CymA and exogenous flavins improve extracellular electron transfer and couple it to cell growth in Mtr-expressing *Escherichia coli*

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Abstract

Introducing extracellular electron transfer pathways into heterologous organisms offers the opportunity to explore fundamental biogeochemical processes and to biologically alter redox states of exogenous metals for various applications. While expression of the MtrCAB electron nanoconduit from *Shewanella oneidensis* MR-1 permits extracellular electron transfer in *Escherichia coli*, the low electron flux and absence of growth in these cells limits their practicality for such applications. Here we investigate how the rate of electron transfer to extracellular Fe(III) and cell survival in engineered *E. coli* are affected by mimicking different features of the *S. oneidensis* pathway: the number of electron nanoconduits, the link between the quinol pool and MtrA, and the presence of flavin-dependent electron transfer. While increasing the number of pathways does not significantly improve the extracellular electron transfer rate or cell survival, using the native inner membrane component, CymA, significantly improves the reduction rate of extracellular acceptors and increases cell viability. Strikingly, introducing both CymA and riboflavin to Mtr-expressing *E. coli* also allowed these cells to couple metal reduction to growth, which is the first time an increase in biomass of an engineered *E. coli* has been observed under Fe_2O_3 (*s*) reducing conditions. Overall, this work provides engineered *E. coli* strains for modulating extracellular metal reduction and elucidates critical factors for engineering extracellular electron transfer in heterologous organisms.

Keywords: synthetic biology, dissimilatory metal-reducing bacteria, bioelectrochemical systems, energy conservation, multi-heme cytochrome *c*

Introduction

Some metal-reducing bacteria, such as those from the *Geobacter* and *Shewanella* genera, have extracellular electron transfer pathways that can route electrons across the cell membrane to alter the redox state of exogenous metals. This extracellular metal reduction by microorganisms plays a key role in microbial-driven mineral transformations (*1*) and can be used to drive nanoparticle synthesis under mild conditions (*2, 3*) or alter the phase of metals for bioremediation (*4*) or biomining (*3*). The ability to modulate extracellular electron transfer then offers the opportunity to dissect and control these processes. For example, systematically varying the rate of extracellular electron transfer should allow synthetic control over the number and size of nanoparticles and should elucidate the role of redox kinetics on mineral transformation. However, the sparse availability of genetic tools in metal-reducing bacteria and the multiple overlapping pathways for extracellular electron transfer make it challenging to modulate electron transfer and/or introduce other functions of interest. In response to this challenge, we (*5-7*) and others (*8-10*) have taken the complementary approach of engineering portions of extracellular electron transfer pathways into the well-studied industrial microbe *Escherichia coli* (*11*). While this work has shown that the Mtr pathway can route electrons to extracellular metal oxides (*6*), the low electron flux and the inability of cells to maintain biomass using solid minerals as the terminal electron acceptor has hampered the use of these strains for applications such as nanoparticle synthesis and bioremediation (*7*).

Since the extracellular electron transfer pathways of *Shewanella oneidensis* MR-1 have been extensively studied at the molecular level (*12, 13*), they have provided the basis for these engineering efforts (**Figure 1A**). When *S. oneidensis* is grown under metal-respiring conditions, reducing equivalents from oxidation of electron donors are directed to the menaquinol pool and

71 then to the inner membrane tetraheme cytochrome *c* CymA (*14*). When extracellular metals or
72 electrodes are present, electrons from CymA are passed to the periplasmic decaheme cytochrome
73 *c* MtrA. The re-oxidation of CymA completes the Q-cycle, increasing the proton motive force
74 (*15*) and allowing the cell to conserve energy. Notably, it is debated whether CymA directly
75 reduces MtrA (*9, 16*) or whether FccA, STC (also known as CctA), or another redox protein,
76 serve as an intermediate to pass electrons from CymA to MtrA (*17-19*). MtrA reduces MtrC, a
77 decaheme cytochrome *c* located on the extracellular face of the outer membrane, as part of the
78 MtrCAB complex. This complex is proposed to form a porin cytochrome complex (*12*) that
79 spans the outer membrane and allows MtrA to directly contact MtrC (*20*). There are
80 approximately 70,000 MtrC proteins per *S. oneidensis* MR-1 cell (*21*), and these proteins can
81 either directly reduce a mineral or electrode surface or use a flavin-dependent process to
82 indirectly reduce the solid electron acceptor. While the mechanism underpinning flavin-mediated
83 electron transfer is still being elucidated (*22-24*), it is clear that ~80% of the electron transfer
84 occurs through a flavin-dependent process and ~20% of the electron transfer occurs directly (*25-*
85 *27*). Electron transfer through MtrCAB does not affect the proton motive force; however, it
86 indirectly contributes to energy conservation by re-oxidizing CymA.

87 *E. coli* strains expressing combinations of MtrA and CymA can reduce soluble Fe(III),
88 but not extracellular Fe(III) oxides (*8, 9*). In contrast, expression of the MtrCAB electron
89 nanoconduit in *E. coli* confers the ability to reduce Fe_2O_3 (*s*) (*6*). However, this strain reduces
90 Fe(III) several orders of magnitude more slowly than *S. oneidensis* MR-1. Likely as a result of
91 this slow reduction rate, this *E. coli* strain is unable to conserve energy and its biomass sharply
92 decreases, which poses a particular challenge for further applications. Introduction of
93 extracellular electron transfer pathways into another industrial microbe, *Pseudomonas putida*,

has not translated into cell growth (*28-30*), indicating this may be a common challenge. Our previous work identified re-reduction of MtrA as a rate-limiting step in Fe(III) reduction (*6*). This strain also lacks CymA, extracellular flavins, and has fewer Mtr electron conduits (30,000 (*5*) vs. 70,000) (**Figure 1A**). Here we systematically test how co-expression of CymA, changing the number of electron conduits per cell, and introduction of flavins affect the flux of electrons to extracellular iron and *E. coli* survival. We found that co-expression of CymA with the Mtr nanoconduit and introduction of exogenous flavins significantly increased the electron transfer rate over the original strains. We also found that *E. coli* strains expressing CymA coupled these improvements in extracellular reduction to enhanced cellular viability. Importantly, the combination of CymA and riboflavin allowed Mtr *E. coli* cells to couple Fe(III) reduction to growth under Fe_2O_3 (*s*)-reducing conditions.

