

# Electrochemical Analysis of *Shewanella oneidensis* Engineered To Bind Gold Electrodes

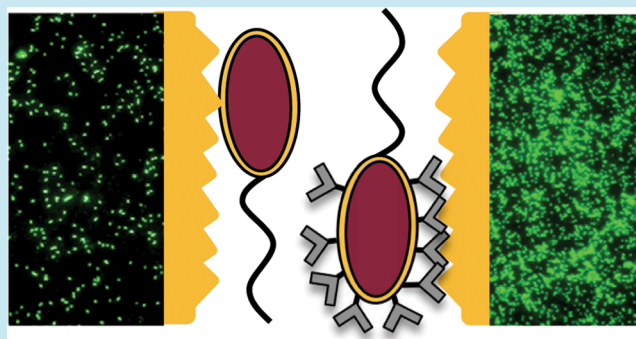
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## Supporting Information

**ABSTRACT:** Growth in three-electrode electrochemical cells allows quantitative analysis of mechanisms involved in electron flow from dissimilatory metal reducing bacteria to insoluble electron acceptors. In these systems, gold electrodes are a desirable surface to study the electrophysiology of extracellular respiration, yet previous research has shown that certain *Shewanella* species are unable to form productive biofilms on gold electrodes. To engineer attachment of *Shewanella oneidensis* to gold, five repeating units of a synthetic gold-binding peptide (SrGBP) were integrated within an *Escherichia coli* outer membrane protein, LamB, and displayed on the outer surface of *S. oneidensis*. Expression of LamB-SrGBP increased cellular attachment of *S. oneidensis* to unpoised gold surfaces but was also associated with the loss of certain outer membrane proteins required for extracellular respiration. Loss of these outer membrane proteins during expression of LamB-SrGBP decreased the rate at which *S. oneidensis* was able to reduce insoluble iron, riboflavin, and electrodes. Moreover, poisoning the gold electrode resulted in repulsion of the engineered cells. This study provides a strategy to specifically immobilize bacteria to electrodes while also outlining challenges involved in merging synthetic biology approaches with native cellular pathways and cell surface charge.

**KEYWORDS:** gold-binding peptide, biocatalysis, metal reduction



*Shewanella* are Gram-negative, facultative anaerobes derived from aquatic or subsurface environments at the oxic/anoxic interface. Survival in such environments is aided by the ability to respire a diverse set of electron acceptors, a phenotypic hallmark of the *Shewanella* genus.<sup>1</sup> *Shewanella* species are able to respire more than 20 organic and inorganic compounds in the absence of oxygen, examples of which include trimethylamine-*N*-oxide, fumarate, iron and manganese oxides, nitrate, uranium, and electrodes.<sup>1,2</sup> Several of these substrates are insoluble at or near neutral pH ranges, so *Shewanella* require a specialized system to transfer excess electrons produced during carbon oxidation to extracellular substrates in a process termed extracellular respiration.<sup>3</sup>

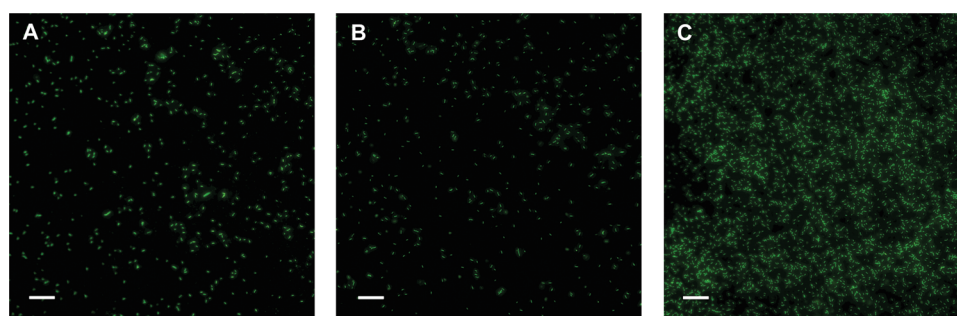
Respiratory electron transfer generally occurs at the inner membrane, so extracellular respiration without metal chelation necessitates a specialized system to transfer electrons across the inner membrane, periplasmic space, and outer membrane. For the reduction of insoluble metals and electrodes, *Shewanella oneidensis* utilizes the Mtr pathway, which includes the *omcA-mtrCAB* gene cluster, its paralogs, and the tetra-heme cytoplasmic membrane protein CymA.<sup>4,5</sup> Excess electrons produced during carbon oxidation are transported by the menaquinone pool to CymA.<sup>6–8</sup> These electrons are then passed to the periplasmic *c*-type cytochrome MtrA and finally to the terminal *c*-type cytochromes MtrC and OmcA located in

the outer membrane.<sup>9,10</sup> Interactions between MtrA and MtrC are facilitated by the integral outer membrane protein MtrB.<sup>9,11</sup> Electrons can then reduce insoluble acceptors by direct contact or via soluble mediators. *S. oneidensis* secretes redox-active flavins that have been shown to increase the rate of insoluble iron and electrode reduction.<sup>12–14</sup> This respiratory pathway not only is important in biogeochemical cycling but has also garnered interest for utilizing *S. oneidensis* in applications for bioremediation and biotechnology.

