Extracellular Electron Transfer Enables Cellular Control of Cu(I)-Catalyzed Alkyne−Azide Cycloaddition

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ABSTRACT: Extracellular electron transfer (EET) is an anaerobic respiration process that couples carbon oxidation to the reduction of metal species. In the presence of a suitable metal catalyst, EET allows for cellular metabolism to control a variety of synthetic transformations. Here, we report the use of EET from the electroactive bacterium Shewanella oneidensis for metabolic and genetic control over Cu(I)-catalyzed alkyne−azide cycloaddition (CuAAC). CuAAC conversion under anaerobic and aerobic conditions was dependent on live, actively respiring S. oneidensis cells. The reaction progress and kinetics were manipulated by tailoring the central carbon metabolism. Similarly, EET-CuAAC activity was dependent on specific EET pathways that could be regulated via inducible expression of EET-relevant proteins: MtrC, MtrA, and CymA. EET-driven CuAAC exhibited modularity and robustness in the ligand and substrate scope. Furthermore, the living nature of this system could be exploited to perform multiple reaction cycles without regeneration, something inaccessible to traditional chemical reductants. Finally, S. oneidensis enabled bioorthogonal CuAAC membrane labeling on live mammalian cells without affecting cell viability, suggesting that S. oneidensis can act as a dynamically tunable biocatalyst in complex environments. In summary, our results demonstrate how EET can expand the reaction scope available to living systems by enabling cellular control of CuAAC.

INTRODUCTION

Biological catalysis provides several advantages over traditional chemical catalysis including milder operating conditions, self-regeneration, and the ability to optimize activity via genetic manipulation. Whole-cell biocatalysts can leverage additional dynamic control over such reactions by coupling activity to cellular growth and metabolism. However, reactions catalyzed by whole cells are typically limited to known metabolic transformations. While efforts to augment the substrate scope of enzymatic reactions via directed evolution have been highly successful, there is still an ongoing need to expand the synthetic capabilities of live cells.

Recently, we and others have connected cellular metabolism to exogenous synthetic reactions via hydrogen generation, the secretion of reactive cellular metabolites, and extracellular electron transfer (EET). Among these approaches, EET is particularly advantageous, because it provides a tunable protein bridge between central carbon metabolism and extracellular redox reactions, including those controlled via metal catalysts. Specifically, EET in the model electroactive bacterium Shewanella oneidensis (wild-type MR-1) is regulated through a set of well-defined heme-containing cytochromes in the Mtr-pathway (metal-reducing pathway). This pathway allows S. oneidensis to use oxidized metal ions, including organo-metallic catalysts, as terminal electron acceptors under anaerobic conditions. Indeed, bacterial reduction of metals including iron(III) and copper(II) by E. coli and S. oneidensis has previously been used to perform atom-transfer radical polymerization (ATRP) and transcriptional regulation of specific EET proteins in S. oneidensis has enabled

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dynamic control over metal reduction and resulting catalysis.24,25 Given these results, we hypothesized that additional synthetic reactions involving the Cu(II/I) redox couple could be metabolically controlled using EET from S. oneidensis.

Cu(I)-catalyzed alkyne−azide cycloaddition (CuAAC) is an example of bioorthogonal click chemistry that exhibits fast kinetics and high specificity in complex environments.26,27 The reaction involves a [2 + 3] cycloaddition between a terminal azide and terminal alkyne to create a product with a 1,4-disubstituted 1,2,3-triazole.28,29 CuAAC has been used for almost two decades in applications including drug design, delivery, and synthesis;30−33 polymer synthesis;34−36 tissue engineering;36−38 and bioorthogonal labeling.39−43 As a result, expanding whole-cell catalysis to include this ubiquitous chemical transformation could yield significant control over triazole formation in unique environments.

Finally, we demonstrate that EET-controlled CuAAC is effective in complex environments, such as a eukaryotic co-culture, without negatively impacting cell viability. Overall, our results highlight EET’s ability to enable non-enzymatic catalysis and place synthetic chemical reactions under living control.

