidity, 100%; and temperature, 4°C), and vitrified in liquid ethane in a Vitrobot Mark IV system (ThermoFisher Scientific). Image acquisition used the EPU (Thermo Scientific) software in linear mode at a 22,000x magnification (pixel size 6.6 Å) with a defocus range of – 10 µm and a dose rate of 3.3 e/Å²/s for ~ 2 sec. Periplasm widths were measured in ImageJ (“Analyze>Measure” function) by fitting a rotated rectangle (~ 5 nm wide) on the envelope every 15–20 nm, excluding the mid-center and poles (each ~ 1/5th of the cell length).

Fluorescence assays of outer membrane permeability and cell viability. Mid-exponential cells were harvested by centrifugation (8000xg, 7 min), washed twice (5 mM HEPES-5 mM glucose buffer pH 7.2), and resuspended in ½ vol of the same buffer before dispensing 100 µl of sample and buffer controls (blanks) in triplicate wells of a white-walled, clear-bottom 96-well plate (Alkali Scientific). A stock of 500 M N-Phenyl-1-naphthylamine (NPN) (Sigma-Aldrich) in acetone was diluted to 20 µM in assay buffer before adding 100 µl to each well, pipetting to mix, and incubating in the dark (room temperature, 10 min) before measuring (350 nm excitation, 420 nm emission) fluorescence from the hydrophobic fluorescent NPN probe after intercalation into the outer membrane phospholipids.^{75,76}

Cell viability was assayed with the Live/Dead® BacLight™ Bacterial Viability Kit (ThermoFisher Scientific) in cells harvested by centrifugation (5,000xg for 10 min) from 0.5-1 ml early-stationary phase cultures or resting cell suspensions, washed twice in 0.85% NaCl, and dispensed (100 µl) in triplicate wells of a 96-well plate (Wuxi NEST), avoiding peripheral wells (rows A and H, columns 1 and 12) for improved reproducibility. A 2X staining solution (4 µl of 5 mM Syto9 and 6 µl of 20 mM propidium iodide per 2 ml nuclease-free water) was added to each sample, pipetting to mix, before dark incubation (room temperature, 15 min) and fluorescence measurements in the green (485/530nm excitation/emission)

and red (485/630nm excitation/emission) channels. The ratio of green over red fluorescence was used as a proxy of cell viability.

Resting cell assays with uranium. Resting cell suspensions (OD_{600} 0.1) used mid-exponential cells osmotically adjusted in sequential steps to maximize viability, as previously described^{16,77} except that the suspensions were prepared in 20 ml of buffer with 500 μ M of uranyl acetate. Samples collected before or after 6-h incubation (35°C) were filter-sterilized (0.22 μ m), acidified in 0.8 N nitric acid, and diluted 200-fold in 3% nitric acid before measuring the uranium concentration in an Agilent 8900 triple quadrupole tandem inductively coupled proton mass spectrometer (QQQ-ICP-MS).

Proteomics and RNA-Seq analysis. Proteins and RNA transcripts extracted from triplicate 10-ml, mid-exponential NBAF and DBAF cultures were sequenced at Michigan State University Research Technology Support Facility (RTSF) and the SeqCenter (Pittsburg, PA), respectively, following standardized protocols. Protein extraction was from cells harvested by centrifugation (3,220 $\times$ g, 20 min) and washed three times in 10 mM Tris-HCl buffer (pH 7.7) before resuspending in 500 μ l of buffer and centrifuging again in 2-ml cryotubes (8,000 $\times$ g, 10 min) to obtain a pellet for storage at -80°C. Cell lysis was initiated in 2x SDS-PAGE sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 50 mM DTT; 95°C for 5 min) and, after cooling to room temperature, continued in a water bath sonicator (3 cycles of 1 min sonication and 1 min rest). Protein (100 μ g, measured with Pierce BCA assay) was digested with trypsin/LysC (Promega) using S-traps (ProtiFi) according to manufacturer's instructions before resuspending 20 μ l of the digest in 2% acetonitrile/0.1% trifluoroacetic acid and calculating the final peptide concentration with a Pierce Quantitative Colorimetric Peptide Assay Kit. Samples were then run through Data-Independent Acquisition (DIA) LC/MS/MS to obtain the peptide spectra and analyzed as described in the Supplementary Methods and elsewhere.^{78,79} Missing ('NA') values were separately imputed in NB and DB replicates with the R studio missForest package (v1.5).⁸⁰ Differentially expressed proteins (DEPs) (**Data S1**) were determined in Microsoft Excel, as described in the Supplementary Methods.