Results and Discussion

The *cymA, cymAmtrA, and cymAmtr* strains express the full-length and redox active cytochromes *c*.

To systematically explore the effect of CymA on soluble iron and solid iron oxide reduction, we created three separate plasmids for expression of *cymA*, *cymAmtrA*, and *cymAmtrCAB* (**Figure S1**); in these plasmids, the RBS sequences were designed to be approximately the same as the corresponding native RBS strength in *S. oneidensis* (*31*). The *cymA*, *cymAmtrA*, and *cymAmtrCAB* plasmids were co-transformed with the cytochrome *c* maturation (*ccm*) plasmid (*32*) into *E. coli* to create the *cymA, cymAmtrA, and cymAmtr* strains, respectively (**Table 1**). The *mtrA* strain, co-expressing *ccm* and *mtrA*, and the *mtr* strain, which expresses *ccm* and *mtrCAB*, (**Table 1**), were used as previously described (*6*).

We next characterized the expression and localization of each cyt *c* to validate each strain. Proteins from whole cell extracts were resolved by SDS-PAGE, and heme *c* containing proteins were identified by enhanced chemiluminescence (ECL) analysis. These whole cell extracts show that the MtrA and MtrC proteins in all the strains containing *mtrA* and *mtrC*, i.e. the *cymAmtrA, mtrA*, *mtr*, and *cymAmtr* strains, are full-length (35 kD and 76 kD, respectively; **Figure 1B**). The *cymA* strain also clearly shows a band corresponding to full length CymA (18 kD, **Figure 1B**). While difficult to discern by eye, densitometry analysis of the ECL demonstrates the CymA band is also present in the two other strains containing *cymA*, the *cymAmtrA* and *cymAmtr* strains (**Figure 1C**). ECL analysis of fractionated cells shows CymA, MtrA, and MtrC in *E. coli* are localized (**Figure S1**) as they are in *S. oneidensis* (*33-35*). Lastly, diffuse reflectance spectroscopy of all these strains under oxidizing and reducing conditions showed characteristic changes in the α- and β- bands, indicating that the cyt *c* proteins are redox active. Thus, each strain expresses its respective redox active, full length and correctly localized cyt *c*.

To characterize the concentration range of cyt *c* that could be expressed, we increased the promoter activity (as defined in (*5*)) in *mtr* and *cymAmtr* strains via IPTG induction (**SI Table 1**) and measured the total heme *c* content in these cells using diffuse reflectance as described previously (*5*). In brief, diffuse reflectance measures absorbance of visible light with limited scattering, thus allowing quantitative measurement of the α-bands in whole cell suspensions (**Figure S2**). From these spectra, we can determine the concentration of heme *c*, and thus infer relative abundance of electron conduits in whole cell suspensions. From the 0 to 0.37 promoter activity, the cyt *c* concentration in the *mtr* and *cymAmtr* strains both increase ~5-fold (**Figure 1D**). The maximum heme *c* measured in *mtr* and *cymAmtr* strains are 140 and 106 μM heme *c*

OD_{600nm}^{-1}, respectively (**Figure 1D**), which is about half the total heme *c* content of *S. oneidensis* when grown under the same conditions (228 μM heme *c* OD_{600nm}^{-1}). ECL analysis shows that the relative abundance of each cyt *c* within a strain is very similar across different induction levels (**Figure S3**), indicating that induction boosts the abundance of each cyt *c* rather than a single cyt *c*. Thus, tuning the promoter strength via induction allows us to vary the number of electron transfer proteins in the *mtr* and *cymAmtr* strains.

Fe(III) citrate reduction and cell survival are improved by co-expression of CymA.

Previous work by Schuetz *et al.* (*9*) has shown that co-expression of CymA with MtrA from genomically-integrated constructs can improve Fe(III)-NTA reduction in *E. coli* over expression of CymA alone, strongly suggesting that CymA would improve Fe_2O_3 reduction in the *cymAmtr* strain. However, co-expression of CymA alongside other *S. oneidensis* MR-1 cyts *c* dramatically reduces its expression level in our strains (**Figure 1B-D**), and we have previously observed that subtle changes in expression levels can profoundly impact extracellular electron transfer capabilities (*5*). Thus, we first tested cell suspensions of the *cymA, mtrA,* and *cymAmtrA* strains for the ability to reduce soluble Fe(III) citrate in anaerobic conditions with D,L-lactate as a carbon source to determine the effect of limited expression of CymA. Since lysed *E. coli* cells can reduce Au^+ (*36*), we also included a homogenized sample of the *mtrA* strain to determine if cell lysis contributed to Fe(III) reduction (**Figure S4**). The lysed *mtrA* sample reduced at a similar rate as the *ccm* strain (**Figure S4**), demonstrating cell lysis is not a major contributor to Fe(III) citrate reduction in this system. While the *mtrA* strain reduced Fe(III) slightly faster than the *ccm* control, the *cymA* and *cymAmtrA* strains increased the Fe(III) reduction rate over the *mtrA* strain by a substantial ~3-fold and ~4-fold, respectively (**Figure 2A**).