■ RESULTS

Extracellular Electron Transfer from Live S. oneidensis Catalyzes Alkyne−Azide Cycloaddition via Cu(II) Reduction. To monitor CuAAC reaction progress, we utilized a fluorogenic azide, CalFluor 488, which undergoes a click-activated quenched-to-fluorescent shift46 (Figure 1a). This substrate allowed reaction progress to be monitored in real time with standard well plates. A typical EET-controlled CuAAC reaction consisted of CalFluor 488 and propargylated-PEG (alkyne-PEG4-acid) with Cu(II)Br₂ and a Tris-(benzyltriazolylmethyl)amine (THPTA) ligand in Shewanella basal medium (SBM). We initially compared anaerobic reaction conversion between wild-type S. oneidensis (inoculating OD₆₀₀ = 0.1) and a chemical reducing agent, sodium ascorbate (NaAsc, 200 μM). After 5 h, reactions containing NaAsc or S. oneidensis showed comparable conversion by fluorescence turn-on (Figure 1b). The presence of the desired triazole product was also confirmed with LC-MS (Figure S2), while no CalFluor 488 starting material was detected. Utilizing a standardized curve to relate fluorescence and conversion, we measured a reaction yield of 97 ± 16% for n = 18 microbial CuAAC reactions after 10 h. As expected, reactions lacking
NaAsc, MR-1, or catalyst did not show any detectable conversion. While Cu(II/I) exhibits anti-microbial activity above certain concentrations, complexation to various ligands has been shown to mitigate the cytotoxic effects in E. coli.47 Nevertheless, to account for the possibility of cell toxicity, we measured the cell viability of S. oneidensis under typical reaction conditions containing 50 μM of CuBr2 and 300 μM of THPTA. Colony counting confirmed that CuAAC reagents did not cause cell death. Additionally, when employing a lower inoculating density (OD600 = 0.01) in equivalent reaction conditions, cells grew to anaerobic saturation (Figure S3). Finally, both mechanically lysed and heat-killed cells failed to achieve appreciable conversion, suggesting that actively respiring whole cells are required for the reaction to proceed. (Figure S4).

Next, we monitored CuAAC kinetics in real time using fluorescence turn-on. Reactions containing wild-type S. oneidensis achieved comparable conversion to CuAAC using a chemical reductant over nearly identical time scales (Figure 1c). By comparison, wild-type Escherichia coli MG1655, which lacks specific EET proteins,48 drove conversion more slowly than NaAsc or S. oneidensis (Figure 1c), consistent with previous evidence that non-specific Cu reduction is possible but lacks comparable kinetics to EET.24,25 Together, these results confirm that S. oneidensis MR-1 remains viable under our reaction conditions and can drive the CuAAC reaction via Cu(II) reduction.

**S. oneidensis Catalyzes Aerobic CuAAC without Requiring Dedicated Oxygen Removal.** CuAAC often requires air-free conditions since Cu(I) is rapidly oxidized to Cu(II) in the presence of oxygen.39 Because S. oneidensis is a facultative anaerobe and preferentially respires on oxygen, we hypothesized that our system could tolerate oxygen exposure and still drive CuAAC. To examine this possibility, anaerobically prepared reactions with either NaAsc, S. oneidensis, or E. coli were periodically unsealed, manually aerated, and left shaking under ambient conditions. Plates were then resealed, and the reaction was allowed to proceed. The aeration was repeated four times (Figure 1d). As expected, the reducing power of NaAsc was depleted after the first aeration event. Similarly, E. coli, which exhibited significant background activity under anaerobic conditions, was hindered by the repeated Cu(I) oxidation and was unable to achieve substantial conversion. In contrast, S. oneidensis drove the reaction to completion despite repeated oxygen exposure, presumably consuming dissolved oxygen through aerobic respiration before resuming EET.25 Next, we challenged S. oneidensis to perform microbial CuAAC with an aerobic pre-growth and benchtop setup. Under these conditions, S. oneidensis achieved comparable conversion to anaerobic reactions (Figure 1e). Standard concentrations of NaAsc failed to achieve any significant conversion aerobically, and reactions with E. coli were again significantly arrested. In fact, greater than 500 μM of sodium ascorbate was required to achieve any notable aerobic conversion and greater than 1000 μM, a 20-times excess relative to Cu(II), was required to achieve comparable conversion to S. oneidensis (Figure S6). In contrast to chemical reductants, the lag time between cell inoculation and reaction turn-on could be modulated by changing the initial density of S. oneidensis (Figure S6). Together, these results indicate that S. oneidensis is unique in its ability to sustain CuAAC conversion under benchtop conditions by upregulating EET and Cu(II) reduction in response to oxygen depletion.

**Observed Rate of Copper Reduction Can Be Modeled with Simple Reactions.** To compare the kinetics of microbial CuAAC, we developed a simplified reaction model that accounts for oxygen consumption, copper reduction/oxidation, and cycloaddition. This model facilitated parameter estimation from time course kinetics by monitoring the concentration of the triazole product (eq S1, Figure 2a). We calculated the observed parameter fitting for rate constants for our standard S. oneidensis CuAAC system from the following simplified reactions (eqs S2–S5).

\[
\text{azide} + 2^*\text{Cu(I)} \rightarrow \text{triazole} + 2^*\text{Cu(I)} \quad (1)
\]
\[
\text{Cu(II)} + \text{cells} \rightarrow \text{Cu(I)} + \text{cells} \quad (2)
\]
\[
\text{Cu(I)} + \text{O}_2 \rightarrow \text{Cu(II)} \quad (3)
\]
\[
\text{O}_2 + \text{cells} \rightarrow \text{cells} \quad (4)
\]

