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Extracellular Electron Transfer by *Shewanella oneidensis* Controls Palladium Nanoparticle Phenotype

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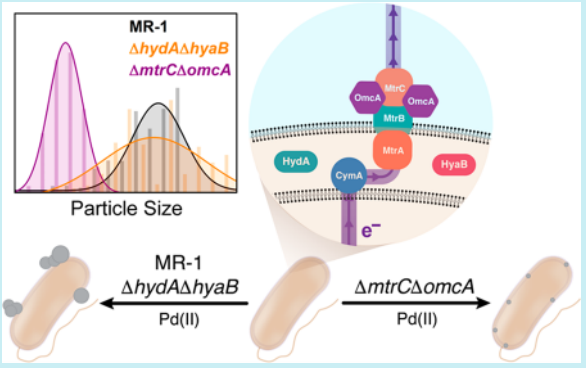
Supporting nformation

ABSTRACT: The relative scarcity of well-de ned genetic and metabolic linkages to material properties impedes biological production of inorganic materials. The physiology of electroactive bacteria is intimately tied to inorganic transformations, which makes genetically tractable and well-studied electrogens, such as *Shewanella oneidensis* attractive hosts for material synthesis. Notably, this species is capable of reducing a variety of transition-metal ions into functional nanoparticles, but exact mechanisms of nanoparticle biosynthesis remain ill-de ned. We report two key factors of extracellular electron transfer by *S. oneidensis*, the outer membrane cytochrome, MtrC, and soluble redox shuttles (flavins), that affect Pd nanoparticle formation. Changes in the expression and availability of these electron transfer components drastically modulated particle synthesis rate and phenotype, including their structure and cellular localization. These relationships may serve as the basis for biologically tailoring Pd nanoparticle catalysts and could potentially be used to direct the biogenesis of other metal nanomaterials.



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Control over cellular machinery has enabled the production of diverse compounds including pharmaceuticals, fuels, ne chemicals, and soft materials.[1 3] However, microbial engineering of metal and metal oxide products remains a signi cant challenge. Attributes such as electron transfer and nucleation are typically absent from organic biosyntheses, and their presence adds increased complexity to inorganic transformations. As a result, the enzymatic and metabolic factors that drive material formation in biological systems have been challenging to study and manipulate.[4] Despite the paucity of bioengineered inorganic products, microbial transformation of inorganics occurs quite frequently in nature. Several organisms generate highly functional and ordered materials, including metal nanoparticles, silicas, calcium carbonates, and metal oxides.[5 7] In these systems, coordinated protein and metabolite networks exert control over material morphology, composition, and function. For instance, magnetotactic bacteria (e.g., *Magnetospirillum* spp.) use several proteins that govern nucleation, electron transfer, and vesicle formation to generate size-controlled magnetite nanoparticles.[8] This example highlights the capability of living systems to tailor inorganic structure function relationships and suggests that exploiting naturally occurring pathways may provide a means for designer material biosynthesis.

Electroactive bacteria are attractive hosts for inorganic materials engineering, as a diversity of soluble and insoluble inorganic substrates can be incorporated into their metabolism.[9] Many electrogens can transfer respiratory electron flux onto metal species, including Cu(II), U(VI), Ag(I), Au(III), and Pd(II), to generate functional nanoparticles.[5] However, it is generally unclear how electroactive physiology dictates the structural and functional properties of produced nanoparticles. One electroactive bacterium, *Shewanella oneidensis* MR-1, is poised to address this issue, as it directs metabolic electron flux onto metals using a well-characterized electron transport pathway.[10] The organism s genetic tractability has also facilitated understanding and control of this network, with knockout, complementation, and overexpression studies leading to identi cation of important redox-active metalloproteins and small-molecules.[11] Notably, this electron transport pathway can reduce substrates located outside the bacterial outer membrane in a process known as extracellular electron transfer (EET). Despite signi cant progress in applying *Shewanella* electroactivity toward bioremediation and microbial fuel cell engineering, elucidating the function of EET components in nanoparticle formation has proven challenging. Whereas the outer membrane cytochromes, MtrC and OmcA, are primary mediators of EET to iron oxides and electrodes,[12,13] their activity in nanoparticle formation appears largely dependent on culture conditions and the identity of metal reduced. For example, a knockout strain de cient in

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these proteins changed the cellular localization of produced UO_2 nanoparticles relative to those produced by MR-1,[14] but had no measured effect on the kinetics of Pd or Cu nanoparticle formation.[15,16] Additional proteins have been identi ed as influencing nanoparticle formation rates, including hydrogenases and other periplasmic reductases,[17,18] but little is known of their influence on particle properties. Furthermore, the importance of soluble redox shuttles (flavins) in EET by *Shewanella* has become increasingly apparent,[19] but their impact on nanoparticle synthesis is wholly unexamined.