In these iron reduction assays, the *E. coli* strains have only D,L-lactate as a carbon source, and the only available terminal electron acceptor for respiration is Fe(III) citrate. Therefore, we hypothesized that aspects which increase Fe(III) reduction would also improve energy conservation and cell survival. Interestingly, while the cell density of the slowest Fe(III)-reducing strains, the *ccm* and *mtrA* strains, sharply decreased, the cell density of the fastest Fe(III)-reducing strains, the *cymA* and *cymAmtrA* strain, stayed the same or slightly increased (**Figure 2B**). Indeed, a direct comparison between the rate of Fe(III) citrate reduction and the linear rate of change of the cell density in CymA-expressing strains (**Figure S5**) reveals that the iron reduction rate and strain fitness are strongly, positively correlated (Pearson correlation coefficient, R = 0.93). This strong positive correlation strongly suggests that increasing the iron reduction rate in these *E. coli* strains also improves energy conservation under our experimental conditions. The correlation also suggests that a minimum iron reduction rate (~0.4 mM day^{-1}, **Figure S5**) is necessary to maintain or increase cell biomass. Taken together, these observations show that even low levels of CymA co-expressed with MtrA are sufficient to improve Fe(III) citrate reduction and strongly suggest that increasing the Fe(III) reduction rate helps maintain biomass in *E. coli* by increasing energy conservation.

Fe_2O_3 (*s*) reduction and cell survival are improved by co-expression of CymA.

We next set out to probe the effects of CymA co-expression on solid Fe_2O_3 reduction and cell viability in the *ccm*, *cymAmtr*, and *mtr* strains. In the solid iron oxide reduction assays, the *E. coli* strains have only D,L-lactate as a carbon source, and the only available terminal electron acceptor for respiration is solid Fe_2O_3. To survey a wide set of conditions, we varied the number of electron conduits with a range of promoter activity (**Figure 1D, Table S1**), and we additionally examined the effect of exogenous riboflavin. The promoter activity is indicated in

superscript throughout. Due to the relatively slow reduction of solid Fe_2O_3 by these engineered *E. coli* in comparison to *S. oneidensis*, many strains required 21 day incubations before statistically significant measurements of Fe(II) by ferrozine could be attained. In contrast, changes in colony forming units (cfu) were apparent over much shorter time scales than changes in Fe(II) concentration.

We first summarize the effects of CymA co-expression with the Mtr electron nanoconduit in *E. coli*. The *ccm* only strain, which accounts for basal Fe_2O_3 (*s*) reduction, reduced only 62 ± 19 μM Fe(II) over 21 days (**Figure 3A**). The best reducing *mtr* strain, $mtr^{0.37}$, showed a statistically significant ~2-fold improvement over the *ccm* strain (**Figure 3A**). Interestingly, of all the strains and induction conditions tested, the uninduced *cymAmtr* strain, $cymAmtr^{0.001}$, reduced the greatest amount of Fe_2O_3, ~3.5 fold more Fe_2O_3 (*s*) than the *ccm* strain. The $cymAmtr^{0.001}$ strain reduced statistically more Fe_2O_3 (*s*) than the $mtr^{0.37}$ strain, demonstrating that co-expression of CymA improves the reduction of solid Fe_2O_3 (**Figure 3A**) as well as soluble Fe(III) (**Figure 2A**).

CymA also significantly enhanced the survival of *cymAmtr* in the iron reduction assay conditions. The *ccm* strain decreased to 7% of its initial cfu mL^{-1} over 4 days **(Figure 3B)**, consistent with the inability of the native *E. coli* strain to conserve energy when Fe_2O_3 (*s*) is the sole terminal electron acceptor available. The $mtr^{0.37}$ strain maintained 27% of the initial cfu mL^{-1}, indicating that *mtr* improved survival somewhat over native *E. coli*. The most robust strain was the $cymAmtr^{0.001}$ strain, which increased its cfu mL^{-1} 5% above its initial value (**Figure 3B**). More broadly, comparing the amount of Fe(II) produced to the relative cell density after 21 days (**Figure S5**), shows there is a strong, positive correlation between the degree of reduction and cell survival in CymA-containing strains (Pearson correlation coefficient, R = 0.74). Taken

together, these observations indicate that these CymA-expressing *E. coli* strains use metal reduction to maintain biomass under both Fe(III) citrate and Fe_2O_3*(s)* reducing conditions.

The expression of CymA significantly increases extracellular electron transfer by enabling catalytic turnover of Mtr.

Since CymA can reduce MtrA *in vitro*, we hypothesized that CymA co-expression increased Fe_2O_3 (*s*) reduction and cell viability by more rapidly reducing the MtrCAB complex than native *E. coli* inner membrane quinone dehydrogenases. To directly investigate this, we turned to bioelectrochemical techniques that can monitor the redox turnover of MtrCAB. Since these techniques use a carbon felt electrode as the terminal electron acceptor instead of Fe_2O_3 (*s*), we first monitored current production from the *ccm*, $mtr^{0.18}$, and $cymAmtr^{0.18}$ strains in bioelectrochemical reactors. The working electrode chamber contained cell suspensions in M1 media supplemented with 40 mM D,L-lactate, and the counter electrode chamber contained 50 mM PIPES buffer (pH 7.4), and was separated by a cation exchange membrane. As expected, the *ccm* strain, which does not express any *S. oneidensis* cyts *c*, produced very low current (**Figure 4A**). The $mtr^{0.18}$ strain did not produce significantly more current than the *ccm* strain, while in contrast, the $cymAmtr^{0.18}$ strain produced an average of ~4-fold more current than the $mtr^{0.18}$ strain (**Figure 4A**). This increase between these two strains is comparable to the increase in Fe_2O_3 (*s*) reduction, suggesting that the cause of improved extracellular electron transfer routes in these strains is independent of the identity of the terminal electron acceptor. Next, we monitored reduction of the Mtr pathway by intracellular reducing equivalents using turnover cyclic voltammetry, scanning at 2 mV/s. Both the *ccm* and $mtr^{0.18}$ strains exhibited an irreversible oxidation peak of unknown origin, but did not show a catalytic wave, i.e. an s-shaped peak, indicative of re-reduction. This indicates that the catalytic turnover of the Mtr pathway is