RNA extraction and purification was as previously described.³⁵ Illumina library preparation and sequencing (paired end 150bp reads on a NovaSeq X Plus instrument) used protocols standardized at SeqCenter. Demultiplexing, quality control, and adapter trimming used Illumina's bcl-convert (v4.2.4) software, whereas read mapping used HISAT2 (v2.2.0) with default parameters and '-very-sensitive'.⁸¹ Read quantification used Subread's featureCounts (v2.0.1) functionality with default parameters and '-Q20'.⁸² Read counts loaded into R (v4.0.2) were normalized using edgeR's (v1.14.5) Trimmed Mean of M values (TMM) algorithm with default parameters⁸³ before conversion to counts per million (CPM). Differential expression analysis used edgeR's glmQLFTest. The data was then filtered for a $|\log_2 FC| > 0.58$ and p -value < 0.05 to determine differentially expressed genes (DEGs) (**Data S2**). Adjusted p -values were not used given the low number of DEGs.

Functional assignments to DEPs and DEGs used the KEGG and UniProt⁸⁴ databases. Analysis of transmembrane regions and subcellular localization was via DeepTMHMM (v1.0) and PSORTb (v3.0.3),

respectively. Data visualization (heatmaps, dispersion plots, and volcano plots) used the pheatmap (v1.0.12) and ggplot2 (v3.5.1) packages in R studio.

Statistical Analysis. The statistical significance ($p < 0.05$) of phenotypic differences in the various cultures was assessed with either a Student's or Welch's t -test (chosen based on the results of a F-test for variance) in Excel, as described in the corresponding figure legends. Where indicated, we used the Grubbs' test for outliers from GraphPad's online outlier calculator to remove up to one outlier per condition.

Data and materials availability. All data are available in the main text or the supplementary materials. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD069850. The RNA-Seq raw data are available at the NCBI Gene Expression Omnibus (GEO) repository under accession number GSE285971.

Declarations

Data and materials availability

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Author contributions

Conceptualization: GR, KK. Methodology: MMC, GR. Investigation: MMC. Visualization: MMC, GR. Supervision: GR, KK. Writing—original draft: MMC, GR. Writing—review & editing: GR, MMC, KK.

Competing interests

Authors declare that they have no competing interests.