To better inform the design of material syntheses using *S. oneidensis*, we explored the role of key EET factors in the formation of Pd nanoparticles. Pd was chosen as it is one of the more well-studied nanoparticles generated by *S. oneidensis* and enabled us to draw comparisons with previous reports.[15,20 22] Biobased stabilization of speci c Pd crystal facets was previously demonstrated via peptide-directed synthesis,[23] and we reasoned that *S. oneidensis* cells may exhibit similar control of particle nucleation and growth. In contrast to previous works, the EET machinery of *S. oneidensis* also affords the opportunity to genetically and metabolically instruct electron transfer to Pd(II) ions during nanoparticle synthesis. Thus, we tested the effect of *S. oneidensis* genotype and the role of redox-active small molecules in Pd nanoparticle formation. Here, we report two factors of *S. oneidensis* electron transport that strongly influence nanoparticle phenotype: the outer membrane cytochrome, MtrC, and soluble redox shuttles (flavins). After manipulating the concentration of these species in the bacterial environment, through control of gene expression and exogenous supplementation, respectively, we observed drastic effects on the synthesis rate and phenotype (i.e., size and cellular localization) of biogenic nanoparticles. Identi cation of these factors provides proof-of-principle for using genetic and metabolic manipulation to tune the properties of Pd and potentially other metal nanoparticles generated by *S. oneidensis*.

RESULTS AND DISCUSSION

Optimizing *S oneidensis* Control over Nanoparticle Biosynthesis and Cellular Viability. To drive *S. oneidensis* control over Pd nanoparticle formation, we rst identi ed nanoparticle synthesis conditions that minimized abiotic effects and exhibited unambiguous nanoparticle phenotypes. Specifically, we examined how reaction media formulation and method of anoxic culture influenced nanoparticles formed by *S. oneidensis* MR-1. The general procedure for Pd nanoparticle biosynthesis was as follows: MR-1 was anaerobically pregrown overnight to stationary-phase in *Shewanella* Basal Medium (SBM) containing lactate/fumarate, anaerobically washed with degassed SBM, and nally used to inoculate a Pd nanoparticle reaction mixture (nal $OD_{600} \sim 0.2$). Whole mount transmission electron microscopy (TEM) enabled initial assessment of different conditions, as the bacteria became palladized upon exposure to Pd(II) and adherent nanoparticles could be visualized by electron micrographs.

Choice of electron donor can greatly affect bacterial nanoparticle formation, as many metabolically accessible electron donors (e.g., formate, hydrogen) also abiotically reduce palladium ions.[24] Indeed, we found that the hydrogenous atmosphere (3) of a humidi ed anaerobic glovebox caused autocatalytic reduction of palladium in the absence of bacteria. Thus, we used butyl rubber-stoppered Hungate Tubes purged with argon to maintain anaerobicity for bacterial pregrowth and nanoparticle biosynthesis reactions. As lactate is relatively inert to palladium and generates metabolic electron flux in *Shewanella* we used it as our primary electron donor. Reaction mixtures omitting lactate or any other electron donor showed no Pd(II) reduction (Supporting Information, Figure S1). We also assessed the influence of culture medium components that could cause abiotic reduction of Pd(II). Prior to bacterial inoculation of the reaction mixture, *S. oneidensis* was pregrown in SBM supplemented with casamino acids. When the nanoparticle reaction mixture was similarly supplemented, small Pd nanoparticles were formed on bacteria (<10 nm) (Figure S2). In contrast, mixtures omitting casamino acids had a larger nanoparticle size distribution, with particles generally falling within one of two populations: small (<10 nm) and large (~50 nm) particles. We speculated that the presence of free cysteines may cause abiotic reduction of Pd(II) and lead to fewer large particles formed on bacteria. Thus, to minimize abiotic effects we utilized reaction mixtures that lacked casamino acids.

The intracellular space is a reducing environment and leakage of promiscuous reductants (e.g., NADH, glutathione) through compromised membranes could also contribute to Pd(II) reduction.[25,26] To address this, we performed viability measurements to quantify the effect of increasing concentration of Pd(II) in the *S. oneidensis* reaction mixtures. Viability was quanti ed using the BacLight Live/Dead stain after exposure to 10, 100, and 1000 M Pd(II) for 2 h under our standard nanoparticle synthesis conditions. *S. oneidensis* viability exhibited a dose-dependent response for the concentration range tested, varying from ~80 to ~30 viable with increasing Pd(II) concentration (Figure S3). We also examined how cell viability affects reduction rate when cells were mixed with 100 M Pd(II) and given lactate as a carbon source. Viable MR-1 samples demonstrated initial adsorption and sustained reduction of Pd(II) over 4 h (Figure S4), similar to what has been observed by others.[15] In contrast, Pd(II) levels for heat-killed MR-1 and fully lysed MR-1 were lower at the initial time point and did not signi cantly vary over time. We interpreted this result as a con rmation that promiscuous cell reductants can reduce Pd(II), but that continuous reduction requires active metabolism.

As MR-1 palladization increases the 600 nm light scattering signal for single cells,[27] we hypothesized that cellular nanoparticle formation could be assessed by flow cytometry side scattering. To further probe the relationship between palladization and *S. oneidensis* viability, we used flow cytometry to simultaneously measure side scattering and fluorescence from a dye (propidium iodide, PI) that indicates membrane permeability of palladized bacteria.[21,28] At both 100 and 1000 M Pd(II), increases in side scatter were detected for palladized *S. oneidensis* strains, relative to a Pd-free control (Figure S6). At the higher Pd(II) concentration, there was a corresponding increase in both side scatter and PI fluorescence, indicating the presence of a dead population that is highly palladized. However, the same increase in PI fluorescence was not detectable at the lower Pd(II) concentration (Figure S7). This result corroborates our microscopic viability measurements and indicates that the majority of palladized cells remain viable. On the basis of these results, we chose 100 M Pd(II) as our primary concentration for nanoparticle synthesis reactions (except where noted) as to mitigate the effects of promiscuous reducing agents and cell death, while balancing sufficient nanoparticle yield for characterization and analysis.

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