extremely slow in the absence of CymA. In contrast, the turnover cyclic voltammogram of the *cymAmtr*$^{0.18}$ strain (**Figure 4B**) showed a clear catalytic wave, centered just below 0.0 $V_{Ag/AgCl}$, which is consistent with previous measurements of the formal potential of the Mtr pathway in *S. oneidensis* MR-1 (*37*). The presence of this wave indicates that the Mtr pathway can be re-reduced in the presence of CymA under turnover conditions. This rapid turnover additionally shows that there are properly assembled electron conduits in the engineered *E. coli*, however we have not yet determined what fraction of the electron conduits are correctly assembled.

Taken together, these data support our initial observation that re-reduction of MtrA is a rate-limiting step for Fe(III) reduction in *mtrA E. coli* (*6*) and show that CymA increases the rate of Fe(III) reduction in both MtrA- and MtrCAB-expressing *E. coli* by increasing the rate of re-reduction of MtrA. The ~15 nm thick periplasm in *E. coli* (*38*) is too thick to allow CymA to directly contact MtrA within an MtrCAB complex, however, we observe a significant amount of MtrA in the periplasmic fraction (**Figure S6**). If MtrA diffuses in the periplasm with a diffusion constant similar to comparably sized *E. coli* proteins, i.e. ~2 $\mu m^2 s^{-1}$ (*39*), it would only require 3 μs on average to diffuse from the inner membrane to the outer membrane. This timescale is 3 orders of magnitude faster than the ms required for electron transfer across the MtrCAB conduit (*20*). Coupled with the observation that CymA can reduce MtrA *in vitro* (*40*), the simplest explanation of these data is that, in *E. coli*, CymA directly reduces MtrA and MtrA diffuses across the periplasmic space to shuttle electrons to MtrC.

The number of electron nanoconduits does not significantly affect solid Fe_2O_3 reduction and cell survival.

Using the data we gathered on Fe(III) reduction for *cymAmtr* and *mtr* strains at different induction levels, we next probed the relationship between the number of electron conduits in the

mtr and *cymAmtr* strains and iron reduction. We use [heme *c*] as a measure of the number of electron conduits because the stoichometry of cyts *c* is unchanged with different induction levels (**Figure S3**) in our *E. coli* strains. Pearson correlation analysis shows a strong correlation between [heme *c*] and [Fe(II)] in both strains, with the *mtr* strain showing a positive correlation and the *cymAmtr* showing a negative correlation (Pearson correlation coefficients R_{mtr} = 0.95 and $R_{cymAmtr}$ = -0.84, **Figure S7**). However, the slope of these correlations are fairly small (m_{mtr} = 0.31 [Fe(II)] [heme *c*]$^{-1}$ and $m_{cymAmtr}$ = -0.52 [Fe(II)] [heme *c*]$^{-1}$): for example, the Fe(III) reduction rate in the *mtr* strain changes only ~1.5-fold when the number of *mtr* electron conduits changes ~5-fold. This indicates that, for either strain, the number of conduits is not a strong determinant of the reduction of solid iron oxide.

The finding that the number of electron conduits in engineered *E. coli* is not a strong determinant of Fe_2O_3 (*s*) reduction suggests that electron transfer through the Mtr complex is not a rate-limiting step in iron reduction in these strains. To further test this idea, we estimated what the rate of iron reduction in *E. coli* would be if transport through the MtrCAB conduit were rate-limiting. Using our diffuse reflectance data of our best performing *cymAmtr*$^{0.001}$ strain (**Figure 1D**) and assuming that 90% of the heme *c* is in MtrCAB complexes (**Figure 1B**), we estimate the number of Mtr electron nanoconduits per *E. coli* cell is 50,000 complexes cell^{-1}. This is comparable to the number of MtrC in *S. oneidensis*, which is ~70,000 cell^{-1} (*21*). Together with estimates on the maximal rate of electron transfer through the Mtr electron nanoconduit (10^3 e- s^{-1} complex^{-1}) (*20*), we can approximate the maximal rate of iron reduction per *cymAmtr*$^{0.001}$ *E. coli* cell would be ~8 x 10^{-17} M Fe(II) s^{-1} cell^{-1} (**Supplemental Calculation 1**). This theoretical maximum rate is ~2000-fold higher than what we observe (~4 x 10^{-20} M Fe(II) s^{-1} cell^{-1}). Thus,

we conclude that electron transfer through the Mtr conduit is not rate-limiting for extracellular electron transfer in these strains of engineered *E. coli*.

Flavins couple solid Fe_2O_3 reduction to transient growth in the *cymAmtr* strain.

