Enabling Unbalanced Fermentations by Using Engineered Electrode-Interfaced Bacteria

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ABSTRACT

Cellular metabolism is a series of tightly linked oxidations and reductions that must be balanced. Recycling of intracellular electron carriers during fermentation often requires substrate conversion to undesired products, while respiration demands constant addition of electron acceptors. The use of electrode-based electron acceptors to balance biotransformations may overcome these constraints. To test this hypothesis, the metal-reducing bacterium Shewanella oneidensis was engineered to stoichiometrically convert glycerol into ethanol, a biotransformation that will not occur unless two electrons are removed via an external reaction, such as electrode reduction. Multiple modules were combined into a single plasmid to alter S. oneidensis metabolism: a glycerol module, consisting of glpF, glpK, glpD, and tpiA from Escherichia coli, and an ethanol module containing pdc and adh from Zymomonas mobilis. A further increase in product yields was accomplished through knockout of pta, encoding phosphate acetyltransferase, shifting flux toward ethanol and away from acetate production. In this first-generation demonstration, conversion of glycerol to ethanol required the presence of an electrode to balance the reaction, and electrode-linked rates were on par with volumetric conversion rates observed in engineered E. coli. Linking microbial biocatalysis to current production can eliminate redox constraints by shifting other unbalanced reactions to yield pure products and serve as a new platform for next-generation bioproduction strategies.

IMPORTANCE

All reactions catalyzed by whole cells or enzymes must achieve redox balance. In rare cases, conversion can be achieved via perfectly balanced fermentations, allowing all electron equivalents to be recovered in a single product. In most biotransformations, organisms must produce a mixture of acids, gasses, and/or alcohols, and no amount of enzyme or strain engineering can overcome this fundamental requirement. Stoichiometric conversion of glycerol, a waste product from biodiesel transesterification, into ethanol and CO2 with no side products represents such an impossible fermentation, due to the more reduced state of glycerol than of ethanol and CO2. The unbalanced conversion of glycerol to ethanol has been viewed as having only two solutions: fermenting glycerol to ethanol and potentially useful coproducts or “burning off” excess electrons via careful introduction of oxygen. Here, we use the glycerol-to-ethanol example to demonstrate a third strategy, using bacteria directly interfaced to electrodes.

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The need to efficiently convert feedstocks into products or fuels, combined with the development of tools for metabolic engineering, has produced many novel strains able to catalyze useful fermentations. However, the need to achieve redox balance limits the range of possible bioconversions, and the synthesis of mixtures of end products increases bioseparation costs. One possible solution to this issue is to harness the ability of bacteria able to transfer electrons to electrodes. Some microorganisms have previously been shown to utilize exogenous redox mediators (e.g., thionine, neutral red, or ferricyanide) to balance redox reactions (1–4). The recent discovery that certain bacteria can natively transfer electrons directly to electrodes (5, 6), a process called extracellular electron transfer, has enabled a strategy for balancing bioconversions. Extracellular electron transfer allows bacteria to utilize an electrode as an external sink for electrons, eliminating the necessity for the production of undesired side products, thereby facilitating stoichiometric conversion of substrate to product while simultaneously generating electrical current.

To demonstrate the principle of such an electrobiocatalyst, we sought to achieve production of ethanol (and CO2) from glycerol without the need to excrete redox-balancing end products, such as formate or 1,2 propanediol, using the facultative anaerobe Shewanella oneidensis. S. oneidensis possesses the ability to respire electrons to insoluble substrates, such as electrodes (7, 8). With a well-studied, diverse metabolism and a sequenced genome (9) amenable to genetic manipulations, S. oneidensis is well suited to demonstrate a concept applicable to a variety of environmentally diverse electrode-respiring organisms (5, 6).

Unlike common industrial hosts, such as Escherichia coli, S. oneidensis is naturally equipped for electron transfer to elec-
and while our work focuses on solving this specific unbalanced redox reaction, in principle, our strategy can be broadly applied to any reaction where the substrate is more reduced than the desired product(s).

**RESULTS**

Engineering of *S. oneidensis* for glycerol uptake and utilization. No *Shewanella* isolates tested to date have been shown to utilize glycerol as a sole carbon and energy source (17), and growth experiments confirmed this inability in *S. oneidensis* strain MR-1. To create a respiratory pathway linked to quinone reduction, unlike fermentative pathways developed for *E. coli* (18), three genes were predicted to be required: *glpF*, *glpK*, and *glpD*, which encode a glycerol facilitator, a glycerol kinase, and a membrane-bound quinone-linked glycerol-3-phosphate dehydrogenase, respectively (19). When these genes were introduced under the control of a lac promoter, glycerol kinase and glycerol dehydrogenase activities were detected in whole-cell lysates, but no growth or utilization of glycerol was observed by whole cells under any condition tested (data not shown). Only after introduction of *tpiA*, which encodes a triosephosphate isomerase responsible for the isomerization of dihydroxyacetone phosphate (DHAP) and 3-phosphoglycerate, was glycerol consumption and cell growth observed. A requirement for increased TpiA was consistent with the known competitive inhibition of GlpD by DHAP (20) and the use of Entner-Doudoroff glycolysis by *S. oneidensis* (21), which requires only low TpiA activity for gluconeogenic flux during utilization of its preferred substrate (lactate) as a carbon source.