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Figures

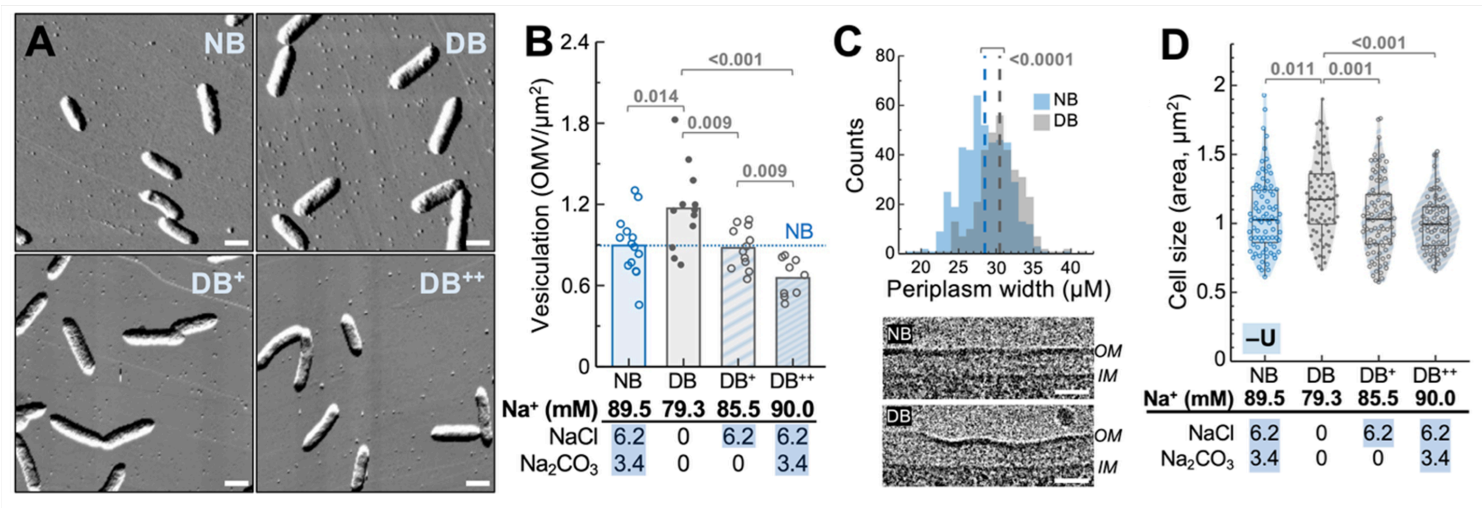


Figure 1

Na⁺ ionic strength modulates vesiculation. (A-B) AFM micrographs (A; scale bar, 1 μm) used to quantitate OMV formation (B; per μm² and OD₆₀₀ of 0.5) in NB, DB and salt-amended DB (DB⁺ and DB⁺⁺) cultures. (C) Histograms showing average periplasmic widths in 8-10 cells imaged via cryo-electron microscopy (representative images shown below). (D) Cell size (AFM area) in NB, DB or salt-amended DB cultures (DB⁺, DB⁺⁺) imaged in AFM scans such as those shown in (A). Statistical analyses were conducted with a two-tailed *t*-test (*p* < 0.05 in bold).