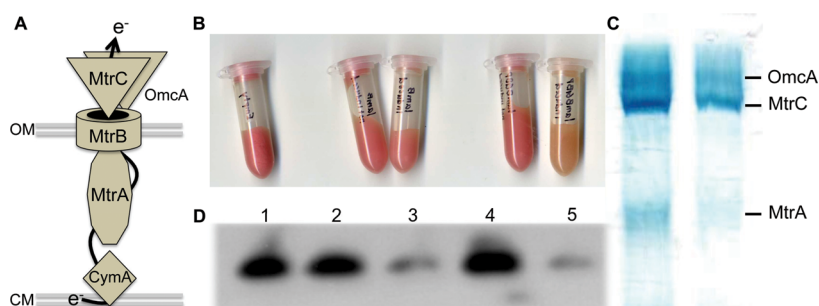
Three-electrode electrochemical cells allow constant monitoring of bacteria-mediated electron transfer reactions at redox-controlled electrode surfaces and can be used to examine the mechanisms of electron transfer from bacteria to insoluble electron acceptors. In these systems, bacteria typically oxidize a supplied carbon source serving as an electron donor and utilize an anodic electrode as the terminal electron acceptor in the absence of oxygen. These systems differ from microbial fuel cells (MFCs) in that electrochemical cells allow a user-defined potential to be set at the working electrode via a reference electrode and potentiostat. Electrodes used in these systems differ greatly, both chemically and physically, compared to substrates that *S. oneidensis* encounters naturally, and certain

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**Figure 1.** Attachment to gold(111)-plated silicon wafers. Gold wafers were added to arabinose-induced stationary phase (A) empty vector, (B) LamB, and (C) LamB-5rGBP cultures incubated at room temperature for 16 h. Bound cells were stained using the LIVE/DEAD BacLight Kit and were visualized under an inverted fluorescent microscope. Six images were taken of each wafer, and representative images are shown. Scale bars are 10  $\mu\text{m}$ .



**Figure 2.** Changes in protein profiles associated with expression of LamB and LamB-5rGBP and schematic of the Mtr pathway. (A) Simple scheme depicting key components of the Mtr pathway in *S. oneidensis* that is used to reduce Fe(III) and riboflavin (OM indicates outer membrane and CM indicates cytoplasmic membrane; see text for more information). (B) Color changes associated with LamB and LamB-5rGBP expression. From left to right: empty vector, LamB (not induced), LamB (induced), LamB-5rGBP (not induced), and LamB-5rGBP (induced). (C) Heme stain of sonicated whole cell extracts of *S. oneidensis* LamB-5rGBP cultures. Lanes include 10  $\mu\text{g}$  of total protein from not induced (left lane) and induced (right lane) samples separated by SDS-PAGE on a 4–12% bis-tris gel. Staining is due to the peroxidase activity of heme-containing proteins. (D) Western blot of outer membrane fractions utilizing an antibody against MtrB. Lanes 1–5 correspond to empty vector, LamB (not induced), LamB (induced), LamB-5rGBP (not induced), and LamB-5rGBP (induced), respectively.

anode materials have shown to be incompatible for growth of *Shewanella* biofilms.<sup>15,16</sup>

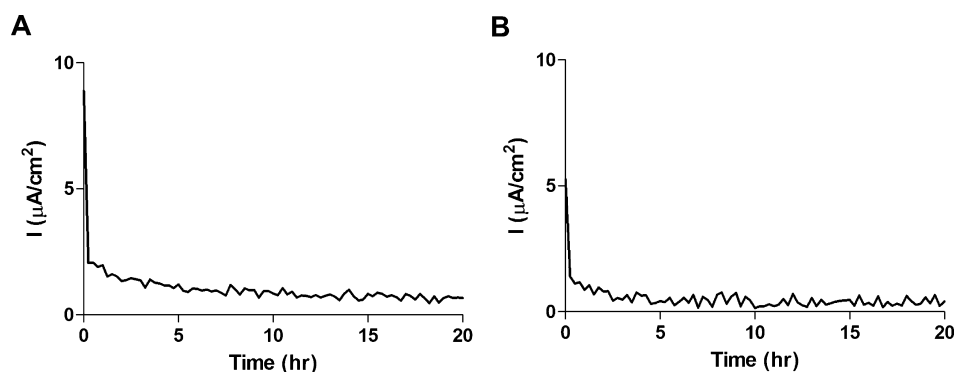
Gold is highly conductive and resistant to oxidation and can be manufactured into versatile structures and surface topologies, making it an ideal material for anode construction. However, despite these aforementioned qualities, research has indicated that bare gold is a poor anode material in studies utilizing certain *Shewanella* species.<sup>15,16</sup> Multiple theories explaining why *Shewanella* fail to interact with gold electrodes have been hypothesized and include toxicity of gold, lack of functional groups that mediate microbe/electrode interactions, a block in extracellular electron transport, inability to attach, and instability of flavin mediators in redox reactions with gold. To couple electron transfer between *Shewanella* and gold, previous studies have relied on alterations of the electrode surface. Formation of an acid-terminated self-assembled monolayer on the gold surface allowed short-term current production by *Shewanella putrefaciens* ATCC 49138 in MFCs, whereas bare gold exhibited only a capacitive discharge.<sup>15</sup> In another study with *S. oneidensis*, sputtering of a gold layer atop graphite electrodes increased current production when compared to that of bare gold.<sup>16</sup>

This study sought to engineer a fusion protein to promote attachment of *Shewanella* to gold surfaces. To do so, five repeating units of a synthetically derived gold-binding polypeptide (5rGBP) were displayed on the outer surface of the cell via surface display of a modified *Escherichia coli* outer