Introduction of these four constitutively expressed genes from *E. coli* (Fig. 1, glycerol module) enabled *S. oneidensis* to utilize glycerol as a sole carbon and energy source. It is interesting to note that GlpD encodes a flavoenzyme characterized as the aerobic dehydrogenase of *E. coli* (22), while another enzyme complex (encoded by *glpABC*) is used for the anaerobic utilization of glycerol by *E. coli*. *S. oneidensis* was able to utilize glycerol both aerobically and anaerobically with the GlpD dehydrogenase, suggesting that it is compatible with the menaquinone-dependent transfer of electrons via CymA to electrodes in *S. oneidensis* (11). Moreover, addition of antibiotics was not necessary to maintain the plasmid in cultures growing with glycerol due to the selective pressure presented by the carbon source.

**Engineering of *S. oneidensis* for ethanol production.** Ethanol production has yet to be observed in *S. oneidensis*, despite the presence of numerous putative, annotated alcohol dehydrogenases in its genome. Attempts to increase pyruvate levels in *S. oneidensis* via deletion of acetate-producing pathways, as well as expression of typical acetyl coenzyme A (acetyl-CoA)-dependent alcohol dehydrogenases from *E. coli*, proved unsuccessful. Even in organisms with functional alcohol dehydrogenases, such as *E. coli*, it is well established that expression of two genes from *Zymomonas mobilis* (*pdc*, encoding pyruvate decarboxylase, and *adhB*, encoding alcohol dehydrogenase) can significantly increase ethanol production rates (23, 24). The effectiveness of this route has been attributed to the high affinity of Pdc for pyruvate (25, 26). When *pdc* and *adhB* were cloned and expressed constitutively in *S. oneidensis* (Fig. 1, ethanol module), pyruvate decarboxylase and alcohol dehydrogenase activities were observed in crude extracts, and ethanol production from lactate was observed in cell suspensions (data not shown).
The soluble electron acceptor fumarate were used to evaluate met-
ment for an external electron sink, batch cultures with or without
anaerobic glycerol conversion to ethanol and examine its require-
able conversion of glycerol to ethanol only when a mechanism was
directly downstream of $\text{adh}$. To demonstrate
able conversion of glycerol to ethanol only when a mechanism was
able conversion of glycerol to ethanol only when a mechanism was
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anaerobic growth with glycerol yielded an adapted isolate of
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held at an oxidizing potential (Fig. 3B and C). Conversion of glycerol under these conditions was absolutely dependent upon the oxidizing electrode, demonstrating that this bioconversion was enabled by the flux of electrons to the electrode. Moreover, when additional glycerol was added or when the medium was removed and replaced with fresh glycerol-containing medium, current production and glycerol consumption immediately resumed, showing that cells remained attached to the electrode and that continuous operation was possible (data not shown).

As observed in incubations with fumarate, only ethanol and a small amount of acetate accumulated during glycerol conversion; no succinate, malate, lactate, formate, or other end products were detected for the wild type (Fig. 3B) or the Δpta strain (Fig. 3C) containing pGUT2PET. Mutants lacking pta exhibited a 46% decrease in acetate production compared to wild-type cultures when respiring to electrodes (Fig. 3C; Table 1). Continuous sparging with gas did not enhance conversion, nor was it essential for conversion of glycerol to ethanol in electrochemical reactors (data not shown). To improve analytical measurements of coulombic yield by removing a potential electron donor (H2), a low rate (1 ml/ min) of gas sparging was employed in subsequent experiments. As this flushing also stripped some ethanol from the medium, volatile products were captured in a dry ice trap to confirm that ethanol was the only end product of glycerol bioconversion, as had been observed in our other experiments. In a subsequent series of experiments with the Δpta strain, we used water-jacketed reflux and/or dry ice traps on top of flushed reactors and observed 78% ± 5.6% ethanol and 20% ± 9.4% acetate production from glycerol (total carbon balance, 98% ± 3.6%; n = 3).

