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Tuning Promoter Strengths for Improved Synthesis and Function of Electron Conduits in *Escherichia coli*

Cheryl P. Goldbeck, Heather M. Jensen, Michaela A. TerAvest, Nicole Beedle, Yancey Appling, Matt Hepler, Guillaume Cambray, Vivek Mutalik, Largus T. Angenent, and Caroline M. Ajo-Franklin

Divisions of 1Materials Sciences and 2Biological Biosciences, Lawrence Berkeley National Laboratory, Berkeley, California 94720, United States

3Department of Chemistry, University of California, Berkeley, California 94720, United States

4Department of Biological and Environmental Engineering, Cornell University, Ithaca, New York 14853, United States

5BIOFAB International Open Facility Advancing Biotechnology (BIOFAB), Emeryville, California 94608, United States

6California Institute for Quantitative Biosciences, University of California, Berkeley, California, 94720, United States

Supporting Information

ABSTRACT: Introduction of the electron transfer complex MtrCAB from *Shewanella oneidensis* MR-1 into a heterologous host provides a modular and molecularly defined route for electrons to be transferred to an extracellular inorganic solid. However, an *Escherichia coli* strain expressing this pathway displayed limited control of MtrCAB expression and impaired cell growth. To overcome these limitations and to improve heterologous extracellular electron transfer, we used an *E. coli* host with a more tunable induction system and a panel of constitutive promoters to generate a library of strains that separately transcribe the *mtr* and cytochrome c maturation (*ccm*) operons over 3 orders of magnitude. From this library, we identified strains that show 2.2 times higher levels of MtrC and MtrA and that have improved cell growth. We find that a ~300-fold decrease in the efficiency of MtrC and MtrA synthesis with increasing *mtr* promoter activity critically limits the maximum expression level of MtrC and MtrA. We also tested the extracellular electron transfer capabilities of a subset of the strains using a three-electrode microbial electrochemical system. Interestingly, the strain with improved cell growth and fewer morphological changes generated the largest maximal current per cfu, rather than the strain with more MtrC and MtrA. This strain also showed ~30-fold greater maximal current per cfu than its *ccm*-only control strain. Thus, the conditions for optimal MtrCAB expression and anode reduction are distinct, and minimal perturbations to cell morphology are correlated with improved extracellular electron transfer in *E. coli*.

KEYWORDS: multiheme cytochrome c, extracellular electron transfer, cytochrome c biogenesis, secretion, membrane protein expression, *Shewanella oneidensis* MR-1

Both organisms and technological devices use electrons to carry information and energy. Creating a means for the bidirectional flow of electrons between living and nonliving systems would create new opportunities in energy conversion, biosensing, and biocomputing. However, engineering such a conduit involves overcoming the electrically insulating character of cellular membranes without significantly affecting cell viability. To make electronic connections to cells, most approaches rely on the introduction of diffusible redox species. We recently established a novel synthetic biology approach to overcome this long-standing challenge by genetically introducing a heterologous extracellular electron transport pathway into *Escherichia coli*. This strategy takes advantage of the respiratory capability of the dissimilatory metal reducing bacterium *Shewanella oneidensis* MR-1. Under anaerobic conditions, this bacterium couples the oxidation of organic matter to the reduction of insoluble metals. Because insoluble metals cannot enter the cell, dissimilatory metal reducing bacteria use a transmembrane electron transfer pathway, which transports electrons from the cell interior to the extracellular surface. Genetic and biochemical studies have defined the pathway for anaerobic respiration of lactate with Fe(III) oxides or an anode serving as the terminal electron acceptor in *S. oneidensis* MR-1 (Figure 1a). L-Lactate dehydrogenase oxidizes L-lactate to pyruvate, and then pyruvate is converted to formate and acetate. Reducing equivalents resulting from *l*-lactate oxidation are delivered to the periplasmic reductase CymA via menaquinol. Electrons are transferred from CymA to MtrA and from MtrA to MtrC within the MtrCAB complex. From MtrCAB...

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electrons can be transported to terminal electron acceptors through redox shuttles, direct contact, or possibly through other proteins on the cell surface (e.g., OmcA). The electron conduit of this organism is also reversible and can accept electrons from a cathode to drive intracellular reduction reactions.

Previously, we transferred the MtrCAB electron transfer pathway into the model microbe E. coli and demonstrated that the resultant strain could reduce inorganic solids. In E. coli, NapC and other unidentified proteins partially substituted for the function of CymA in S. oneidensis MR-1. However, close examination of this strain, referred to here as the BL21(DE3) mtrCAB strain, reveals two key shortcomings: (i) this strain has dramatically slowed growth relative to its parent strain, and (ii) inducing transcription of the mtr operon with isopropyl β-D-1-thiogalactopyranoside (IPTG) caused the cell pellet to appear less red, suggesting that fewer c-type cytochromes were produced at higher transcription rates.