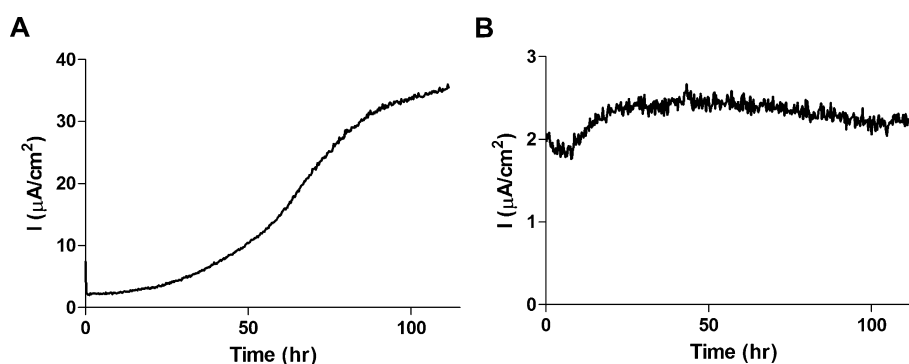
membrane porin, LamB.<sup>17</sup> Employing a synthetic peptide to directly adsorb cells was chosen over modification of the gold surface due to the advantages of being a simple, self-generating, and substrate-specific immobilization method. The engineered strain was then used in gold-electrode electrochemical cells to determine if enhanced attachment could promote increased current production. Since electrode reduction by flavins accounts for much of the current produced by *S. oneidensis* biofilms, the stability of riboflavin in redox reactions with gold electrodes was also confirmed. These studies showed that binding to gold surfaces could be increased via expression of this engineered fusion protein but at the cost of other respiratory proteins in the outer membrane essential for electron transfer.

## RESULTS AND DISCUSSION

**LamB-5rGBP Is Expressed in the Outer Membrane of *S. oneidensis* and Increases Attachment to Gold Surfaces.** Five repeating units of a synthetically derived gold-binding peptide (MHGKTQATSGTIQS) were displayed on the cell surface of *S. oneidensis* by incorporation into LamB.<sup>17</sup> Constitutive expression of LamB was toxic in both *E. coli* and *S. oneidensis*, resulting in small colony size and accumulation of an insoluble white precipitate in liquid cultures. To overcome toxicity issues, *lamB* and *lamB-5rGBP* were placed under control of the  $P_{\text{BAD}}$  promoter in the expression vector pBAD18K, requiring L-arabinose for induction.<sup>18</sup> Induction of exponential



**Figure 3.** Chronoamperometry of gold-electrode reactors prior to medium replacements. (A) Empty vector control and (B) induced LamB-SrGBP strain after inoculation and addition of 20 mM lactate and 1  $\mu$ M riboflavin to the medium. Gold electrodes were poised at 0.24 V versus SHE.



**Figure 4.** Chronoamperometry of graphite-electrode reactors poised at 0.24 V vs SHE. (A) Current produced by *S. oneidensis* expressing the empty vector. (B) Current produced by *S. oneidensis* expressing LamB-SrGBP. Reactors were run in duplicate, and representative data are shown.

phase cells did not adversely affect growth rate, and LamB and LamB-SrGBP were observed only in the outer membrane fractions of arabinose-induced cultures (Supplementary Figure S1).

To determine if the expression of LamB-SrGBP increased attachment to gold surfaces, induced cultures were incubated for 16 h with gold(111)-plated silicon wafers. *S. oneidensis* strains expressing LamB-SrGBP showed increased attachment to gold surfaces compared to strains expressing only LamB or the empty vector control (Figure 1). Bound cells were stained using propidium iodide and Syto9, and attachment to gold was visualized using fluorescence microscopy. Red channel images showed either no or very few red-stained cells, indicating that exposure to gold surfaces or expression of LamB-SrGBP did not compromise membrane integrity (data not shown).

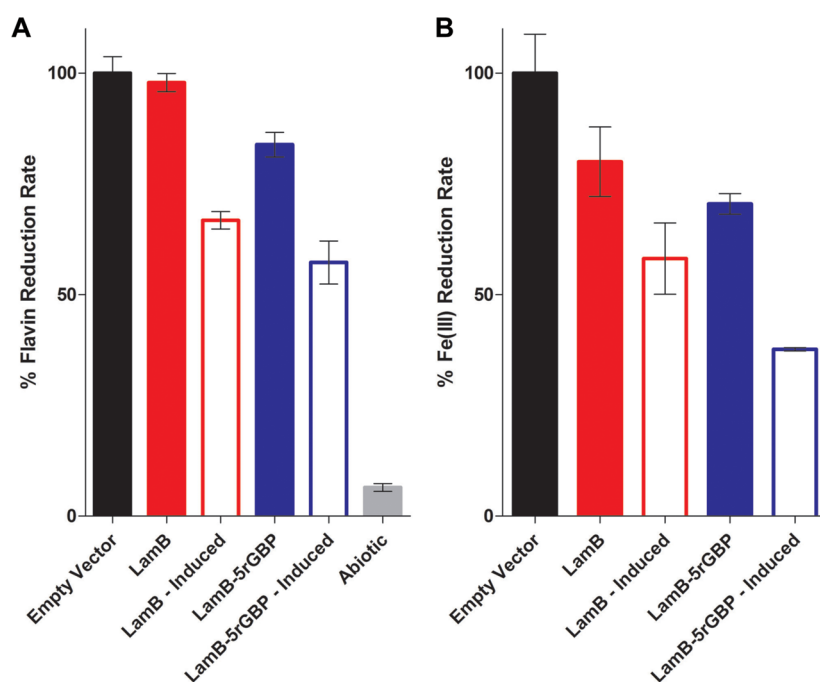
#### Expression of LamB and LamB-SrGBP Is Associated with the Loss of Outer Membrane Cytochromes Required for Extracellular Respiration.