To further confirm that growth in electrochemical reactors led to stoichiometric glycerol-to-ethanol conversion and to eliminate the possibility that glycerol was being completely oxidized to CO2 or other unknown compounds, a comparison of predicted and actual electron recoveries of the engineered glycerol-to-ethanol pathway was performed. Electrochemical measurements record every electron equivalent excreted, allowing current output to be integrated over time (Fig. 4A and B) and compared with changes in glycerol and acetate levels over the same period. Electron amounts recorded agreed with the pathway in Fig. 1, where glycerol conversion to ethanol releases 2 electrons and acetate production releases 6 electrons. Comparisons between predicted and measured charge transfer values in every experiment deviated no more than 10% (Fig. 4C), and the Δpta strain with pGUT2PET behaved similarly (Fig. 4D). As only endpoint measurements were possible via dry ice trapping of gas-phase metabolites, ethanol production values at intermediate time points shown in Fig. 3B and C are based on levels calculated from nonvolatile metabolite measurements and coulombic recovery. Taken together, the carbon and electron recovery data showed that the outcomes of the engineered glycerol-to-ethanol pathway were identical when fumarate or an electrode was used as the electron acceptor.

**DISCUSSION**

The field of biocatalysis relies heavily on certain yeasts (Saccharomyces spp., Pichia spp., and Candida spp.) and bacteria (E. coli and Zymomonas spp.) to convert feedstocks into fuels (32). E. coli has been touted as an efficient and easily modifiable biocatalyst, with ethanol titers reaching 40 g/liter (33) and with volumetric productivities of strains engineered to ferment glycerol to ethanol, with H2 as a coproduct, as high as 4.7 mmol/liter/h (31). With most
Microbe-electrode systems, the fact that the key reaction occurs at a surface makes electrode surface area a crucial factor. These experiments with our first engineered strains of *Shewanella* in non-optimized reactors contained only 3 cm² electrode/ml, yet they already approached volumetric production rates of 1 mmol ethanol/liter/h. Recent work has shown that high-surface-area electrodes, such as treated carbon brush electrodes, can easily achieve surface/volume ratios on the order of 30 to 70 cm²/ml (34) and dramatically increase rates of current collection from *Shewanella*.

Electrode-linked microbial catalysis has many potential benefits; it can act as an electrochemical “lever” to drive an unfavorable reaction, allow generation of electricity via operation of a microbial fuel cell, or serve as reducing equivalents for additional products. In the case of glycerol, the shift from aerobic respiration (1, 2, 46) to anaerobic fermentation, with formate or H₂ as an end product (18, 37), represents a 3.5-fold decrease in the ΔG available to the cell. This low energy yield explains why anaerobic strategies are greatly enhanced by stripping of inhibitory by-products, such as H₂. In contrast to what occurs during fermentation, use of an oxidizing electrode can accelerate metabolism, as it directly increases the thermodynamic driving force and avoids oxidative losses (and costs) associated with oxygen (36). In a microbial fuel cell-like reactor, passing these captured electrons to oxygen offers the possibility for additional energy recovery of ~150 to ~80 kJ/mol glycerol, depending on the set potential of the anode.

The challenges facing large-scale bioelectrochemical production of electricity, fuels, and chemicals have been summarized in recent reviews (38, 39). In general, the low cost of electricity and the low volumetric rates of electricity generated by natural bacterial communities present significant barriers to adoption of microbial fuel cells. However, when synthesis of higher-value fuels or chemicals is the goal, the economics become more favorable (39). In addition, electrons flowing from oxidative reactions can be boosted by a small (0.25- to 0.5-V) potential, which significantly increases overall volumetric rates of electron flow and can be used to power H₂- or CH₄-evolving microbial electrolysis chambers (5, 40) at energetic yields superior to those of water-splitting re-actors. More recent work suggests that valuable bio-oxidations could be coupled in this way to electrodes used as electron donors for a wider variety of processes (41–44), such as was recently demonstrated by acetogenesis by biofilms of *Sporomusa ovata* on graphite electrodes (45).

It has previously been proposed that electrodes may alter fermentative pathways, but these strategies required high concentrations of toxic redox mediators to extract electrons from fermentative cells unable to transfer electrons to their outer surfaces for electrode respiration (1, 2, 46). *S. oneidensis* proved a tractable organism for linking native electron transfer ability to synthetic biology. Our work here shows that the landscape of metabolic engineering and synthetic biology strategies for biofuel and bioproduct synthesis (47) can be expanded through the use of engineered electrode-interfaced bacteria.