These challenges likely arise from the high degree of post-translational modifications required to make c-type cytochromes (Figure 1b). MtrA is a 32-kD periplasmic decaheme cytochrome c, and MtrC is a 69-kD cell-surface-exposed lipid-anchored decaheme cytochrome c. MtrC requires MtrB, a 72-kD predicted twenty-eight strand β-barrel outer membrane protein, for correct assembly in the outer membrane (Figure 1a). MtrA also appears to be necessary for MtrB stability. MtrC and MtrA biogenesis requires the cytochrome c maturation (ccm) genes, which encode the eight membrane proteins CcmABCDEFGH. Because transcription of the genomic ccm operon in E. coli is repressed under aerobic conditions, the ccm genes must be expressed from a plasmid to mature MtrC and MtrA. This is traditionally done with cotransformation of the helper plasmid, pEC86, which uses a tet promoter to constitutively express the E. coli ccm genes. After their synthesis, the Ccm and Mtr polypeptides are secreted into the periplasm (Figure 1b). The Ccm system plays a role in translocating heme into the periplasm and then catalyzes the formation of thioether bonds that link heme to two cysteine residues. Once these processes are complete, the axial ligands (typically histidine) are coordinated to the heme iron and the holocytochrome c is folded (Figure 1b). Finally, MtrC and MtrB must be translocated to the outer membrane, presumably by type II secretion.

This series of post-translational modifications creates multiple points for misregulation of the synthesis and maturation of the MtrCAB complex in E. coli. High levels of leader-sequence bearing polypeptides can saturate the signal recognition particle, leading to the misfolding of the unsecreted polypeptides and accumulation of cytoplasmic aggregates. This results in pleiotropic cellular impairments including an increase in the size and granularity of E. coli cells, decreased growth, and decreased expression of the inner membrane proteome. Since L-lactate dehydrogenase is an inner membrane protein, secretion impairment has the potential to negatively impact the flux of electrons to menaquinone from lactate metabolism as well as the synthesis of MtrCAB (Figure 1).

Misregulation of cytochrome c synthesis can also be caused by inadequate heme, which will result in degradation of apocytochromes c by the periplasmic proteases, such as DegP (Figure 1b). Thus c-type cytochrome biogenesis requires an appropriate balance between the rates and levels of polypeptide synthesis, heme biosynthesis, polypeptide secretion, and cytochrome c maturation. Reflecting this, other groups have found that heterologous multiheme cytochrome c expression in E. coli can be increased in two ways: (i) by enhancing heme biosynthesis with heme precursors, such as δ-aminolevulinic acid (δ-ALA) or rich media, and (ii) by lowering apolypeptide synthesis through weaker transcriptional promoters, different E. coli hosts, and lower copy number of the cytochrome c genes.

Figure 1. Multiple post-translational modifications are required to synthesize the components of the Mtr electron conduit, and misregulation of these processes has the potential to negatively impact extracellular respiration. (a) Route for electrons from lactate to an anode via the Mtr pathway. L-lactate is oxidized into pyruvate, and the resulting reducing equivalents are used to reduce menaquinone (MQ) to menaquinol (MQH2). In S. oneidensis MR-1, CymA is the inner membrane cytochrome c that accepts electrons from menaquinol; in E. coli, this function is achieved by NapC, as well as yet unidentified proteins. From the inner membrane protein, electrons are passed through the periplasm and outer membrane to extracellular electron acceptions via the MtrCAB complex. (b) After transcription and translation, the Mtr and Ccm polypeptides are secreted into the periplasm by the Sec secretion system. Excessive production of signal sequence-containing polypeptides can overload the Sec translocon, resulting in aggregation and/or retention of the polypeptides in the cytoplasm. The Ccm machinery then loads heme into the apocytochromes c to form mature MtrC and MtrA. Low production of heme or inadequate concentrations of Ccm biogenesis machinery relative to the steady-state apocytochrome c concentration can trigger degradation of the apocytochrome c by periplasmic proteases such as DegP.
The slow growth of BL21(DE3) mtrCAB and decrease in cytochrome c expression upon induction suggests that the basal transcription rate of mtr and the constitutive expression of ccm outpace the rates of heme synthesis, polypeptide secretion, and/or cytochrome c maturation. Here, we hypothesize that by balancing the ccm and mtr transcription to avoid misregulation of post-transcriptional modifications, we can improve cytochrome c expression, cell growth, and/or extracellular electron transfer relative to the BL21(DE3) mtrCAB strain. Through the use of a more tightly regulated and tunable induction system and a panel of constitutive promoters, we created strains with varying expression of the ccm and mtr operons. We show that cytochrome c expression and cell growth are strongly affected by the mtr promoter activity. Surprisingly, we also find that the ccm and mtr promoter strengths for optimal cytochrome c expression are distinct from those for improved anode reduction.