A schematic of the Mtr extracellular electron transfer pathway is shown in Figure 2A to provide context for the following results. Cell pellets revealed a color change in arabinose-induced LamB and LamB-SrGBP samples as compared to the empty vector control (Figure 2B). A slight color change was seen in strains expressing LamB, while LamB-SrGBP expression led to the greatest color shift. Membranes of *S. oneidensis* contain multiple heme-containing *c*-type cytochromes essential to extracellular respiration and give *S. oneidensis* its characteristic pink color. Therefore, it was hypothesized that strains expressing either LamB or LamB-SrGBP were less pink due to a decrease in the concentration of these *c*-type cytochromes. Sonicated whole-cell extracts were separated by PAGE, and cytochromes were

visualized with tetramethylbenzidine.<sup>19</sup> For strains expressing LamB-SrGBP, protein levels decreased for outer membrane cytochromes MtrC and OmcA in induced cultures (Figure 2C). MtrA levels also appeared to decrease upon induction (Figure 2C).

Previous work has shown that MtrA co-purifies with MtrC and MtrB as a complex and fractionates with the outer membrane in sucrose density gradients.<sup>20</sup> Research concerning this complex has also found that while the MtrAB complex forms in the absence of MtrC, the MtrBC complex cannot form in the absence of MtrA.<sup>9</sup> Furthermore, MtrA was able to associate only with the outer membrane in the presence of MtrB.<sup>9</sup> Therefore, the reduction in MtrA levels observed in LamB and LamB-SrGBP-expressing strains could potentially be explained by a decreased concentration of MtrB in the outer membrane. Since MtrB is a non-heme-containing integral membrane protein, it could not be visualized by heme staining; therefore, a Western blot was performed using outer membrane fractions and an antibody against MtrB. This data demonstrated that MtrB levels were also severely diminished in both LamB- and LamB-SrGBP-expressing strains (Figure 2D).

The Mtr complex is essential for electron transfer to electrodes.<sup>14,21</sup> Excess electrons produced during carbon source oxidation are transported from within the cell to the outer surface via the Mtr pathway. As reduction of electrodes could be tracked in real time through the use of a potentiostat interfaced with a computer, three-electrode electrochemical cells were used to test the effects of LamB-SrGBP expression and diminished levels of Mtr components on current production.



**Figure 5.** Comparison of (A) riboflavin and (B) Fe(III) reduction by *S. oneidensis* expressing LamB and LamB-SrGBP. Rates were calculated as  $\Delta$ RFU/s and mM Fe(II)/h for riboflavin and Fe(III) reduction, respectively, by fitting the linear portion of reduction assay curves from triplicate experiments and normalized to the empty vector control (100%). Error bars represent standard error.

**Expression of LamB-SrGBP Does Not Significantly Increase Electron Transfer to Gold Electrodes.** After inoculation into electrochemical cells and poisoning of the electrode surface, a capacitive discharge is typically observed. This is generally followed by an increase in current resulting from cell attachment and active biofilm formation. Planktonic cells contribute little, if any, to current production despite active stirring of the bulk medium since active mediator turnover occurs within a diffusion layer on the order of micrometers from the electrode.<sup>22</sup> In electrochemical cells inoculated with either the empty vector control or the strain engineered to express LamB-SrGBP, data were dominated by a capacitive discharge and showed no following increase in current (Figure 3). This indicated that a productive biofilm was not forming on gold electrodes even with expression of LamB-SrGBP (Figure 3B).

**Expression of LamB-SrGBP Also Reduces Electron Transfer to Graphitic Carbon Electrodes.** In contrast to gold, graphitic carbon electrodes have been shown to support robust attachment by *Shewanella* species. To test if expression of the LamB-SrGBP construct was compromising the ability of *S. oneidensis* to transfer electrons beyond the cell membrane, graphitic carbon electrodes were used in place of gold in electrochemical cells. Maximum current was diminished over 10-fold for strains expressing LamB-SrGBP as compared to the empty vector control,  $2.6 \pm 0.1$  and  $30.5 \pm 5.5 \mu\text{A}/\text{cm}^2$ , respectively (Figure 4). Thus, expression of LamB-SrGBP and the subsequent disruption of the Mtr respiratory pathway appeared to significantly hinder the cells' ability to utilize electrodes as terminal electron acceptors (Figure 4).

Three main strategies have been posed to account for extracellular electron transfer by *S. oneidensis*: direct transfer via heme-containing cytochrome complexes, by transfer mediated by soluble redox-active shuttles, and by intercytochrome transfer along electrically conductive pili.<sup>12–14,23–28</sup> Redox-

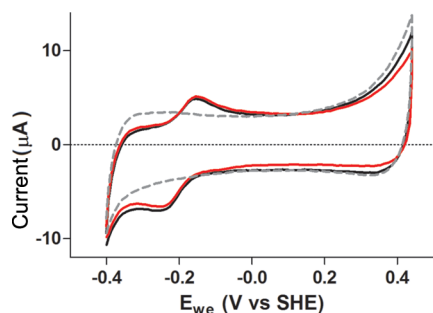
active flavins typically account for greater than 70% of the current generated by *S. oneidensis* in graphitic carbon-electrode experiments.<sup>12</sup> To better explain the lack of current at gold and graphitic carbon electrodes following inoculation, we examined the effect of LamB-SrGBP expression and diminished outer membrane cytochromes on flavin reduction by *S. oneidensis*.