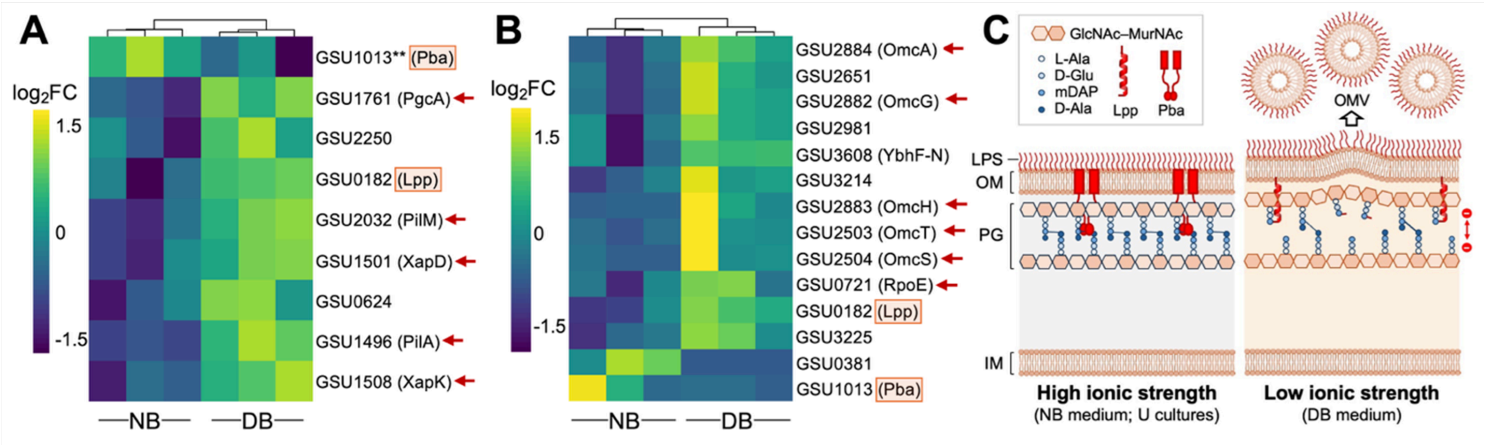


Figure 2

Na⁺-induced reprogramming of cell envelope proteins. (A-B) Heat maps show differentially expressed proteins (A) and RNA transcripts (B) in DB vs. NB cultures, including outer membrane-cell wall anchors (arrows) and proteins involved in extracellular electron transfer (asterisks). One of the Pba values in the proteomic dataset could not be retrieved under the experimental conditions (NA, “not available”), and was imputed. (C) Model illustrating how cell wall distortions due to reduced linking outer membrane-cell wall anchoring to cell wall distortions at low ionic strengths and vesiculation. Abbreviations: amino acids in the peptide chain (L-Ala, *L*-alanine 1; D-Glu, *D*-glutamic acid 2; mDAP, meso-diaminopimelic acid 3; D-Ala, *D*-alanine 4), peptidoglycan sugars (GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid); outer membrane-peptidoglycan anchors Lpp (lipoprotein trimer, adapted from the *E. coli* crystal structure PDB: 1EQ7) and Pba (peptidoglycan-binding anchor, dimer representation); other abbreviations (IM, inner membrane; LPS, lipopolysaccharide; OM, outer membrane; OMV, outer membrane vesicle; PG, peptidoglycan).

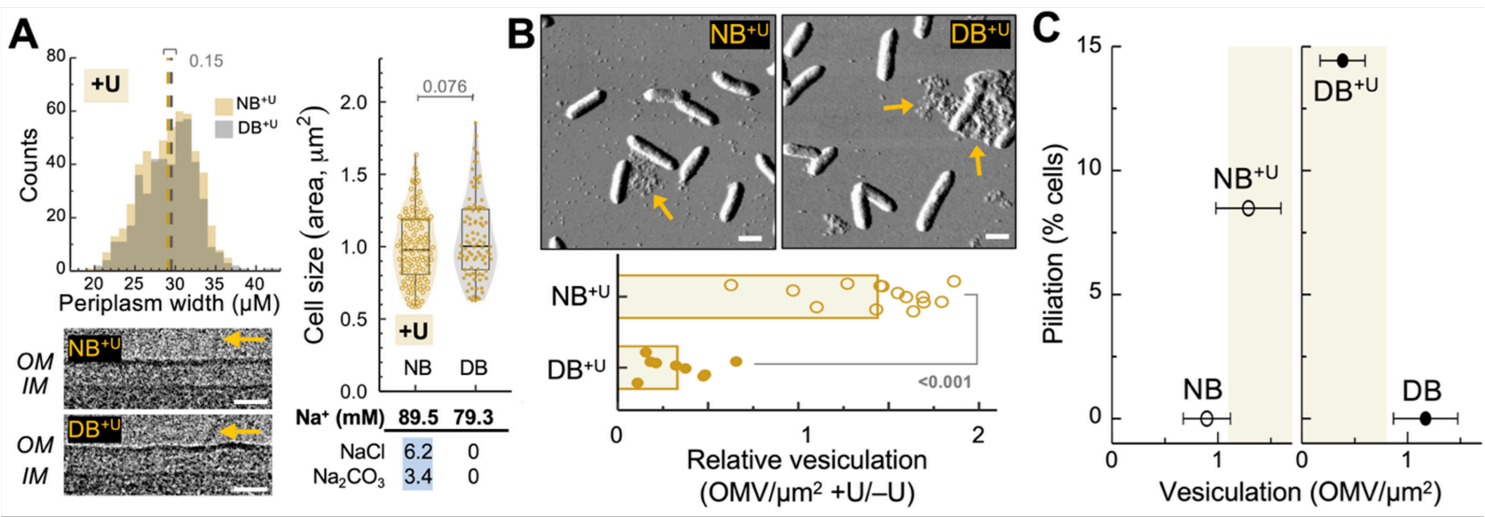


Figure 3

Impact of uranium on envelope structure, vesiculation, and piliation. (A) Histograms showing average periplasmic widths in uranium-treated NB and DB cells (8-10 per condition) imaged via cryo-EM

(representative images shown, with arrow pointing at protruding pili; scale, 50 nm). The violin plots show cell sizes measured as AFM area for each culture. **(B)** Representative AFM images of uranium-treated NB and DB cultures showing cells, OMVs, and monolateral mineralization of uranium by piliated cells. The relative vesiculation of the uranium-treated versus untreated cultures is also shown. **(C)** Correlation between vesiculation and piliation in the uranium-treated and untreated NB and DB cultures. Statistical analyses were conducted with a two-tailed *t*-test ($p < 0.05$ in bold).