**RESULTS AND DISCUSSION**

**Design of Strains with Variable Levels of Transcription of the ccm and mtr Operons.** The overall goal of this work is to provide controllable and improved synthesis and function of the MtrCAB pathway in *E. coli*. The BL21(DE3) mtrCAB strain contains a pET30a+-derived plasmid containing mtrCAB under the control of a T7 lac promoter and a cytochrome c maturation plasmid containing the ccm operon regulated by the constitutive tet promoter (pEC86).11 Designed as a control for the ccm operon, the BL21(DE3) ccm strain only carries the pEC86 plasmid. Interestingly, the BL21(DE3) mtrCAB and BL21(DE3) ccm strains grew 27 ± 8% and 7 ± 1% more slowly than the BL21(DE3) WT strain, respectively (Supporting Information Figure 1a). Moreover, the BL21(DE3) mtrCAB strain shows 2-fold greater forward scatter relative to BL21(DE3) WT strain (Supporting Information Figure 1b), indicating that MtrCAB expression causes an increase in cell size. Additionally, induced BL21(DE3) mtrCAB appear less red than uninduced cell pellets,11 indicating that increased transcription of mtrCAB led to decreased production of holocytochrome c.

Because expression of multiheme c-type cytochromes and membrane proteins have previously been improved by lowering transcriptional levels,49,50 we hypothesized that a similar strategy would improve control or levels of cytochrome c expression or both. To achieve controlled expression, we changed the host strain to C43(DE3), which has lower basal and induced levels of T7 RNA polymerase relative to BL21(DE3) due to mutations in the lacUV5 promoter region.50 Using the methodology of Kelly et al.,51 we calibrated the relative promoter activity by varying the concentration of IPTG. We found that induction of the T7 lac promoter can modulate transcription over ~3 orders of magnitude in the C43(DE3) strain (Supporting Information Figure 2a–c and Supporting Information Table 1). We used this promoter (in the previously described mtrCAB plasmid11) in the C43(DE3) strain to control transcriptional levels of mtrCAB.

Since the BL21(DE3) ccm strain showed slower cell growth, which has been correlated with misregulation of membrane protein expression,50 we hypothesized that decreasing the level of ccm expression could improve cell growth and possibly functional expression of the MtrCAB pathway. Thus, we calibrated a series of constitutive promoters of varying strength generated by the BioFAB (Emeryville, CA; Mutalik et al., unpublished data), again using the methodology of Kelly et al.51

This library spanned 3.5 orders of magnitude in promoter strength (Supporting Information Figure 2d–f and Supporting Information Table 2), providing a wide window over which to tune ccm transcription. Golden Gate cloning52 was used to introduce the BioFAB promoters in front of the ccm operon, creating a library of plasmids with variable ccm transcription.

**High Transcriptional Levels of ccm and mtr Decrease Cytochrome c Expression and Cell Growth.** The ccm plasmids were co-transformed with the previously described mtrCAB plasmid11 into C43(DE3) cells (Figure 2a) to generate a series of mtrCAB-expressing strains (Supporting Information Table 3). We notate the different transcriptional levels of ccm and mtr operons in these cultures as ccm\(^{\text{fi}}\) mtrCAB\(^{\text{ff}}\) where the superscripts indicate the ccm promoter strength and mtr promoter activity based on our calibrated scale in Supporting Information Tables 2 and 1, respectively. As a control, the
C43(DE3) WT strain was generated by co-transformation of the empty pET30a+ and pACYC184 plasmids.

To screen the effects of simultaneously tuning the ccm promoter strength and the mtr promoter activity on cytochrome c expression, the ccm
mtrCAB
strains were grown in 96-well plates with varying IPTG concentrations to generate 192 different transcriptional combinations. This number of combinations precluded direct analysis of cytochrome c content. Instead, because c-type cytochromes are red in color, we quantified the relative red intensity of the cell pellets (calculated as described in Methods) and used this value as a metric for cytochrome c production. To measure the relative red intensity, equal volumes of each culture were centrifuged, and images of the resulting cell pellets were acquired using a scanner. The heatmap shown in Figure 2b summarizes the results of varying the transcriptional levels of both the ccm and mtr operons on the relative red intensity of the cell pellet. Unlike the BL21(DE3) mtrCAB strain, the ccm
mtrCAB
strains show little or no relative red intensity in the absence of induction (Figure 2b, leftmost column). This increased control reflects the lower basal transcription of T7 RNA polymerase in the C43(DE3) strain. The pellets with the greatest relative red intensities were localized in the narrow range of mtr promoter activities between ~0.1 to 0.3 arb. units and ccm promoter strengths between ~0.01 to 0.2 arb. units (Figure 2b). At ccm or mtr transcription levels outside this optimal range, the relative red intensity of the cell pellets decreased sharply. This decrease suggests that there is misregulation in the post-transcriptional processes. The ccm
mtrCAB
culture with the greatest relative red intensity (0.22 ± 0.02) was more intensely red than the BL21(DE3) mtrCAB cells (0.11 ± 0.03), indicating that our screen produced a strain with higher expression levels of cytochrome c than the original strain.