Lastly, we considered how addition of 5 μM riboflavin affected iron reduction and cell survival in the engineered *E. coli* strains. We found that riboflavin increased the total amount of reduced iron by 2 and 2.5 fold in the $mtr^{0.18}$ and $cymAmtr^{0.18}$ strains over those strains without riboflavin added, respectively (**Figure 5A, Figure S8**). To quantitatively assess the fraction of electrons that reduce *via* direct contact or flavin-dependent mechanisms, we performed experiments in M1 medium, a trace medium with no riboflavin or casein added (See Supplemental Information), with and without supplementation of riboflavin. We assumed that cultures without riboflavin reduce iron solely by direct contact, while cultures supplemented with riboflavin reduce iron by both direct contact and through a riboflavin-mediated mechanism. This allowed us to calculate the percentage of flavin-dependent iron reduction by comparing the iron reduction in the supplemented and non-supplemented cultures. The resulting percentage of iron reduced in a flavin-dependent process in the $mtr^{0.18}$ and $cymAmtr^{0.18}$ strains was 72% and 77%, respectively (**Supplemental Calculation 2**). These percentages closely match the ratio observed in *S. oneidensis* MR-1 (*25, 26*). This indicates that, like in the native organism, MtrC is able to utilize solid metal oxides as terminal electron acceptors *via* both direct contact and through flavin-mediated mechanisms.

Interestingly, while riboflavin had similar relative effects on iron reduction in both the *mtr* and *cymAmtr* strains, it had strikingly different effects on cell growth in these strains. The addition of riboflavin did not statistically change the survival of the $mtr^{0.18}$ strain (**Figure S8**). This observation indicates that riboflavin by itself does not support growth under metal reducing

conditions. In the case of the *cymAmtr*$^{0.18}$ strain, supplementation with riboflavin allowed the cells to grow, increasing the cfu mL^{-1} 10-fold over the original suspension after 4 days (**Figure 5B**). This cfu increase is temporally associated with an initial period of rapid reduction of Fe(III) in the *cymAmtr*$^{0.18}$ strain but not the *ccm* strain (**Figure 5C, Figure S9**). Following this period, the cfu decrease and the increase in Fe(II) concentration slows (days 4-10). After 10 days, the cell density drops below its initial value and the Fe(II) concentration remains constant, even though a large excess of Fe(III) is still present. Given the correlations between iron reduction and cell viability, we suggest that the initial high rate of Fe(III) reduction conferred by riboflavin and CymA is enough to allow the *E. coli* to conserve energy and grow. However, as the Fe(III) reduction rate in this strain slows, perhaps due to a depletion of energy stores and slow oxidation of lactate, the *E. coli* can no longer conserve energy and the cell density decreases correspondingly. Eventually, the cell density is so low that there is no detectable change in Fe(II) concentration. This scenario would be consistent with observations in *E. coli*, *Shewanella sp.*, and other microbes that the respiratory rate is positively correlated with the growth rate (*37, 41*).

The *cymAmtr*$^{0.18}$ *E. coli* strain generates 200 μM Fe(II) over the course of 10 days, which corresponds to a cell normalized reduction rate of ~8x10^{-20} M Fe(II) s^{-1} cell^{-1}. Also, this strain can maintain cell density at or above its initial level over this same period, which marks the first time an engineered *E. coli* strain has been shown to grow under solid metal-reducing conditions. These are significant improvements over previously described *E. coli* strains capable of solid metal reduction. Additionally, this work strongly suggests that further improvements in extracellular metal reduction will also boost cell growth in these strains.

Opportunities for additional engineering of metal-reducing *E. coli.*

The fastest iron reduction rate in the engineered *E. coli* cultures described here is still ~50 times slower than *S. oneidensis* MR-1 (~$8x10^{-20}$ M Fe(II) s^{-1} $cell^{-1}$ vs. ~$4x10^{-18}$ M Fe(II) s^{-1} $cell^{-1}$) (*42*). We attribute the strain's inability to sustain growth for long periods of time to this slower rate and suggest further work is still required in strain engineering for demanding applications. While our cyclic voltammetry data indicates that a significant fraction of the MtrCAB electron nanoconduits are correctly assembled in the outer membrane of *E. coli*, we cannot rule out that some fraction of MtrCAB complexes are misassembled and non-functional in *E. coli*. Assessing and potentially improving this assembly may improve extracellular metal reduction. Additionally, the difference between our *cymAmtr E. coli* strains and *S. oneidensis* MR-1 may reflect our still-evolving understanding of electron transfer in *S. oneidensis*. While this work was in preparation, new data generated by Sturm et al. (*19*) and Alves et al. (*17*) strongly suggested that Stc and/or FccA may be involved in shuttling electrons across the periplasm. Thus, co-expression of Stc and/or FccA may help extracellular electron transfer in *Mtr E. coli*.

The difference between the electron transfer rates in engineered *E. coli* and *S. oneidensis* MR-1 may also arise from the fact that that efficient extracellular electron transfer relies on many processes besides transport of electrons from inner membrane to extracellular acceptors (*43*). Specifically, extracellular electron transfer also depends on the import and oxidation of an electron donor, transfer of the electrons *via* intracellular redox carriers to the transmembrane pathway, and adhesion of the bacterium to a metal oxide. Several of these processes are slow or disrupted in the *E. coli* background used here. For example, the reaction rates of the L- and D-lactate dehydrogenases in *E. coli* are ~10 and ~20-fold slower, respectively, than their counterparts in *S. oneidensis* (*44*). Additionally, since the *Mtr E. coli* are grown aerobically before being introduced into anaerobic metal-reducing conditions, these cells may lack the

dehydrogenases and quinones needed to efficiently direct reducing equivalents to the Mtr pathway. Lastly, while *Shewanella sp.* can rapidly attach to Fe_2O_3 surfaces (*45, 46*), the BL21(DE3) derivatives used in this work are disrupted in their ability to initiate attachment to surfaces because they are non-motile (*47*). Future work will focus on testing these multiple possibilities to increase the Fe(III) reduction rate and cell growth rates in Mtr-expressing *E. coli*.