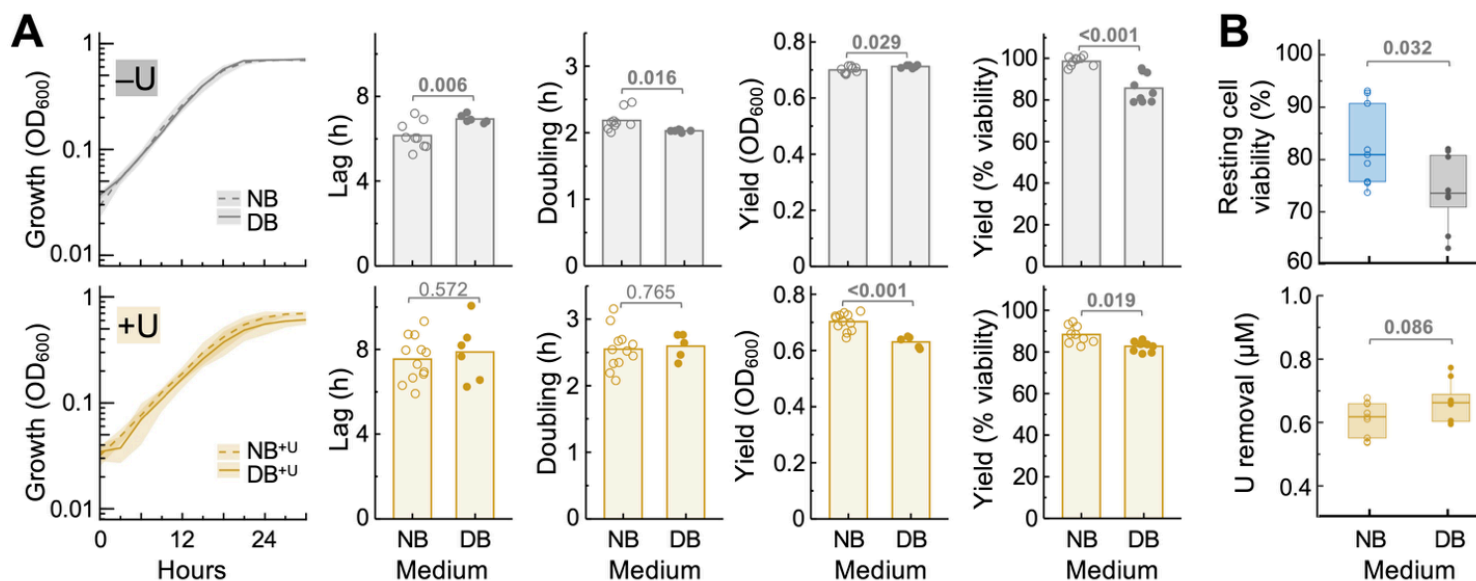


Figure 4

Effect of uranyl cations on growth and detoxification. **(A)** Growth curves (optical density at 600 nm, OD_{600} , of >6 biological replicates at 35°C) and efficiency indicators (lag phase, doubling time, and maximum growth yields as OD_{600} and percentage of viable cells measured with the LIVE/DEAD[®] BacLight[™] kit) in the absence (gray) or presence (ochre) of 500 mM uranyl acetate. **(B)** Resting (growth-arrested) cell suspension assays with mid-exponential cells from **(A)**. Top panel shows viability (BacLight fluorescence dyes ratio) of resting cells from NB and DB cultures. Bottom panel shows average uranium removal (μ M) per viable cell in triplicate biological replicates and two technical replicates after incubating (35°C) the resting cells in the presence of 500 μ M uranyl acetate for 6 h. Statistical significance of treated versus untreated controls was assessed with a two-tailed *t*-test ($p < 0.1$ in bold) after a Grubb's outlier test.

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