After fixing all other observed reaction rate constants, we performed a parameter estimation on the triazole formation curves to determine the rate of copper reduction (kobs Cu Reduction μM\(^{-1}\) s\(^{-1}\)). In cases where cell growth was relevant to the parameter estimation, as occurs with a lower starting inoculum, the observed rate of cell growth was also fit using cell growth curves that were experimentally monitored through absorption at 600 nm. The agreement between the fit and the experimental data is outlined in Figure 2a. This simple model provides a convenient handle for quantifying and comparing kinetic rate differences between various reaction conditions and strains.
Carbon Metabolism Controls the Rate of S. oneidensis Catalyzed CuAAC. The requirement for viable cells and the relationship between inoculating density and reaction lag time in our aerobic reactions suggested that cellular respiration influences conversion and that manipulating carbon metabolism may exert control over the reaction. Anaerobic carbon metabolism in S. oneidensis is well-defined; it cannot utilize acetate as a carbon source under these conditions but generates four electron equivalents per molecule of lactate and two electron equivalents per molecule of pyruvate. 40,40 To test the effect of the carbon source on conversion, microbial CuAAC reactions were performed in reactions that contained either lactate, pyruvate, or acetate as the carbon source (Figure 2b). As expected, the cycloaddition reaction proceeded at the greatest rate when cells were grown on lactate \( (k_{\text{obs}} = 674 \pm 264 \, \mu\text{M}^{-1} \cdot \text{s}^{-1}) \). With starved or acetate-fed cells, the reaction was significantly attenuated. Consistent with our hypothesis, pyruvate-fed cells proceeded with a greater lag time and slower rate \( (k_{\text{obs}} = 22 \pm 3 \, \mu\text{M}^{-1} \cdot \text{s}^{-1}) \) compared to lactate (Figure 2c). These results indicate that live cell carbon metabolism is critical for both aerobic respiration and copper reduction through EET and that the CuAAC reaction rate can be directly controlled by the carbon source provided to S. oneidensis.

Specific Mtr-Pathway Proteins Control the Rate of S. oneidensis-Catalyzed CuAAC. In the absence of oxygen, S. oneidensis expresses the cytochromes in the CymA/Mtr-pathway (Figure 1a). 51 Electrons are transported via the cytoplasmic membrane protein, CymA, which reduces periplasmic proteins that provide electrons to the Mtr-pathway. Electrons are then shuttled through MtrA and onto MtrC, where they are deposited onto a terminal electron acceptor. 16,52 Given the importance of the Mtr-pathway in regulating EET, we tested a series of EET-deficient strains for their ability to perform aerobic CuAAC. As expected, the observed reaction rate significantly decreased upon the removal of mtrC (Figure 3a,b). Additionally, removal of mtrF, which encodes an MtrC homologue, further decreased the observed reaction rate from 402 \( \pm 66 \) to 193 \( \pm 33 \, \mu\text{M}^{-1} \cdot \text{s}^{-1} \). Finally, complete removal of the Mtr-pathway caused the reaction kinetics to closely resemble the background reduction observed in E. coli. Similar to wild-type S. oneidensis, the lag time of the knockouts was also dependent on inoculating density. Lag times for reactions involving E. coli did not show a similar dependence (Figure S7). Together, these results confirm that the rate of CuAAC is primarily controlled by EET via the Mtr-pathway.

Transcriptional Regulation of the Mtr-Pathway Enables Controllable CuAAC. Our results using EET-deficient knockouts suggested control of CuAAC rates and conversion could be achieved using genetic engineering. Thus, we aimed to control CuAAC activity via inducible transcription of EET genes. Specifically, we used a S. oneidensis \( \Delta \text{mtrC} \Delta \text{omcA} \Delta \text{mtrF} \) strain harboring mtrC on a plasmid under control of the \( \text{P}_{\text{lac}} \) promoter 23 (Figure 3c). The signaling molecule isopropyl \( \beta\)-D-1-thiogalactopyranoside, IPTG, activates mtrC gene expression, which we predicted would regulate CuAAC rate and conversion. All genetic constructs tested were grown overnight in the absence of IPTG and induced upon inoculation into CuAAC reaction mixtures (Figure 3d). At the same initial inoculum and in the presence of 1 mM IPTG, CuAAC reaction kinetics using a complemented mtrC knockout in a \( \Delta \text{mtrC} \Delta \text{omcA} \Delta \text{mtrF} \) strain closely resembled those of wild-type S. oneidensis. However, conversion kinetics resembled those of the parent-knockout strain in the absence of IPTG (Figures S10 and S11). Reactions using empty vector controls in both wild-type S. oneidensis and \( \Delta \text{mtrC} \Delta \text{omcA} \Delta \text{mtrF} \) closely agreed with their parent strains. (Figure S8).

We similarly regulated two other EET-relevant genes, mtrA and cymA, in their cognate knockouts and saw comparable differences in observed rate constants in response to IPTG (Figure 3e,f). For each of these inducible constructs, the observed rate of Cu reduction was rescued by the addition of IPTG and cymA for fully induced and uninduced constructs. Data show a mean \( \pm \) SD of \( n = 3 \) biological replicates. * * * * P < 0.0001.