To assess the impact of mtr and ccm transcriptional levels on cell growth, we calculated (as described in Methods) the change in apparent cell pellet area relative to the C43(DE3) WT strain. This area is proportional to the pellet volume and thus the total cell biomass. As shown in the heatmap in Figure 2c, the apparent cell pellet area was unchanged in strains with both very low mtr and ccm transcriptional levels. The pellet area slightly decreased with increasing ccm promoter strength and steeply decreased with increasing mtr promoter activity, such that the apparent pellet area decreased by up to 35% (Figure 2c). Thus, transcription of the mtr operon negatively impacts the maximal cell density. Moreover, cytochromes c expression and cell growth were impacted in distinct manners by the transcriptional levels of the ccm and mtr operons.

Increasing the ccm Promoter Activity Results in Decreased Levels of CcmE and Cytochrome c Matura- tion. The initial screen (Figure 2) suggested that ccm and mtr transcription levels impact cytochrome c expression and total cell biomass, both of which are likely to be important for extracellular electron transfer in E. coli. To confirm these trends and to understand their molecular underpinnings, we determined the cytochrome c abundance and cell density for a subset of cultures that showed high relative red intensities in the initial screen. These strains were grown as 50 mL cultures in flasks, and the final cell density was measured using traditional UV−vis spectroscopy. Diffuse reflectance spectroscopy was performed on intact cells to quantitatively determine [MtrC+MtrA] per unit cell density using the unique absorption of c-type cytochromes at 552 nm (see Methods, Supporting Methods, and Supporting Information Figure 3). Spectroscopic measurements (Supporting Information Figure 4a) and hemestaining of whole-cell lysates (Supporting Information Figure 4b) show that only MtrC and MtrA, rather than endogenous cytochromes c, contribute to the absorption at 552 nm.

Measurements of relative red intensity and [MtrC+MtrA] from the same culture are highly correlated (r = 0.9, Pearson’s correlation coefficient, Supporting Information Figure 5a,b), confirming that relative red intensity is an effective screen for high cytochrome c production per cell. As in the initial screening experiments, all the ccm
mtrCAB
strains measured have a low [MtrC+MtrA] (2.5 μM/OD
DR
) in the absence of induction by IPTG. The [MtrC+MtrA] increased to the 10−20 μM/OD
DR range in the ccm
mtrCAB
strains measured (Supporting Information Figure 4a) and heme-
Figure 4. mtr promoter activity sharply affects the efficiency of MtrC + MtrA synthesis and final cell density. (a) The total concentration of MtrA and MtrC per OD

mtrCAB

levels in the membrane. In turn, the low level of Ccm biogenesis machinery decreases the maturation of apo-proteins into holo-MtrC and holo-MtrA.

Efficiency of MtrC and MtrA Synthesis and Cell Growth Decrease Strongly with Increasing mtr Promoter Activity. We next investigated the effect of modulating the mtr promoter activity on decaheme cytochrome c expression and cell growth. We probed these trends as described above in the ccm

mtrCAB

promoter activity on decaheme cytochrome c production with increasing mtr promoter strength. (c) The normalized CcmE intensity as a function of mtr promoter activities, showing that there is no significant decrease in the membrane-associated CcmE levels with increasing mtr promoter activity. (d) The final cell density as a function of mtr promoter strength in the presence of 1 mM δ-ALA (black filled circles) and absence of δ-ALA (dotted black circles), showing a strong decrease in cell density with mtr promoter activity (r = −0.97, Pearson’s correlation coefficient). In contrast, the final cell density of a C43(DE3) strain harboring the pET30a+ GFP plasmid (filled green squares) increases slightly with IPTG induction.