Conclusions

This work shows that replicating certain features of the electron transfer pathway of *S. oneidensis* MR-1 are critical for boosting extracellular electron transfer in *E. coli*, while others are not. Specifically, we found that increasing the number of Mtr complexes did not increase extracellular electron transfer, but the presence of CymA and riboflavin had a significant impact on extracellular electron transfer and viability. Additionally, we show that as the rate of metal reduction in CymA-expressing *E. coli* increases, these strains gradually transition from rapidly losing biomass to transiently growing under metal-reducing conditions. This knowledge will allow metal-reduction to be more readily introduced into new heterologous hosts.

Despite the relatively modest iron reduction in these newest *E. coli* strains relative to *Shewanella sp.* or *Geobacter sp.*, the ability of the *cymAmtr E. coli* strain described here to maintain biomass or, in the presence of riboflavin, grow over limited timescales makes a useful new tool for both basic and applied studies. Since *E. coli* uses many different electron donors compared to *Geobacter* or *Shewanella*, it may provide a more versatile tool for bioremediation or biomining. Also, it is well known that both cell surface structures and metal reduction play a role in formation and transformation of metal-containing solids such as metallic nanoparticles and metal-oxide minerals. The ability of our *E. coli* strains to reduce solid and chelated Fe(III) with a significantly different cell surface chemistry than *Shewanella sp. or Geobacter sp.* offers an

367 opportunity to dissect these separate effects (*48*). Thus, new *E. coli* strains described herein and
368 those enabled by the design rules described herein, will fuel both basic and applied studies.
369

Methods

Plasmids and strains.

The primers, plasmids, and strains used in this study are listed in **Tables S2, S3, and Table 1**, respectively. The *ccm* (pEC86), *mtrA* (I5024), and *mtrCAB* (I5023) plasmids were described previously (*6*). The *cymA* (I5040), *cymAmtrA* (I5052), and *cymAmtrCAB* (I5049) plasmids were constructed for this work. In brief, these plasmids were constructed using PCR amplification of the genes from genomic DNA of *Shewanella oneidensis* MR-1 using Pfx Platinum polymerase (Invitrogen), digestion of the pSB1ET2 plasmid and PCR fragment(s) with restriction endonucleases (New England Biolabs), ligation of these fragments with T4 DNA ligase (Roche) and, in some cases, site directed mutagenesis (QuikChange II, Agilent Technologies). Detailed descriptions of the assembly of I5040, I5052, and I5049 can be found in the Supporting Information. After sequence verification, the resulting *cymA* plasmid and *cymAmtrA* were co-transformed with *ccm* into BL21(DE3) to make the *cymA* and *cymAmtrA* strains, respectively. In contrast, the *mtrCAB* and *cymAmtrCAB* plasmids, were co-transformed with pEC86 into the *E. coli* strain C43(DE3) (Lucigen, Middleton, WI) to make the *mtr* and *cymAmtr* strains, respectively.

Growth conditions and medium composition.

All strains, unless otherwise specified, were grown in 2xYT medium at 30 °C with 50 μg mL^{-1} kanamycin; strains containing the pEC86 plasmid were grown with an additional 30 μg mL^{-1} chloramphenicol. Glycerol stocks were used to inoculate 5 mL 2xYT medium, and cultures were grown overnight at 37 °C with 250-rpm shaking. Then, 500 μL of overnight cultures were back-diluted into 50 mL 2xYT medium and grown with 200-rpm shaking for 16 h at 30 °C.

When indicated, strains were induced with IPTG at an OD_{600nm} of 0.5-0.7. IPTG concentration is displayed here as promoter activity defined in Goldbeck, et al *(5)*.

Cell suspensions used for iron reduction assays were resuspended in anaerobic defined M1 medium supplemented with 40 mM D,L-lactate and 0.2% casamino acids. The composition of the M1 medium can be found in the Supporting Information.

Subcellular Fractionation.

Periplasmic and membrane preparations were performed as previously described (*6*). Membrane samples were solubilized in a solution of 5% (w/v) Triton X-100, 50 mM HEPES pH 7.4, and 200 mM NaCl.

Visible spectra of cytochrome samples by diffuse reflectance.

The concentration of cytochromes in whole cells was determined by diffuse reflectance as previously described by Goldbeck et al. (*5*). The reduced samples were chemically reduced with sodium dithionite crystals (Sigma, St. Louis, MO). The extinction coefficients at 552 nm of 104 mM^{-1} cm^{-1} (personal communication, Prof. Julea Butt) and 280 mM^{-1} cm^{-1} (*35*) were used to determine the CymA and MtrA concentrations, respectively.

ECL to detect *c*-type cytochromes in whole cell lysates.

Cell pellets from 1.5 mL of culture was resuspended in 0.1 mL Bacterial Protein Extraction Reagent (B-Per, ThermoScientific, Grand Island, NY). The cells were frozen at -20 °C immediately after growth and then thawed immediately before analysis. Cells were lysed for 30 minutes at room temperature with 6 μg mL^{-1} chicken egg white lysozyme (Sigma), 1 μg mL^{-1} DNAase, 3.9 mM $MgSO_4$, 0.96 mM EDTA, and 0.98 mM phenylmethylsulfonyl fluoride. Total protein of the cell lysates was determined by BCA Protein Assay Kit (ThermoScientific). Cells were diluted in 100 mM HEPES, pH 7.4, to normalize to equal total protein concentration.

Samples were prepared with NuPAGE 4x Sample Buffer (Bio-Rad) and heated at 95 °C for 5 minutes. A total of 8 µg protein was loaded and run in a 4-20% Tris-HCl polyacrylamide gel (Bio-Rad) at 200 V for 1 hour. The gel was rinsed twice in water and then equilibrated in cold Pierce Western Transfer buffer (ThermoScientific) for 15 minutes. The proteins were transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad, Hercules, CA) in Pierce Western Transfer buffer at 30 V for 100 minutes. Ponceau S staining was used to confirm uniform transfer across all lanes.