Figure 3. Control over CuAAC using genetic engineering of the CymA/Mtr-pathway. (a) Aerobic kinetic curves performed with various S. oneidensis knockouts (OD\text{600} = 0.1) and an E. coli MG1655 negative control. (b) Quantification of the observed rate of copper reduction for varying S. oneidensis knockouts or an E. coli negative control. (c) Diagram of a generic buffer gate circuit used to control expression of the gene of interest (mtrC, mtrA, or cymA) in S. oneidensis knockout strains. 18 Quantification of the observed rate of copper reduction for raw kinetic curves inoculated at an OD\text{600} = 0.025 (5 \times 10^7 \text{ CFU/mL}) with (d) JG596 + mtrC, (e) \Delta \text{mtrA} + mtrA, and (f) \Delta \text{cymA} + cymA for fully induced and uninduced constructs. Data show a mean \( \pm \) SD of \( n = 3 \) biological replicates. * * * * P < 0.0001.
Figure 4. The effect of the copper ligand on S. oneidensis catalyzed CuAAC and the accessible substrate scope. (a) Chemical structures for THPTA, BTTAA, and TPMA. (b) Aerobic kinetic curves for CuAAC between 0.6 μM CalFluor 488 and 100 μM alkyne-PEG-alkyne partners in a 3T3 murine fibroblast MR-1 and sodium ascorbate (NaAsc, 1 mM).

To demonstrate this, we first conducted a CuAAC reaction during which S. oneidensis adhered to the bottom of a Nunc-coated 96-well plate (Figure 5b). After completion of the reaction, the product-containing supernatant was decanted and replaced with fresh starting material, leaving the adhered cells intact. Each cycle of the system yielded consistent conversion (Figures 5c, S13), and cycles III and IV reflect remarkably similar reaction rates to the initial reaction. The increase in observed rate for cycle II likely reflects an increase in cell inoculum from a partial transition to aerobic respiration during oxygen exposure and corresponding growth. In subsequent cycles, the cells appear to perform the reaction at the same rate as the initial turnover. To further demonstrate the robustness of the system and highlight the capability to tune reaction kinetics in situ, the carbon source provided to S. oneidensis was changed between cycles, alternating between pyruvate and lactate. Cycles with S. oneidensis grown on pyruvate maintained microbial viability but did not display significant conversion over the course of the 11 h cycle. When the reaction material was replaced with a solution containing lactate, the CuAAC conversion increased (Figures 5d, S13). This OFF/ON cycling highlights how S. oneidensis can dynamically control CuAAC by sensing and reacting to changes in reaction conditions.

S. oneidensis Enables CuAAC in Mammalian Coculture. CuAAC is notable for its orthogonality in complex biological settings, and bacteria are well-suited for long-term or responsive applications in these environments. However, for applications involving eukaryotic organisms, it is critical that S. oneidensis controls CuAAC without affecting eukaryotic viability. To assess this, we first performed CuAAC with our standard azide and alkyne partners in a 3T3 murine fibroblast and S. oneidensis coculture. Under our conditions, S. oneidensis...
was as effective at performing CuAAC as 1 mM of chemical reductant NaAsc (Figure 6a). After 90 min, neither the CuAAC reactants nor the bacteria negatively impacted viability (Figure 6b). A microscopy time-series revealed that bacteria remained distributed and motile in co-culture (Supplementary Video 1). Together, these results indicate that \textit{S. oneidensis} can exert dynamic control over CuAAC in mammalian co-culture without diminishing viability.

Next, \textit{S. oneidensis} was used to label the membranes of fibroblast cells. Fibroblast cells were NHS-ester functionalized with alkyne-PEG4. Following esterification, cells were washed, and CalFluor 488 probe, Cu(II), and THPTA were added along with \textit{S. oneidensis} to the reaction vessel. After completion of the reaction, fibroblast cells were washed and imaged using epi-fluorescence microscopy. Increased fluorescence intensity along the cell membranes confirmed formation of the triazole product on mammalian cell surfaces (Figure 6c–e). In a bacteria-free control, no notable fluorescence could be detected, indicating that the reaction was dependent on the presence of \textit{S. oneidensis} (Figure 6f–h). In a subsequent experiment to demonstrate the modularity of the system, fibroblast cells were functionalized with 6-azidohexanoic acid (a terminal azide), and the CuAAC reaction was successfully performed with a carboxyrhodamine 110 terminal alkyne probe (Figure S14). Together, these results suggest that \textit{S. oneidensis} CuAAC is compatible with mammalian cells and can potentially be applied in traditional CuAAC settings including biorthogonal labeling,56,57 -omics,38,58,59 and tissue engineering.60,61

### DISCUSSION

Overall, we successfully developed a whole-cell microbial redox biocatalyst for small-molecule CuAAC click reactions. Employing EET for non-enzymatic conversion significantly expands the substrate scope available to bacteria and facilitates genetic and metabolic control over this important chemical transformation. We first leveraged \textit{S. oneidensis} as a dynamic actuator for controlling an aerobic and aerobic CuAAC reactions. Our results highlight how the central metabolism of \textit{S. oneidensis} can be manipulated to control reaction lag time, kinetics, and conversion. Similarly, after repeated oxygen exposures, we showed that our system was less susceptible to oxygen challenges compared to traditional chemical reductants. Furthermore, transcriptionally regulating \textit{mtrC} and other EET genes increased kinetic control and conversion. Thus, changes to central metabolism could be used in tandem with genetic engineering techniques to modulate the reaction kinetics over several orders of magnitude (Figures S7 and S9).