**Expression of LamB and LamB-SrGBP Decrease Rates of Flavin and Iron Oxide Reduction.** Expression of LamB-SrGBP correlated with decreased levels of the outer membrane protein MtrB and decaheme cytochromes MtrA, MtrC, and OmcA (Figure 2C and D), which have been shown to be critical for the reduction of flavins.<sup>14</sup> To test rates of flavin reduction in engineered strains, an assay was conducted based on the fluorescence of riboflavin. Due to the presence of an isoalloxazine ring, oxidized riboflavin is fluorescent, whereas the reduced species is not, allowing flavin reduction to be monitored using fluorescence spectroscopy.<sup>29</sup> Compared to the empty vector control, the rate of riboflavin reduction by washed cells was reduced approximately 2-fold for strains expressing either LamB or LamB-SrGBP (Figure 5A). Decreased rates were also seen for the reduction of insoluble iron oxide (Figure 5B). The decrease in reduction rates for uninduced cultures was likely due to basal expression by the  $P_{\text{BAD}}$  promoter even in the absence of arabinose. Regulation of the  $P_{\text{BAD}}$  promoter varies among bacteria, and basal expression has been shown in uninduced cultures of *Escherichia*, *Xanthomonas*, and *Pseudomonas*.<sup>18</sup> Overall, reduction rates for both riboflavin and iron oxide decreased, yet these data demonstrated that the LamB-SrGBP strain was able to reduce riboflavin and insoluble iron oxide in 96-well plates under nongrowth conditions.

**Riboflavin Remains Stable in Electrochemical Cells.** While riboflavin and iron oxide reduction rates in liquid medium are not directly transferrable to biofilm-associated cells, the demonstrated ability of the LamB-SrGBP strain to still

reduce riboflavin left the lack of current production on gold (and the severe inhibition on graphitic carbon electrodes) partially unexplained. Previous research hypothesized that the shuttling mechanism utilized by *Shewanella* may not be conducive to reducing gold surfaces due to chemical instability of flavins in redox reactions with gold.<sup>30</sup> We tested this hypothesis by directly cycling flavin-enriched medium with gold electrodes.

Cyclic voltammetry (CV) was performed in unstirred abiotic reactors containing 10  $\mu\text{M}$  riboflavin in growth medium. After obtaining a baseline CV, riboflavin was added to reactors and cycled 100 times. Data was similar for all CV sweeps conducted, and the baseline, first, and 100th cycle are shown (Figure 6).



**Figure 6.** Cyclic voltammetry of riboflavin in abiotic gold-electrode reactors containing SBM medium at pH 7.0. The gray dashed line represents blank medium. After the addition of 10  $\mu\text{M}$  riboflavin, 100 sweeps were performed, and the first (black) and last (red) are shown. Representative data from three experiments are shown.

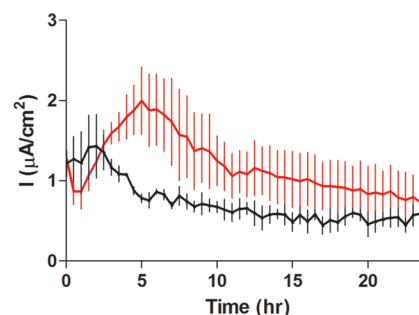
No degradation in anodic current, peak height, or peak separation was observed, and the midpoint potential of riboflavin with gold electrodes was similar to what has been observed with graphitic carbon electrodes ( $-0.21$  V vs SHE). These analyses confirmed that gold electrodes were capable of oxidizing reduced riboflavin in electrochemical reactor experiments provided it was produced near the electrode surface by *Shewanella*.

**Cells Expressing LamB-5rGBP Are Repelled by Gold Surfaces Poised at Positive Potentials.** Since cells expressing LamB-5rGBP demonstrated increased attachment to gold surfaces and retained partial ability to reduce riboflavin, and gold electrodes showed robust ability to oxidize riboflavin, the lack of current obtained from these strains remained unexplained. Previous experiments with *Geobacter* have

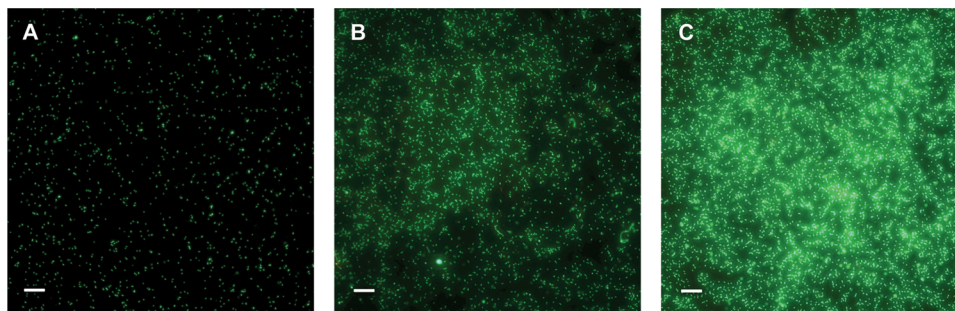
indicated that the poised electrode environment differs from that used for attachment studies since surface charge changes with the potential of the electrode.<sup>31</sup> Gold-binding peptide has an overall positive charge; therefore, it was possible that electrodes poised at a positive potential repelled LamB-5rGBP and hence the biofilm.

To test the effect poising had on LamB-5rGBP binding, a second gold surface was included in reactors. This second gold surface was not connected to the potentiostat and remained unpoised throughout data collection. Cells were allowed to attach to both unpoised electrodes, half of the electrodes were raised to positive potentials, and electrodes were imaged using fluorescence microscopy. Six images were taken of each surface and representative images were chosen (Figure 7). Fewer cells remained attached to the poised LamB-5rGBP electrode as compared to the unpoised electrode (Figure 7B versus C). As predicted, electrodes containing *S. oneidensis* with the empty vector control harbored the fewest cells (Figure 7A).