The nitrocellulose membrane was incubated for 5 minutes in 10 mL of Pierce Pico West Enhanced Chemiluminescence substrate (ThermoScientific), a 1:1 mixture of Pico West Peroxide Solution and Luminol Enhancer solution. The chemiluminescent signal was detected using the ChemiDoc™ XRS system. The chemiluminescent signal and molecular weights were quantified using ImageJ (*49*).

Assaying soluble Fe(III) citrate reduction and cell density of strains.

Fe(III) reduction assays were performed as previously described (*6*). Briefly, cultures grown aerobically were pelleted, washed, and resuspended to an $OD_{600\ nm}$ of 0.5 in anaerobic M1 medium supplemented with 40 mM D,L-lactate as the sole carbon source. All anaerobic media and buffers were sparged with nitrogen and anaerobic conditions were maintained in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere of 2% H_2 balance N_2. Fe(III) citrate (Sigma, St. Louis, MO) was added to a final concentration of 10 mM. The Fe(II) concentration was determined with the ferrozine assay, adapted from Stookey (*50*). The concentration of Fe(II) in each culture was subtracted by abiotic iron reduction observed in sterile medium-only controls at each time point. Additionally, a lysed *mtrA* control was included to determine the extent of non-metabolic Fe(III) reduction (Supporting Information). Cell density

was determined by measuring the optical density at 600 nm and subtracting the scattering of abiotic media with 10mM Fe(III) citrate. Error bars represent the standard deviation across three biological replicates. The rate of Fe(III) citrate reduction and linear rate of change in optical density were calculated by best linear fit for each individual biological replicate.

Bulk Fe_2O_3 (*s*) reduction assay.

Fe_2O_3 (*s*) reduction assays were performed as previously described (*6*). Briefly, cultures grown aerobically were pelleted, washed, and resuspended to an $OD_{600\ nm}$ of 1.0 in anaerobic M1 medium supplemented with 40 mM D,L-lactate as the carbon source, 6.0 mg mL^{-1} Fe_2O_3 (*s*) (Sigma) as the sole terminal electron acceptor, and IPTG, where indicated. Where indicated, the media was supplemented with 5 μM riboflavin.

The colony forming units (cfu) and Fe(II) concentration for each culture was measured after initial anaerobic inoculation and at time points following. Cfu's were determined with kanamycin selection on LB plates grown aerobically at 37 °C. The Fe(II) concentration was determined with the ferrozine assay, adapted from Stookey (*50*). The concentration of Fe(II) in each culture was subtracted by any abiotic iron reduction observed in sterile media-only controls at each time point. Error bars represent standard deviation of triplicate cultures.

Cyclic Voltammetry of *mtr* and *cymAmtr* strains.

Cells from 50-mL cultures were pelleted and washed twice with M1 medium supplemented with 40 mM D,L-lactate. Pellets were resuspended to an OD_{600nm} of 0.7 in the working chamber of 2-chambered bioelectrochemical reactors. The working chamber contained M1 medium supplemented with 40 mM D,L-lactate as the sole carbon source, the counter electrode chamber contained 50 mM PIPES buffer (pH 7.4), and the two chambers were separated by a cation exchange membrane (CMI-7000, Membranes International, Ringwood,

NJ). The working electrode was a 25 x 25 mm square piece of graphite felt (GF-S6-06, Electrolytica, Amherst, NY) and was connected to the potentiostat via a piece of Pt wire. The counter electrode was a piece of Pt wire and the reference electrode was a pre-made Ag/AgCl reference (CH Instruments, Bee Cave, TX). During current stabilization, the working chambers were stirred at ~200 rpm with magnetic stir bars, and stirring was turned off during cyclic voltammetry analysis. Cyclic voltammograms were recorded in 2 cycles from -0.5 $V_{Ag/AgCl}$ to +0.5 $V_{Ag/AgCl}$ at a scan rate of 2 mV/s. Cyclic voltammograms were recorded before and immediately after inoculation. After initial cyclic voltammetry, the working electrodes were held at +0.2 $V_{Ag/AgCl}$ overnight to allow cells to associate with the electrode surface. Cyclic voltammograms were recorded again after the overnight period.

Supporting Information.

SI Figure 1. Schematic of plasmids and localization of MtrC, MtrA, and CymA.

SI Figure 2. Diffuse reflectance spectra show the spectral signatures characteristic of cytochromes *c* in whole cell suspensions.

SI Figure 3. ECL analysis shows that the relative abundance of each cyt *c* within a strain is similar across different induction levels

SI Figure 4. Homogenized MtrA *E. coli* reduce Fe(III) citrate at the same basal rate as *ccm*.

SI Figure 5. Fitness is strongly correlated to iron reduction rate.

SI Figure 6. A scaled schematic of the gram-negative double membrane and Mtr pathway.

SI Figure 7. Correlation analysis of iron reduced against heme *c* concentration.

SI Figure 8. Riboflavin mildly improves Fe_2O_3 reduction, but not survival.

SI Figure 9. The *ccm* strain shows no temporal correlation between cell density and Fe_2O_3 reduction.

SI Table 1. Relative promoter activity as a function of IPTG concentration.

SI Table 2. Primers

SI Table 3. Plasmids

Supporting Calculation 1. Approximation of maximum iron reduction rate.

Supporting Calculation 2. Percent Fe_2O_3 reduced from direct contact or riboflavin-mediated mechanisms.

Supporting Methods.

Abbreviations

Colony forming units: cfu. Cytochrome *c*: cyt *c*. Cytochrome *c* maturation: ccm. Enhanced chemiluminescence: ECL. Isopropyl β-D-1-thiogalactopyranoside: IPTG.