Consistent with previous observations in synthetic systems, the reaction kinetics was strongly dependent on the copper-stabilizing ligand identity.55 Significant conversion was not observed in the absence of a ligand or in the presence of an incompatible ligand (Figure 4a–c). The ligand for Cu(I) influences the midpoint potential, stability, and reactivity of the
metal center (Figure S15). As a result, the ligand influences the interaction between both EET machinery and Cu(II/1) and the alkyne and Cu(I). We previously showed that Cu:TPMA-catalyzed ATRP in the presence of S. oneidensis, indicating that it is reduced by the bacteria.23−25 Previous studies have reported TPMA as a poor ligand for CuAAC, which suggests the lack of reactivity observed in our system is not due to a lack of reduction.55 In contrast, both Cu:THPTA and Cu:BTAA have midpoint potentials well within the range of MtrC (−610 to −110 mV vs Ag/AgCl)62 and have been previously optimized for CuAAC activity. Cu:BTAA has higher reported CuAAC activity than Cu:THPTA, again consistent with our results and with our chemical controls utilizing NaAsc (Figure S12). How metal−ligand complexes interact with MtrC is unknown, but future protein engineering efforts aimed at affecting catalyst docking may be a promising strategy for enhancing metal reduction and CuAAC activity.

Furthermore, in negative controls involving the ΔMtr-pathway knockout or E. coli, we did not observe a complete arrest of CuAAC conversion. It is likely that Cu(II) reduction is tied to other electron transport or reduction pathways such as extracellular flavins,8 glutathione,14,44,64 and copper nanoparticles.65 Despite the presence of some background reduction, our results indicate that conversion is primarily controlled by the Mtr-pathway. Nevertheless, a key focus of future work will be fine-tuning dynamic range and increasing genetic control over the reaction while mitigating background reduction. Potential solutions are to limit reduction of extracellular Cu solely to the Mtr-pathway and could include expression of the Mtr-pathway in non-native host organisms,66 creation of flavin exporter knockouts,63 or a decrease of glutathione production.44 Finally, mutagenesis of MtrC may allow the protein to simultaneously reduce and ligate Cu, which results in a lower background as we observed negligible conversion in the absence of ligand.

We demonstrated substrate robustness of S. oneidensis CuAAC by successfully reacting an alkyne-functionalized sugar, nucleic acid, protein, and several small molecules. In all cases conversion was comparable to treatment with NaAsc. However, our system is limited by a requirement to maintain cell viability, especially under aerobic conditions. We measured significant triazole formation at two times of our standard alkyne (200 μM) and azide concentrations (1.2 μM) but failed to achieve conversion at higher alkyne and azide concentrations (>300 μM and >1.8 μM respectively). These results indicate that there are likely cytotoxic effects due to either the cosolvents or the CalFluor488 and alkyne-PEG2-acid (Figure S6). However, we note that significantly higher concentrations of substrates such as 4-arm-PEG polymers (3.3 mM) can successfully undergo CuAAC when water solubility and cell viability are maintained.67 In contrast to traditional chemical reductants, adherent S. oneidensis performed aerobic CuAAC over multiple cycles. In these experiments, the bacteria did not require regeneration, replenishment, or intervention even after several repeated oxygen exposures. In contrast to traditional chemical or biological reductants (e.g., NaAsc or NADH), CuAAC activity could be dynamically tuned by interchanging simple carbon sources. Changes to the central carbon metabolism of S. oneidensis, such as engineered glucose catabolism, could be further utilized to tune CuAAC kinetics.68 Together, our results demonstrate that S. oneidensis is comparable to traditional CuAAC reductants but with the added benefit of dynamic metabolic and genetic regulation.

Finally, in complex environments such as mammalian cell culture, S. oneidensis enabled CuAAC without impacting cell viability. While more investigations are needed to determine the full effect of S. oneidensis on mammalian cells, our
successful coculture experiments lay the foundation for applying our system toward traditional CuAAC applications such as drug delivery,59 cell encapsulation,56 tissue scaffolding,56 cell labeling,64,65 noncanonical amino acid incorporation,70 and more.58 Excitingly, several of these applications could benefit from the genetic, metabolic, and temporal control available to our system. Because CuAAC activity is primarily controlled through MrC, directed evolution strategies targeted at this protein may be leveraged to enhance reactivity. Finally, CuAAC provides a novel fluorescent output for EET, potentially allowing for high-throughput screening of EET-active microbes, optical sensing of metabolites or environmental signals, and characterization of genetic constructs aimed at controlling EET flux. In summary, our results demonstrate how EET combines the advantages of biological control and synthetic catalysis to expand the chemical reaction space available to microbes.