To further test the hypothesis that attachment to surfaces poised at a positive potential was ultimately preventing current production, gold electrodes were allowed to form biofilms under unpoised conditions. After a 16-h incubation/attachment period, planktonic cells were removed via three medium replacements, and electrodes were switched to oxidizing potentials. For the strain expressing LamB-5rGBP, current increased significantly compared to the empty vector control and peaked after 5 h before falling to a baseline current for the remainder of the analysis (Figure 8). The empty vector control



**Figure 8.** Chronoamperometry of gold-electrode reactors poised at 0.24 V vs SHE after a 16 h unpoised incubation period. Data from *S. oneidensis* expressing LamB-5rGBP is shown in red, and the empty vector control is in black. Error bars indicate standard error of triplicate reactor experiments.



**Figure 7.** Cellular attachment on poised vs unpoised gold electrodes. All reactors were inoculated with induced cells during mid-logarithmic growth and were incubated for 16 h unpoised to allow attachment. After this attachment period, electrodes containing (A) *S. oneidensis* expressing an empty vector control and (B) *S. oneidensis* expressing LamB-5rGBP were poised at 0.24 V vs SHE. (C) A second electrode containing *S. oneidensis* expressing LamB-5rGBP unpoised. Six images were taken of each electrode, and representative images were chosen. Scale bars are 10  $\mu\text{m}$ .

strain fell steadily to a similar background level (Figure 8). This short-term increase in current production was consistent with the increased biofilm attachment of the LamB-5rGBP strain, as well as the repulsion by poised electrodes observed in previous experiments (Figure 8).

Theories to explain bioincompatibility between *Shewanella* and gold surfaces have focused on interactions at the microbe/electrode and mediator/electrode interface. As to interactions between the bacterium and gold, we have shown that *S. oneidensis* expressing LamB-5rGBP increases attachment to gold surfaces. Expression of gold-binding peptide also led to the disruption of Mtr components required for extracellular respiration and repulsion from poised surfaces, both of which inhibited current production on gold and graphitic carbon electrodes. Microscopy indicates that gold surfaces are not toxic at the level of membrane integrity, yet strong interactions with gold, which may denature outer membrane cytochromes, remain possible. This may explain why monolayer assembly, leading to alteration of the surface chemistry of bare gold, enabled brief periods of current production by *Shewanella*.<sup>15</sup> These modifications may act as a buffer zone that inhibits strong interactions between membrane proteins and gold. As to mediator/electrode interactions, abiotic experiments indicate that gold electrodes are able to oxidize reduced flavins, and the reaction is reversible over multiple rounds of CV data collection.

Although LamB-5rGBP expression results in diminished levels of Mtr components and decreases the rate of riboflavin reduction, the strain maintains some capacity to reduce riboflavin and demonstrated measurable increases in biofilm formation on unpoised gold surfaces. When *S. oneidensis* expressing LamB-5rGBP was allowed to attach to unpoised gold surfaces before analysis, a brief period of increased electron transfer was possible but could not be sustained. Although *S. oneidensis* has been engineered to attach to gold by expressing LamB-5rGBP, further work is needed to significantly increase current production. This work must also take into account the unique environment of the poised electrode compared to the inert surface used in phage display, which originally identified the gold-binding peptide. While surface display of a gold-binding peptide highlights the potential of this mechanism to direct microbial attachment to specific electrode surfaces, it also outlines the difficulties of using synthetic biology to incorporate non-native pathways in living cells, especially at the level of the membrane.

## METHODS

**Strains and Growth Conditions.** Wild-type *S. oneidensis* strain MR-1 has been described previously.<sup>32</sup> *E. coli* strains used for cloning (UQ950) and mating (WM3064) procedures have also been described.<sup>33</sup> A complete list of strains and plasmids used in this study can be found in Supplementary Table S1. Single colonies from freshly streaked  $-80\text{ }^{\circ}\text{C}$  stocks were used to inoculate cultures grown for 16 h in Luria–Bertania (LB) medium supplemented with the appropriate antibiotic (50  $\mu\text{g}/\text{mL}$  kanamycin, 15  $\mu\text{g}/\text{mL}$  gentamycin). Unless specified otherwise, cultures were grown in Luria–Bertani broth (LB), shaken continuously at 250 rpm, and incubated at 30 or 37  $^{\circ}\text{C}$  for *Shewanella* or *E. coli* strains, respectively. Anaerobic cultures were flushed with nitrogen for 20 min and were supplemented with a carbon source and electron acceptor as applicable. Cultures were induced with 0.05% L-arabinose during mid-logarithmic phase when indicated. *Shewanella* Basal Medium

(SBM) pH 7.2 was used where specified and contained, per 1 L of medium, 0.225 g  $\text{K}_2\text{HPO}_4$ , 0.225 g  $\text{KH}_2\text{PO}_4$ , 0.46 g NaCl, 0.225 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.117 g  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ , 100 mM HEPES buffer. This medium was supplemented with 25  $\mu\text{g}/\text{mL}$  kanamycin, 5 mL/L vitamins, 5 mL/L minerals, and 0.05% casamino acids (Difco) as described previously.<sup>34</sup>

**Reagents.** Enzymes were obtained from New England Biolabs (Ipswich, MA). *Pfx50* high fidelity polymerase and pCR-BluntII-TOPO kits were acquired from Invitrogen (Carlsbad, CA). Kits for gel purification and plasmid mini preps were purchased from Qiagen (Valencia, CA). All related reactions were carried out according to manufacturer instructions.