Author Information

Authors' contributions

HMJ and CMAF conceived the study. HMJ, MAT, MGK performed the experiments, and CMAF supervised the work. HMJ analyzed the data. HMJ, MAT, MGK and CMAF drafted the manuscript, which was read, revised and approved by all authors.

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Table 1. Strains used in this study.

Strain	Parental Strain	Plasmid(s)	Gene(s)	Source	Request Name
ccm	BL21(DE3)	pEC86, pSB1ET2	*ccmA-H*	Jensen 2010	MFe208
mtrA	BL21(DE3)	pEC86, I5024	*ccmA-H, mtrA*	Jensen 2010	MFe291
cymA	BL21(DE3)	pEC86, I5040	*ccmA-H, cymA*	this work	MFe431
cymAmtrA	BL21(DE3)	pEC86, I5052	*ccmA-H, cymAmtrA*	this work	MFe538
ccm	C43(DE3)	pEC86, pSB1ET2	*ccmA-H*	Goldbeck 2013	MFe408
mtr	C43(DE3)	pEC86, I5023	*ccmA-H, mtrCAB*	this work	MFe409
cymAmtr	C43(DE3)	pEC86, I5049	*ccmA-H, cymAmtrCAB*	this work	MFe444

Figure 1. CymA is co-expressed with MtrCAB to provide an extracellular electron transfer pathway that spans both membranes of *E. coli*. (A) Schematic of the Mtr pathway in *S. oneidensis.* Electrons present in menaquinone are routed across the inner membrane by CymA (blue box) and are then sequentially transferred to MtrA and to MtrC through the MtrCAB electron nanoconduits (red box). Electrons can then be passed from MtrC to Fe_2O_3 either directly or through a flavin-mediated process (green box). (IM = inner membrane. OM = outer membrane.) (B) Heme staining of whole cell lysates of the *cymA*, *cymAmtrA*, and *mtrA* strains show CymA and MtrA are present in the respective strains. Similarly, heme staining of whole cell lysates of the *mtr* and *cymAmtr* strain shows both strains contain MtrA and MtrC. Heme staining was performed via ECL in (B). (C) Densitometry of the *cymAmtrA* (green), *mtr* (red), and *cymAmtr* (blue) containing lanes shown in (B) show that CymA is present only in the *cymAmtr* strain. (D) Heme *c* concentration per cell density for the *mtr* and *cymAmtr* strains as a function of relative promoter activity. Heme *c* concentration was measured by diffused reflectance and relative promoter activity is measured as Goldbeck et al (*5*).

Figure 2. Co-expression of CymA with MtrA improves Fe(III) citrate reduction and maintains biomass in *E. coli*. (A) Fe(II) concentration as a function of time for the *ccm* (open black circles), *mtrA* (closed red circles), *cymA* (half-filled green circles), and *cymAmtrA* (half-filled blue circles) strains. The *cymA* and *cymAmtrA* strains reduce Fe(III) citrate ~3x and ~4x

faster than the *mtrA* strain. (B) Change in cell density over time for the *ccm* (open black circles), *mtrA* (closed red circles), *cymA* (half-filled green circles), and *cymAmtrA* (half-filled blue circles) strains. Only the *cymAmtrA* strain maintains constant biomass under Fe(III) reducing conditions.

Figure 3. Co-expression of CymA with MtrCAB increases Fe_2O_3 (*s*) reduction and maintains biomass in *E. coli*. **(A)** The concentration of bulk α-Fe_2O_3 reduced to Fe(II) by *ccm* and the best performing *mtr* and *cymAmtr* strains, showing that *cymAmtr*$^{0.001}$ significantly increased iron reduction over both the *ccm* and the *mtr*$^{0.37}$ strains ($p = 0.002$ and 0.031, respectively). This indicates that the co-expression of CymA with Mtr increases the flux of electrons to iron oxide. (B) Relative cell density of the *ccm*, *mtr*$^{0.37}$ and *cymAmtr*$^{0.001}$ strains after 4 days under Fe_2O_3 (*s*) reducing conditions, showing that only the *cymAmtr* strain maintains constant biomass. The relative cell density is the cell density after 4 days divided by the initial cell density. Average values (n=3) are plotted and error bars represent standard deviation.

Figure 4. The expression of CymA significantly increases current production by enabling catalytic turnover of Mtr. (A) Current production in electrochemical reactors with working electrodes poised at +0.2 $V_{Ag/AgCl}$ increased significantly when *cymA* was co-expressed with *mtr*. Average values (n=4) are plotted and error bars represent standard deviation. (B) Turnover cyclic voltammetry (representative scans plotted) at 2 mV/s reveals a catalytic wave only when *cymA* is co-expressed with *mtr*, indicating that the inner membrane cytochrome is necessary for catalytic turnover of the outer membrane cytochromes.

Figure 5. Riboflavin improves Fe_2O_3 (*s*) reduction and permits growth in the *cymAmtr* strain. (A) Riboflavin significantly enhanced iron reduction by ~2.5x in the *cymAmtr*$^{0.18}$ strain ($p = 0.001$). Assuming direct contact contributes equally in the cultures with and without the supplementation of riboflavin, 77% of the iron oxide was reduced by riboflavin. (B) The survival of the *cymAmtr*$^{0.18}$ strain dramatically increased in the presence of riboflavin ($p = 3x10^{-5}$), but not in the *mtr*$^{0.18}$ strain ($p = 0.13$). Average values ($n = 3$) are plotted and error bars represent standard deviation. (C) Concentration of Fe(II) produced and colony forming units as a function of time for the *cymAmtr*$^{0.18}$ cultures with riboflavin, showing a that the initial period of rapid

722 Fe(II) production is accompanied by increase in cell density for the *cymAmtr* strain with
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