We sought to explain the decrease in holocytochrome c with high ccm promoter strengths (≥0.34 arb. units) to develop a deeper mechanistic understanding of the controls on decaheme cytochrome c production. We hypothesized that high ccm promoter strengths could reduce the translation or secretion efficiency, which would decrease functional Ccm machinery present in the inner membrane and decrease cytochrome c maturation. To quantitatively compare the membrane-associated CcmE levels across different strains (Figure 3c, inset), we normalized the CcmE band intensity of each strain by the band intensity arising from an equal mass of ccm

mtrCAB

culture lysate. This normalized CcmE intensity was linear with CcmE abundance over the range of our measurements (Supporting Information Figure 6a,b). The membrane-associated CcmE levels dramatically decreased with ccm promoter strengths ≥0.34 arb. units, the same ccm promoter strengths that showed a sharp decline in [MtrC+MtrA]. Thus, this trend is consistent with a translation or secretion defect at high ccm promoter strengths, which decreases Ccm protein levels in the membrane. In turn, the low level of Ccm biogenesis machinery decreases the maturation of apo-proteins into holo-MtrC and holo-MtrA.

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Two known mechanisms could result in decreased efficiency of cytochrome c synthesis with increasing cytochrome c transcript levels: (i) saturation of translation or secretion by high mtr expression or decreased ability to mature protein, or (ii) in adequate heme production could lead to increased degradation of partially matured decaheme cytochrome c. We first probed membrane-associated CcmE abundance as a function of mtr promoter activity (Figure 4c). The membrane-associated CcmE levels were within 1 standard deviation of each while the mtr promoter activity was varied by ~5-fold, indicating that there is no significant decrease in the Ccm biogenesis machinery of the strain (Figure 4c). Next, spent media from highly induced ccm mtrCAB cultures supplemented with the heme biosynthesis precursor δ-ALA was analyzed spectroscopically for heme. The visibly red media contained ~0.3 μM heme b (not shown), ruling out heme limitation as a cause of the decrease in cytochrome c synthesis efficiency. Further supporting this idea, we measured the [MtrC+MtrA] in ccm mtrCAB cultures lacking δ-ALA (dotted red squares, Figure 4a). While diminished bioavailability of heme decreases the efficiency of holocytochrome c synthesis by ~30% across all mtr promoter activities, it does not significantly alter the relationship between efficiency of cytochrome c synthesis and mtr promoter activity (Figure 4b). Thus, our data rule out decreased Ccm biogenesis machinery and inadequate heme production as causes for the stark decrease in efficiency of cytochrome c synthesis with increasing mtr promoter activity.

To probe the impact of the mtr promoter on cell growth, we measured the final cell density in the ccm mtrCAB cultures. We observed that the cell density is negatively correlated with increasing mtr promoter activity in the presence (solid black squares) or absence (hollow black squares) of δ-ALA (r = −0.97, Pearson’s correlation coefficient, Figure 4d). As described above, neither polypeptide synthesis nor heme bioavailability are limited in this strain. Therefore, the decrease in growth could be caused by toxicity of IPTG, MtrC, MtrA, or MtrB. To determine whether the decrease in cell density was caused by IPTG, we measured the final cell density of a C43(DE3) strain expressing GFP from the T7 lac promoter induced with varying [IPTG]. The final cell density of these cultures increased slightly with IPTG (solid green squares, Figure 4d), indicating that IPTG does not negatively influence growth. Additionally, comparison of the cytochrome c abundance data in Figure 4a and 4d shows that [MtrC + MtrA] is not strongly correlated with cell density (r = −0.28, Pearson’s correlation coefficient). For example, the ccm mtrCAB mtrCAB cultures have similar [MtrC + MtrA] (Figure 4a), yet the ccm mtrCAB mtrCAB cultures have a final cell density that is 50% of the ccm mtrCAB mtrCAB cultures (Figure 4d). Therefore, our data suggest that the decrease in cell density with mtr promoter activity is not due to toxicity of IPTG, MtrA, or MtrC.

Anode-Reduction Correlates with Minimal Alterations in Cell Morphology and Moderate MtrC and MtrA Expression. Our central hypothesis is that the number of MtrCAB electron conduits as well as minimal perturbations to cell growth and physiology may be important to achieve extracellular electron transfer in E. coli. From the assays and screens above, we selected several ccm mtrCAB cultures with