**Vector Construction.** Primers used in the construction of plasmids are listed in Supplementary Table S1. All plasmids were sequence verified (Biomedical Genomics Center, University of Minnesota). The gold-binding sequence *5r-gbp*, five repeating units of *gbp1* (MHGKTQATSGTIQS) flanked with *Pst*I and *Xho*I restriction sites, was synthesized by GenScript (Piscataway, NJ) and cloned into pUCsimple.<sup>17</sup> *LamB* was amplified from *E. coli* K12 strain MG1655 genomic DNA and was cloned into pCR-BluntII-TOPO. After restriction enzyme digests of the TOPO vector with *Apa*I and *Sac*I, the *lamB* fragment was gel purified and ligated into the corresponding sites of the phosphatase-treated and gel-purified pBBR1mcs-5. *LamB* was then modified at codons 154–157, utilizing a QuikChange kit from Agilent (Santa Clara, CA) and primers LamB-mod and LamB-mod-anti, to introduce *Pst*I and *Xho*I sites to allow for the future incorporation of *5r-gbp*. The *5r-gbp* fragment was excised from pUCsimple-5r-GBP with *Pst*I and *Xho*I. This fragment was then gel purified and ligated into the corresponding sites of pLAMB-5 to create pLAMBGBP-5.

Constitutive expression of LamB in the pBBR1mcs-5 vector was toxic in both *E. coli* and *S. oneidensis*. Sequencing results for the plasmid often indicated base deletions and additions that altered the reading frame of *lamB*. The addition of *5r-gbp* further compounded this instability. To overcome these issues, *lamB* and *lamB-Srgbp* were transferred to the L-arabinose-inducible vector pBBAD18K. Primers LamB-Fwd and LamB-Rev were used to reamplify the *lamB-Srgbp* sequence from purified and sequenced pLAMB-5 by the high-fidelity polymerase *Pfx50*. This fragment was cloned into pCR-BluntII-TOPO, which contains an *Eco*RI site at the point of insertion. Both *lamB* and *lamB-Srgbp* sequences were then digested via *Sac*I and *Eco*RI, gel purified, and ligated into the corresponding sites of pBBAD18K generating the final plasmids utilized in this study: pLAMB and pLAMB-5rGBP.

**Membrane Fractionation and Characterization.** One liter LB cultures, supplemented with 20 mM lactate and 40 mM fumarate, were inoculated with 10 mL of aerobic LB overnights. These cultures were induced during mid-logarithmic growth with 0.05% L-arabinose. After a 2.5-h induction period, cells were harvested by centrifugation for 10 min at 10,000 rpm in a Beckman Avanti J-E centrifuge, and subsequent steps were conducted at 4  $^{\circ}\text{C}$ . Pellets were washed in 10 mM Tris-HCl, pH 8.0 with 1 mM EDTA and 10  $\mu\text{M}$  PMSF and centrifuged again at 12,000g for 10 min. These cells were resuspended in 40 mL of 10 mM Tris-HCl, pH 7.5 containing 10  $\mu\text{M}$  PMSF and then passed 3 times through a French press at 15,000 psi. To remove cell debris and unlysed cells, the suspension was centrifuged at 12,000g for 20 min. The total membrane fraction was isolated from the cleared suspension by centrifugation for 1

h at 100,000g to separate the soluble fraction from the crude membrane pellet. This pellet was resuspended in 1 mL of 10 mM HEPES and added to a 10% step sucrose gradient (35–55%) which was spun at 25,000 rpm for 17 h in a Beckman SW28 rotor resulting in the separation of the outer and inner membrane fractions.<sup>35</sup> Aliquots of bands corresponding to each membrane were collected, pooled, and dialyzed in 10 mM HEPES, pH 7.5 overnight with one exchange of buffer. Samples were then concentrated using Millipore columns, and a BCA assay was performed to determine the resulting protein concentrations. To visualize cellular localization of LamB and LamB-SrGBP, membrane fractions were subjected to SDS-polyacrylamide gel electrophoresis and bands were visualized using the Colloidal Blue Staining Kit (Invitrogen).

**Preparation of Gold(111) Surfaces.** Gold(111)-coated silicon oxide purchased from Sigma was machined to 2.54 cm × 0.635 cm. Prior to cleaning with piranha solution, gold pieces were placed in acetone overnight followed by ethanol for 1 h to remove all organic material. The gold was then placed into a 3:1 (concentrated sulfuric acid/30% hydrogen peroxide) piranha solution for 20 min and washed three times in filtered and autoclaved ddH<sub>2</sub>O. These surfaces were then dried under filtered nitrogen gas and used immediately. Gold surfaces were used once and then discarded.

**Gold-Binding Assays.** Freshly cleaned gold surfaces were added to induced stationary phase cultures and incubated at room temperature for 16 h to allow biofilm formation. Following this incubation, gold wafers were removed and rinsed three times in phosphate buffered saline. To ensure the removal of all unbound cells, gold pieces were placed in buffered minimal medium and washed for 1 min on a rocker (Boekel Scientific, Feasterville, PA). Cells bound to the gold surface were stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Kit L7012) from Invitrogen according to manufacturer instructions. Fluorescence was then visualized using an Olympus IX70 inverted fluorescence microscope and analyzed utilizing the SPOT Basic imaging software.