## MATERIALS

CalFlour 488 (Click Chemistry Tools), alkyne-PEG₉-acid (Click Chemistry Tools), copper(II) bromide (CuBr₂, Sigma-Aldrich, 99%), tris(2-pyridylmethyl)amine (TPMA, Sigma-Aldrich, 98%), Alkyne Tris(benzyltriazolylmethyl)amine (THPTA, Sigma-Aldrich, 95%), 2-(4-(bis((1-((tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid (BTCAA, Click Chemistry Tools >95%), Click-IT Fucose Alkyne (Invitrogen), 6-azidohexanoic acid (Click Chemistry Tools >95%), 1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid (THPTA, Sigma-Aldrich, 95%), Click-IT Fucose Alkyne (Invitrogen), 6-azidohexanoic acid ester (Click Chemistry Tools, >95%), Alkyne-PEG₂-NHS Ester (Click Chemistry Tools >95%), bovine serum albumin (Sigma-Aldrich, >96%), Alkyne Tris(benzyltriazolylmethyl)amine (THPTA, Sigma-Aldrich, 95%), K₂HPO₄ (Sigma-Aldrich), (NH₄)₂SO₄ (Fisher Scientific), 1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid (BTCAA, Click Chemistry Tools >95%), Alkyne Tris(benzyltriazolylmethyl)amine (THPTA, Sigma-Aldrich, 95%), tris(2-pyridylmethyl)amine (TPMA, Sigma-Aldrich, 98%), 1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid (BTCAA, Click Chemistry Tools >95%), Alkyne Tris(benzyltriazolylmethyl)amine (THPTA, Sigma-Aldrich, 95%), 2-(4-(bis((1-tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid (BTCAA, Click Chemistry Tools >95%), Alkyne Tris(benzyltriazolylmethyl)amine (THPTA, Sigma-Aldrich, 95%), Alkyne-PEG₂-NHS Ester (Click Chemistry Tools >95%), bovine serum albumin (Sigma-Aldrich, >96%), Alkyne Tris(benzyltriazolylmethyl)amine (THPTA, Sigma-Aldrich, 95%), 38°C, 70°C, and more.38 Excitingly, several of these applications could benefit from the genetic, metabolic, and temporal control available to our system. Because CuAAC activity is primarily controlled through MtrC, directed evolution strategies targeted at this protein may be leveraged to enhance reactivity. Finally, CuAAC provides a novel fluorescent output for EET, potentially allowing for high-throughput screening of EET-active microbes, optical sensing of metabolites or environmental signals, and characterization of genetic constructs aimed at controlling EET flux. In summary, our results demonstrate how EET combines the advantages of biological control and synthetic catalysis to expand the chemical reaction space available to microbes.

## METHODS

### Analysis and Measurement.

Fluorescence emission was collected on a BMG LABTECH CLARIOstar plate reader with a 491 (±14) nm and an emission collection at 538 (±38) nm. After the addition of all plate components, the 96-well plate was sealed with a sterile and optically transparent sealing film (PCR-SP-S, AxySeal Scientific) and covered with a polystyrene plate lid (Eppendorf) lined with silicone grease and sealed with Teflon tape. The plate reader was held at 30 °C and collected emissions every 90 s for 10 to 24 h.

**Microscopy.** All microscopy was performed using a Nikon Ti2 Eclipse inverted epifluorescence microscope. Fluorescence was measured using a GFP excitation/emission filter cube on the Nikon Ti2.

**Bacteria Strains and Culture.** Bacterial strains and plasmids are listed in Table S1. Cultures were prepared from bacterial stocks stored in 20% glycerol at −80 °C streaked onto LB agar plates (for wild-type and knockout strains) and grown overnight at 30 °C for *Shewanella* and 37 °C for *E. coli*. Overnight cultures were grown by picking single colonies and innoculating into *Shewanella* basal medium (SBM) (Table S2) supplemented with 0.05% w/v trace mineral supplement, 0.05% w/v casamino acids, and 20 mM sodium lactate (2.85 μL of 60% w/w sodium lactate per 1 mL culture) as the electron donor. Aerobic cultures were grown in 15 mL culture tubes at 30 °C and 250 rpm shaking. Anaerobic cultures were grown using the same procedure but in argon sparged growth medium supplemented with 40 mM sodium fumarate as the electron acceptor in a Coy Anaerobic Glovebox containing a humidified atmosphere at 3% hydrogen content and the balance nitrogen. Plasmid-harboring strains were grown with the addition of 25 μg/mL of kanamycin diluted from a 1000x stock in water. Cultures were washed 3X after overnight growth using SBM supplemented with 0.05% casamino acids (degassed for anaerobic cultures).23 OD₆₀₀ was measured using a NanoDrop 2000C spectrophotometer and normalized to an OD₆₀₀ of 0.75 before dilution into reaction mixture. All CuAAC reactions used 26.7 μL of OD₆₀₀ = 0.75 concentrated cell culture into 173.3 μL of reaction mixture to give a final OD₆₀₀ = 0.1, ca. (1.8 ± 0.5) × 10⁸ CFU·mL⁻¹, unless otherwise noted.