Figure 5. A C43-derived mtrCAB strain with a less perturbed cellular morphology can transfer electrons to an anode. (a) Forward scatter versus [MtrC+MtrA] for ccm mtrCAB cultures. Highlighted are the BL21(DE3) WT (black triangle), C43(DE3) WT (black circle), BL21(DE3) mtrCAB (red triangle), least perturbed (blue circle), and most cytochrome c (magenta circle) strains. (b) Representative electrochemical measurements of BL21(DE3) mtrCAB culture (red line), the least perturbed culture (blue line), and most cytochrome c culture (magenta line). (c) Maximal current per cfu for the BL21(DE3) mtrCAB cells (solid red bar), the least perturbed culture (solid blue bar), and most cytochrome c culture (solid magenta bar) and their respective ccm-only strains: the BL21(DE3) ccm (cross-hashed red bar), least perturbed ccm (cross-hashed blue bar), and most cytochrome c ccm (cross-hashed magenta bar). There is a significant difference in maximal current per cfu between the least perturbed culture and its control, the least perturbed ccm strain (p-value = 0.01, Student’s one-tailed t test with equal variances). (d) Lactate consumption per cfu for the BL21(DE3) mtrCAB cells (solid red bar), the least perturbed culture (solid blue bar), and most cytochrome c culture (solid magenta bar) and their respective ccm-only strains BL21(DE3) ccm (hashed red bar), least perturbed ccm (hashed blue bar), and most cytochrome c ccm (hashed magenta bar). There is a significant difference in lactate consumed per cfu between the least perturbed and least perturbed ccm strains (p-value = 0.03, Student’s one-tailed t test with equal variances).
high cytochrome c levels ([MtrC+MtrA] ≥ 10 μM/OD_{600}^{DR} and high final cell density (cell density ≥2 OD_{600}^{UV-vis}). To provide an additional metric of overall cell morphology, we examined the forward scatter of these strains by flow cytometry (Figure 5a). Just as cells from the BL21(DE3) mtrCAB strain (Figure 5a, red filled triangle) showed increased scatter relative to the BL21(DE3) WT strain (Figure 5a, black filled triangle), cells from all of the ccm^p mtrCAB^p cultures (Figure 5a, circles) also showed increased forward scatter compared to the C43(DE3) WT strain (Figure 5a, black filled circle). These measurements indicate expression of MtrCAB causes significant morphological changes without substantially altering the final cell density.

We sought to understand the correlation of cellular cytochrome c levels and cellular morphology to the ability of cells to transfer electrons to an electrode. Using a comparison of forward scatter versus [MtrC+MtrA] (Figure 5a), we selected a culture that shows less morphological perturbations (solid blue circle, ccm^p mtrCAB^p, henceforth referred to as the "least perturbed" culture) and a culture that shows higher [MtrC+MtrA] (solid magneta circle, ccm^p mtrCAB^p, henceforth referred to as the "most cytochrome c" culture), relative to the original BL21(DE3) strain. These strains and their respective ccm-only controls were inoculated at a high cell density into a three-electrode microbial electrochemical system.\(^34\) The working electrode was poised at 0.2 V versus Ag/AgCl (saturated KCl), L-lactate was supplied as the electron donor, and the electric current production was recorded over several days (Figure 5b). Interestingly, the least perturbed strain achieved higher current than either the BL21(DE3) mtrCAB strain or most cytochrome c strain. Additionally, the least perturbed strain also produced ∼30-fold greater maximum current per cfu than its counterpart ccm-only strain (significantly different with a p-value = 0.01, Figure 5c), indicating that reduction of the electrode in the least perturbed strain is enhanced by the presence of the MtrCAB electron conduit. In contrast, both the BL21(DE3) mtrCAB and most cytochrome c strains showed low maximal current per cfu, which were not statistically significant from their respective ccm-only strains (p-value = 0.25, Figure 5c). Although S. oneidensis MR-1 typically produces ∼1000 times higher cytochrome c than the least perturbed strain in these three-electrode electrochemical systems, the difference in current per cfu between the least perturbed strain in comparison to the other E. coli strains indicates it is possible to engineer anode reduction into new organisms using the MtrCAB electron conduit.

Additionally, HPLC analysis of the spent media (Figure 5d) shows that the most lactate per cfu was consumed by the least perturbed culture, supporting the hypothesis that electrons delivered to the electrode originate from E. coli metabolism of lactate. These observations indicate that heterologous expression of MtrCAB allows E. coli to transfer metabolic electrons to an electrode and that phenotypic features and [MtrC+MtrA] are correlated with the efficiency and rate of extracellular electron transfer.

**Transcriptional Balance Is Critical for Optimal in Vivo Synthesis and Function of Electron Conduits.** Using our library of transcriptional variants, we are able to quantitatively define regimes in which different mechanisms limit further synthesis of MtrC and MtrA (Figure 6). Below a threshold level of ccm transcription, decaheme cytochrome c is not produced because insufficient Ccm complexes are available to mature the apoproteins (Figure 6a). Above a certain limit, increasing the ccm transcriptional level actually decreases the membrane-associated Ccm biogenesis machinery (Figure 6b), setting a maximum level of Ccm biogenesis machinery available to mature apocytochrome c. Most critically, holocytochrome c synthesis was sharply dependent on the mtr promoter activity. Increased mtr transcription (Figure 6a) is outweighed by the decreased efficiency of MtrC and MtrA synthesis, resulting in decreased decaheme cytochrome c abundance.