**Heme Staining.** Five milliliters of aerobically grown cultures was collected after 16 h by centrifugation and resuspended in 500  $\mu$ L of 10 mM HEPES buffered at pH 7.5. Samples were lysed by sonication and subsequently centrifuged to clear cell debris. Protein content was quantified using a BCA assay. Standard SDS buffer without reductant was then added, and samples were heated at 80 °C for 10 min. Total protein (10  $\mu$ g) was added to individual wells of a 4–12% bis-tris gradient gel along with 10  $\mu$ L of a broad range prestained protein ladder (New England Biolabs). Proteins were visualized due to the peroxidase activity of heme-containing cytochromes using a staining technique outlined in Thomas et al.<sup>19</sup> Briefly, gels were placed in a 30:70 6.3 mM tetramethylbenzidine/0.25 M sodium acetate solution pH 5.0 for 2 h in the dark, after which bands were visualized by adding 30 mM hydrogen peroxide. Proteins were identified based on molecular weight as previously published.<sup>20</sup>

**Western Blots.** Membrane fractions were collected as described previously, and 8  $\mu$ g of total protein was added to individual wells of a 12% bis-tris gel. The gel was then transferred to a PVDF membrane for western analysis using an antibody against MtrB. Specificity of this enzyme for MtrB has been shown in previous work.<sup>36</sup> Addition of the secondary antibody and the developing process were carried out according to manufacturer instructions (Pierce).

**Riboflavin Reduction Assay.** Stationary phase cells were spun down and resuspended to an OD<sub>600 nm</sub> of 1 in SBM containing 0.05% arabinose. In an anaerobic chamber, 20  $\mu$ L of the resuspended samples was added to black 96-well plates containing 280  $\mu$ L of SBM supplemented with 20 mM lactate, 133  $\mu$ M riboflavin, 5 mL/L vitamins, 5 mL/L minerals, and 0.05% L-arabinose. Fluorescence was read (excitation = 440 nm, emission = 525 nm) for 24 h at 30-s intervals, and reduction was measured by the decrease in riboflavin fluorescence over time. Experiments were performed in triplicate to obtain accurate reduction rates, and data were normalized to OD.

**Bioreactor Construction.** Bioelectrochemical reactors utilized in this study consisted of a 25 mL glass cone (Bioanalytical Systems, West Lafayette, IN) sealed by a Teflon top modified to hold electrodes and a gas line. Anaerobic conditions were maintained by flushing reactors with humidified nitrogen. A silicon wafer electroplated with 1000 Å of gold(111) machined to 2.54 cm × 0.635 cm served as the working electrode and was connected to a platinum wire using a plastic clamp and Teflon bolts (Small Parts, Inc., Miramar, FL). Prior to cleaning, gold electrodes were stored in acetone overnight, followed by ethanol for at least 1 h to remove organic material. Gold electrodes were then further cleaned in a 3:1 (concentrated sulfuric acid/30% hydrogen peroxide) piranha solution for 20 min followed by 3 washes in filtered and autoclaved ddH<sub>2</sub>O. These electrodes were dried under filtered nitrogen and used immediately. Graphitic carbon electrodes were prepared as described previously.<sup>23</sup> A platinum wire served as the counter electrode and was cleaned for 24 h in 1 N HCl prior to use. The reference consisted of a glass body saturated calomel electrode (Fischer Scientific, Pittsburgh, PA) connected to the system via a glass capillary tube filled with 1% agarose in 0.1 M sodium sulfate and capped with a vycor frit. Reactors were autoclaved following construction. Electrochemical data was monitored using a 16-channel VMP potentiostat (Bio-Logic, Claix, France).

**Oxidation of Riboflavin by Gold(111) Electrodes.** Baseline cyclic voltammetry (CV) was conducted using sterile anaerobic SBM medium, pH 7.0 with electrodes poised at 0.24 V versus a standard hydrogen electrode (SHE) from –0.4 to 0.44 V at a scan rate of 50 mV/s. Anaerobic, sterile filtered riboflavin stock was then added to a final concentration of 10  $\mu$ M and was cycled 100 times along the same potential range and scan rate.

**Current Analysis in Reactors Poised During Attachment.** Sealed anaerobic Balch tubes containing 10 mL of LB supplemented with 100 mM HEPES buffer, 30 mM lactate, 40 mM fumarate, and 50  $\mu$ g/mL kanamycin (Km) were inoculated to an OD<sub>600 nm</sub> of 0.05 from an aerobic LB overnight. Cultures were induced with 0.05% L-arabinose during mid-logarithmic growth. After reaching stationary phase, cultures were transferred to anaerobic reactors via a nitrogen-flushed syringe and were stirred continuously and kept at 30 °C. The working electrode was then poised at 0.24 V versus SHE, and 20 mM lactate and 1  $\mu$ M riboflavin were added to the reactor. Reactors were monitored by chronoamperometry (CA).

**Current Analysis in Reactors Not Poised During Attachment.** Aerobic LB overnight cultures were used to inoculate anaerobic Balch tubes containing 10 mL of LB medium buffered with 100 mM HEPES and supplemented with 30 mM lactate, 40 mM fumarate, 0.5  $\mu$ M riboflavin, and 50  $\mu$ g/mL Km to an OD<sub>600 nm</sub> of 0.05. When cells reached mid-logarithmic growth, cultures were induced with 0.05% L-

arabinose and transferred to anaerobic reactors using a syringe flushed with nitrogen. Reactors were kept at 30 °C using a water bath and stirred continuously for 16 h. To remove planktonic cells after this incubation period, 3 medium replacements were conducted with LB containing 100 mM HEPES, 30 mM lactate, and 1  $\mu$ M riboflavin. The working/gold electrode was then poised at 0.24 V versus SHE, and current production was monitored by chronoamperometry using a multichannel potentiostat. Reactors were monitored for approximately 24 h after which electrodes were removed and processed for imaging.

**Imaging of Electrodes.** Electrodes were removed from reactors and immediately rinsed once in SBM. This minimal wash procedure was chosen in an attempt to maintain a surface as similar as possible to the one analyzed during electrochemical measurements. Bound cells were stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Kit L7012) from Invitrogen according to manufacturer instructions. Fluorescence was then visualized using an Olympus IX70 inverted fluorescent microscope and analyzed utilizing the SPOT Basic software.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Supporting figure and table. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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