**Standard Microbial CuAAC.** All reactions were performed in *Shewanella* basal media (SBM) supplemented with 0.05% w/v casamino acids with lactate (20 mM) as a carbon source and fumarate (20 mM) as the primary electron acceptor. Stock solutions of 1 M sodium fumarate and 60 w/v% lactate solutions were stored at 4 °C until use. Aliquots of 1.2 mM of CalFlour 488 were created in DMSO and stored frozen at −80 °C until use. Aliquots of 4 mM alkyne-PEG₂-acid were created in DMSO and stored at −20 °C until use. An 8 mM copper bromide stock in DMF was created and stored at 4 °C and mixed with an equal volume amount of 48 mM freshly made stock of THPTA in sterile water. In alternative copper ligand studies, a 48 mM solution of the ligand in water or DMF was mixed with copper bromide. In order, the following was added to yield a 200 μL reaction in either degassed or ambient SBM supplemented with 0.05% casamino acids with the final concentrations: lactate (20 mM) (or alternative carbon source), fumarate (20 mM), Cu:THPTA 1:6 (50 μM: 300 μM),71 alkyne-PEG₂-acid (100 μM), CalFlour 488 (0.6 μM), and finally *S. oneidensis* (OD₆₀₀ of 0.1) or freshly dissolved NaAsc in water (200 μM). The reaction was then placed into the plate reader for analysis and allowed to react for between 10 and 24 h.

**Microbial CuAAC Controls.** Heat-killed controls were obtained by incubating bacterial cultures (postwash) at an OD₆₀₀ of 0.75 at a temperature of 80 °C for 15 min.24 Upon completion, the cells solution was vortexed to ensure complete mixing, and diluted (26.7 μL into 173.3 μL) into the reaction mixture. Mechanical lysed cells were obtained via sonication with a Branson Model 250 sonicator with a Model 102C Converter. Cell suspensions at an OD₆₀₀ of 0.75 were placed on ice and sonicated at 30% strength for 2.5 min with cycles of 10 and 5 s between cycles. This process was repeated 3X. Upon completion, the transparent solution was vortexed to ensure complete mixing, and diluted (26.7 μL into 173.3 μL) into the reaction mixture.
Oxygen Exposure CuAAC. A standard anaerobic microbial CuAAC reaction was begun utilizing either NaAsc (200 μM), S. oneidensis (OD<sub>600</sub> = 0.1) or E. coli (OD<sub>600</sub> = 0.1). The reaction was sealed and allowed to progress while monitoring the fluorescent output for 6 min before removing the lid and aerating by bubbling 10 mL of ambient air over a 30 s period into each 200 μL reaction. The reactions were then shaken for 20 min, without a lid, and resealed to begin collecting fluorescence for 2 h. This was repeated four times.

Cycling Experiments CuAAC. A standard aerobic microbial CuAAC reaction was begun in a Nunclon Delta-Treated 96-well plate (Thermo Scientific 167008). After sealing and allowing to react for 11 h, the supernatant was removed carefully, as to not disturb the layer of cells on the bottom of the well. Starting materials in fresh SBM (200 μL) was gently added back into the well, the plate was sealed and again allowed to react for 11 h. This was repeated three times for a total of four reactions.

Mammalian Cell Culture. 3T3 fibroblasts (American Type Culture Collection, gifted from the Rosales Lab at UT Austin) were cultured in T75 flasks between 9 and 13 passages in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin. Cells were cultured in a humidified incubator at 37 °C with 5% CO₂. At ∼ 80% confluence, the cells were washed with PBS buffer, cleaved with trypsin solution (0.25% trypsin containing 0.02% ethylenediaminetetraacetic acid in PBS), centrifuged, and seeded at a new confluence of 20%.