To our knowledge, this is the first report systematically documenting a decrease in multiheme cytochrome c production efficiency, although other groups have shown that low induction levels are optimal for heterologous expression of multiheme cytochrome c in E. coli.\(^{36-48}\) Neither low heme abundance nor impairment in secretion account for this drop in efficiency of cytochrome c production. While it is possible that increasing levels of MtrC inhibit its correct localization into the outer membrane, previous work in E. coli\(^{40}\) suggests that mislocalization of MtrC is unlikely to affect the overall levels of decaheme cytochrome c. We hypothesize that the low efficiency is due to an as-yet uncharacterized mechanism. One possible scenario is that the Ccm complex is insufficiently processive to rapidly mature decaheme cytochromes c (Figure 6c). Thus, the apoprotein level increases, CXCH motifs from different polypeptides compete for binding to Ccm complex, resulting in a greater fraction of apoproteins being only partially matured and degraded. Three observations lend support to this idea: (i) heterologous expression of single heme cytochromes c does not show the same dramatic dependence on transcriptional levels,\(^{35}\) (ii) the modest 3.5-fold improvement of cytochrome c production with 67-fold increase in ccm transcriptional levels suggests that the Ccm complex is an inefficient enzyme for
decaheme cytochrome c maturation (Figure 6a), and (iii) in S. oneidensis MR-1 40% of MtrC polypeptides never mature, suggesting that decaheme cytochrome c maturation is intrinsically inefficient.\cite{6}

In addition to the complexity of decaheme cytochrome c synthesis, the function of MtrCAB electron conduits in E. coli is not straightforward. Extracellular electron transfer in E. coli not only is governed by the cellular concentration of decaheme cytochrome c but also is correlated with minimal perturbations to cellular growth and morphology. Both MtrCAB subsystem-specific and more cellular-wide properties could underlie this observation. For example, a difference in ratio of MtrA:MtrB:MtrC or a change in percentage of properly localized MtrC, MtrB, or MtrA could negatively impact extracellular respiration without changing the decaheme content per cell. Alternatively, more cell-wide perturbations may be at root of this. For example, under aerobic growth conditions, the membrane stress response has been shown to decrease the rate of O2(g) consumption and impact the inner membrane proteome.\cite{31,38} Since reducing equivalents from lactate must pass through the inner membrane proteins lactate dehydrogenase and NapC, these alterations could critically affect the availability of electrons to enter the MtrCAB electron conduit. Thus, future investigations will attempt to untangle these many possibilities to more firmly understand the factors critical for extracellular respiration in E. coli. Indeed, given the degree to which this system interfaces with host machinery, we anticipate that additional systems-level analysis will improve heterologous extracellular respiration to levels practical for biosensing and bioenergy applications.

Conclusions. Here we report the impact of tuning ccm and mtr transcription on the synthesis and function of the MtrCAB electron conduit in E. coli. The use of the C43(ΔDE3) strain enables the synthesis of MtrCAB to be controlled and improves MtrC and MtrA expression by 2.2-fold. Surprisingly, the strain that produces the greatest current at the anode does not have MtrC and MtrA expression by 2.2-fold. Extracellular electron transfer in E. coli not only is governed by the cellular concentration of decaheme cytochrome c but also is correlated with minimal perturbations to cellular growth and morphology. Both MtrCAB subsystem-specific and more cellular-wide properties could underlie this observation. For example, a difference in ratio of MtrA:MtrB:MtrC or a change in percentage of properly localized MtrC, MtrB, or MtrA could negatively impact extracellular respiration without changing the decaheme content per cell. Alternatively, more cell-wide perturbations may be at root of this. For example, under aerobic growth conditions, the membrane stress response has been shown to decrease the rate of O2(g) consumption and impact the inner membrane proteome.\cite{31,38} Since reducing equivalents from lactate must pass through the inner membrane proteins lactate dehydrogenase and NapC, these alterations could critically affect the availability of electrons to enter the MtrCAB electron conduit. Thus, future investigations will attempt to untangle these many possibilities to more firmly understand the factors critical for extracellular respiration in E. coli. Indeed, given the degree to which this system interfaces with host machinery, we anticipate that additional systems-level analysis will improve heterologous extracellular respiration to levels practical for biosensing and bioenergy applications.

**METHODS**

Additional details may be found in the Supporting Information.

**Plasmids, Strains, and Growth Conditions.** The strains used in this study are listed in Supporting Information Table 3. The BL21(DE3) WT, BL21(DE3) ccm, and BL21(DE3) mtrCAB strains were described previously.\cite{11} The pFAB#-GFP plasmids were obtained from the BioFAB (Emeryville, CA). To create a library of plasmids containing different promoters in front of the ccm coding sequence, Golden Gate cloning\cite{32} was used to replace the tet promoter in pEC86\cite{35,38} with BioFAB-derived promoter sequences.