MTT Assay. 3T3 cells were used for assays after the ninth passage and before the 13th passage. The cells were plated at 4 × 10<sup>4</sup> cells/well in a Nunclon Delta-Treated 96-well plate (Thermo Scientific 167008). The cells were allowed to adhere overnight, the supernatant was aspirated, and the cells were washed with 1X PBS. Then, 173.3 μL of the appropriate reaction mixture or control was added to the wells and the reaction was initiated through the addition of 26.7 μL of S. oneidensis (OD<sub>600</sub> = 0.75) grown from an anaerobic overnight or appropriate media blank. Control wells contained only 200 μL of SBM + 0.05% w/v casamino acids. A DMEM control was also run to ensure there was no detriment to viability resulting from SBM. The plate was then sealed as described previously and after a 1.5 h incubation, the plates were removed from the plate reader and imaged for 30 min. At this time the supernatant was aspirated and then, 100 μL of DMEM and 10 μL of CyQUANT MTT Cell Viability Assay in 1X PBS (Thermo Scientific V13154) was added. After a 4-h incubation with the MTT the supernatant was aspirated, and the wells were resuspended in 100 μL of dimethyl sulfoxide (DMSO). Absorption measurements were collected at 490 nm using the plate reader.

Cell Surface Functionalization. 3T3 cells used for assays after the ninth passage and before the 13th passage. The cells were plated at 2 × 10<sup>4</sup> cells/well in a Nunclon Delta-Treated 96-well plate (Thermo Scientific 167008). The cells were allowed to adhere overnight, the supernatant was aspirated, and the cells were washed with 1X PBS (pH 7.4) to remove any proteins from the culture media. A 100 μM solution of the desired NHS-Ester in PBS (pH 7.4) was created fresh from a 4 mM stock of the NHS-Ester in DMSO. The solution was added (200 μL) to each well and allowed to react at room temperature for 30 min. The supernatant was then removed, and each well washed with PBS (pH 7.4). To each reaction vessel a solution of: corresponding probe (0.6 μM), lactate (20 mM), fumarate (20 μM), Cu:THPTA 1:6 (50 μM: 300 μM), finally S. oneidensis (OD<sub>600</sub> of 0.1) in SBM supplemented with 0.05% w/v casamino acids was added. The reactions were allowed to incubate for 3 h at 30 °C and washed with PBS (pH 7.4) before being taken to the Nikon T2 Eclipse inverted epifluorescence microscope. Images were taken using a 1 s exposure time using a GFP channel.

Observed Rate of Cu Reduction. Utilizing COmplex PAthway Simulator (COPASI) each of the proposed reactions (Reactions 1−4) was input into the biological model. All fluorescence measurements were converted to CalFluor 488 triazole concentration using the calibration curve outlined in Figure S1. Fitting Reaction 1 first with kinetic data collected from an anaerobic CuAAC using sodium ascorbate, and under the assumption that the triazole formation is the limiting step, the k<sub>1 obs</sub> was obtained through parameter estimation and was fixed. Next, k<sub>2 obs</sub> was obtained by fitting Reaction 2 with kinetic data collected from an anaerobic CuAAC using S. oneidensis (Figure S5) and was fixed. Finally, the observed rate constants for Reaction 3 and 4 were fit simultaneously using parameter estimation and kinetic data from aerobic CuAAC using S. oneidensis. The corresponding observed rate constants for each reaction were fixed and are outlined in Table S4. Each k<sub>obs</sub> (Cu reduction) was obtained by unfixing k<sub>2 obs</sub> and performing a parameter estimation with the fixed rate constants. As this crude model aims to only quantify the observed rate kinetics, it is assumed that only the rate of Cu reduction is changed when changes are made to EET (knockouts, various carbon sources, complementation, etc.).

Statistical Analysis. Unless otherwise noted, data are reported as mean ± SD of n = 3 biological replicates. Significance was calculated in GraphPad Prism 9.0 using a two-tailed unpaired student t test or a one-way ANOVA.

Safety. No unexpected or unusually high safety hazards were encountered.

ASSOCIATED CONTENT

 Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.1c01208.

Table S1. Strains, plasmids, and DNA used in this study.
Table S2. Ingredients in Shewanella Basal Media.
Equations: Fluorescence calibration curve and observed rate constant equations. Table S3. Observed rate constants (k<sub>obs</sub>) as determined by COPASI. Modeling of Inducible Constructions. Table S4. Hill function constants. Figure S1. Calibration curve for CalFluor 488 triazole product. Figure S2. LC-MS results for CalFluor 488 triazole product. Figure S3. Colony counts for CuAAC reaction toxicity. Figure S4. Heat-killed and mechanically lysed cellular control kinetic curves. Figure S5. Anaerobic variable reductant kinetic curves. Figure S6. Aerobic variable reductant and variable copper kinetic curves. Figure S7. Aerobic reaction kinetic curves with various inoculating densities and strains. Figure S8. Empty vector control kinetic curve. Figure S9. Variable carbon source kinetic curves for ΔmtrCΔomcAΔmtrF and E. coli. Figure S10. Inducible mtrC kinetic curves. Figure S11. Hill functions and kinetic curves for mtrC, mtrA, and cymA, inducible buffer gates. Figure S12. Variable copper ligand sodium ascorbate positive control kinetic curves. Figure S13. CuAAC cycling kinetic.
Electrochemical measurements were performed with the generous and kind help of Dr. Yang Gao. Elements of Figures 1, 4, and 5 were created with BioRender.com.

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