All strains were grown in 2xYT media with 50 μg mL\(^{-1}\) kanamycin and 30 μg mL\(^{-1}\) chloramphenicol. Growth of cultures in 96-deep-well plates was done at 37 °C with 275-rpm shaking, and growth in 250 mL flasks was done at 30 °C with 250-rpm shaking. Transcription of mtrCAB was induced by addition of IPTG when the cultures reached an OD\(_{600}\) of 0.5 (typically 2–3 h after inoculation). Cultures were grown for a total of 20 h after inoculation.

**Analysis of Cell Pellets for Assessment of Cytochrome c Expression and Cell Growth.** After growth in 96-well plates, 150 μL of culture was pelleted in clear, V-bottom 96-well polystyrene plates (ThermoScientific, Waltham, MA). The plates containing the cell pellets were scanned using a Scanjet G4050 scanner (HP, Palo Alto, CA). Regions of interest from each pellet were identified using the Matlab (R2009b) Image Processing Toolbox (MathWorks, Natick, MA). The relative red intensity was calculated by taking the ratio intensity in the red channel (I\(_{red}\)) to the grayscale intensity (I\(_{gray}\)) and subtracting the red to gray ratio of C43(DE3) WT cells: relative red intensity = (I\(_{red}\))/(I\(_{gray}\)) − (I\(_{red}\))/(I\(_{gray}\))(WT). To calculate the change in cell pellet area, the difference in area between C43(DE3) WT cell pellet area and the area of the cell pellet of interest was divided by the C43(DE3) WT cell pellet area.

**Quantitative Assessment of Cytochrome c per Cell Density by Diffuse Reflectance Spectroscopy.** Cell suspensions of OD\(_{500}\)~3–4 OD were reduced by sodium dithionite at a final concentration of 10 mM, and ~400 μL was loaded into a transparent chamber consisting of 0.08 cm deep, 22 mm square Secure-Seal hybridization gaskets (Life Technologies, Eugene, OR) adhered to 25 mm wide square glass coverslip. The absorbance spectrum of the cell suspension was measured using Quality Spec Pro UV–vis spectrometer equipped with a Muglight diffuse reflectance attachment (ASD Inc., Boulder, CO). The total decaheme cytochrome c concentration per OD\(_{600}\) mm [MtrC+MtrA], was then calculated by dividing the background corrected absorption at 552 nm normalized for 1 OD\(_{600}\) mm of cells by the effective path length of 0.08 cm and the extinction coefficient for MtrA and MtrC (ε\(_{MtrA,MtrC}\) = 280,000 M\(^{-1}\) cm\(^{-1}\)).\cite{30,57}

**Immunoblotting of CcmE.** The immunoblot of CcmE was done similarly to Mavridou et al.\cite{38} The primary antibody, a rabbit antiserum to E. coli CcmE (gift from Stuart Ferguson) and the secondary antibody, goat anti-rabbit-peroxidase (Sigma, St. Louis, MO), were diluted 1/10,000 and 1/20,000, respectively. Development was performed using SuperSignal West Dura Substrate (Pierce, Rockford, IL). Blots were quantified using the ChemiDoc XRS+ System and software (Bio-Rad, Richmond, CA).

**Electrochemistry.** Cell pellets were resuspended in minimal M4 medium. An equivalent of OD\(_{600}\) ~0.04 was inoculated into sterile bioreactors with 40 mM lactate. Current was measured with a three-electrode electrochemical system with a working electrode maintained at 0.2 V versus Ag/AgCl (saturated KCl) using a potentiostat (VSP, BioLogic USA, Knoxville, TN). The working electrode was a 9 cm square piece of carbon cloth (PANEX 30-PW06, Zoltek Corp, St Louis, MO) attached to a graphite rod with carbon cement, and the counter electrode was a graphite rod. The bioreactors were maintained anaerobically by constant sparging with sterile N2 gas,\cite{54} stirred at ~250 rpm with magnetic stir bars, and maintained at 30 °C.\cite{54} Lactate consumption was measured by HPLC at the end of the experiment.

**ASSOCIATED CONTENT**

**SUPPORTING INFORMATION**

Additional data showing the promoter calibrations, corrections and calibrations for diffuse reflectance, the predominance of MtrC and MtrA over endogenous cytochromes c, the
correlation between the cell pellet color and diffuse reflectance, the linearity of CcmE immunoblotting data, a detailed description of the strains used, and more detailed methods description. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

**Corresponding Author**
*Tel: 510-486-4299. Fax 510-495-2376. E-mail: caio-franklin@lbl.gov.*

**Author Contributions**

**Notes**
The authors declare no competing financial interest.

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**ABBREVIATIONS**

- IPTG, isopropyl β-D-1-thiogalactopyranoside; δ-ALA, δ-aminolevulinic acid; GFP, superfolding green fluorescent protein

**REFERENCES**