**MATERIALS AND METHODS**

**Bacterial strains, culturing, growth, and reagents.** *S. oneidensis* strain MR-1 was previously isolated from Lake Oneida in New York State (48). All strains described in this study can be found in Table 2. Overnight cultures were inoculated from single colonies freshly streaked from a frozen stock into Luria-Bertani (LB) medium (supplemented with 50 µg/ml kanamycin [Km] when required for plasmid maintenance) and incubated for 16 h. *Shewanella* basal medium (SBM) containing 5 ml/liter of vitamins and trace minerals was used where specified below as described previously (49) and supplemented with 0.05% Casamino Acids. Anaerobic cultures were placed in Balch anaerobic tubes sealed with butyl rubber stoppers and flushed with nitrogen for 15 minutes (50). All cultures were maintained at 30°C and shaken continuously at 200 rpm. All molecular biology enzymes were obtained from New England Biolabs (Ipswich, MA), TOPO TA cloning vectors were from Invitrogen (Carlsbad, CA), and PCR cleanup, gel extraction, and plasmid preparation kits were from Qiagen (Valencia, CA). All other chemicals were obtained from Sigma (St. Louis, MO).
optical density at 600 nm (OD600) of ~0.05 into SBM medium containing medium supplemented with Km (when appropriate), washed twice with case, vector inserts were sequenced to verify accuracy and orientation, and then cloned into pGUT2, creating pGUT2PET (Fig. 2A). In every used as a PCR template with primers J3 and J4 to clone the native creating JF3. To clone primers J1 and J2. PCR products were cloned into pBBR1MCS-2 (51), clone glpD to the and J10, designed to incorporate the previously cloned genes in addition described (52), creating JF4. JF4 was used as a PCR template with primers J9 operon. PCR products were cloned into modified pUC19, previously de-6

TABLE 2 Bacterial strains, vectors, and primers used in this study

| Strain, vector, or gene and primera | Relevant characteristic(s) | Reference(s) or source |
|------------------------------------|----------------------------|------------------------|
| **Strains**                        |                            |                        |
| *S. oneidensis*                    |                            |                        |
| MR-1                               | Isolated from Lake Oneida, NY | 17, 48                 |
| JG612                              | Δpta deletion derivative of MR-1 | 27                     |
| *E. coli*                          |                            |                        |
| K-12                               |                            | Laboratory stock       |
| UQ950                              | *E. coli* DHSα for cloning | 57                     |
| WM3064                             | DAP auxotroph donor strain for conjugation | 57                     |
| **Vectors**                        |                            |                        |
| pBBR1MCS-2                         | 5.0-kb broad-host-range vector for cloning; Km | 51                     |
| pPET                                | pBBR1MCS-2 containing *pdc* and *adh* (cloned from Zymomonas mobilis, pLOI297) | This study |
| pGUT2 PET                           | pBBR1MCS-2 containing *glpD*, *glpF*, *glpK*, and *tpiA* (cloned from *E. coli* K-12) | This study |
| pGUT2PET                            | pBBR1MCS-2 containing *glpD*, *glpF*, *glpK*, and *tpiA* (cloned from *E. coli* K-12) and *pdc* and *adh* (cloned from *Z. mobilis*, pLOI297) | This study |
| **Primers for cloning**            |                            |                        |
| *glpD*                             |                            |                        |
| J1 KpnI                            | GGGGTACCACGAAAGTGGAATGAGGCGAGCA | This study |
| J2 XhoI                            | CCGCTCGAGCAGGGCGATTTGAAATCTGA |                        |
| *glpFK*                            |                            |                        |
| J3 XhoI                            | GCTCTAGAAGGATCGTACGAAACGTG | This study |
| J4 NotI                            | ATAGAATGGGGGCGCTGGGCAATACGTTCC |                        |
| *tpiA*                             |                            |                        |
| J5 SacI                            | NNGAGCTCGGCTTATAAACGTTGCGAGA | This study |
| J6 SacI                            | NNGAGCTCGGAAAGTACGATGGCGATAG |                        |
| *glpABC*                           |                            |                        |
| J7 HindIII                         | CCAAGGGTGGCCGCAATCAACATTACA | This study |
| J8 EcoRI                           | CGGAATTCATACATTGGGCACGGAATCG |                        |
| pUCmod Fwd                         |                            |                        |
| J9 XhoI                            | NNCTCGAGCCCGGACTGGAAGCGC | This study |
| pUCmod Rev                         | NNNAGGCTCGACATGCGGTGTAATACCG | This study |
| J10 SacI                           | NNNCTCGAGCTCAGTAACTGATGGGATCCC | This study |
| pBBR1MCS-2 Rev                     |                            |                        |
| J11 XhoI                           |                            |                        |

a Fwd, forward; Rev, reverse.
b DAP, diaminopimelic acid.

Plasmid construction. Oligonucleotides used are listed in Table 2. To clone *glpD*, genomic DNA of *E. coli* K-12 was used as a PCR template with primers J1 and J2. PCR products were cloned into pBBR1MCS-2 (51), creating JF3. To clone *glpF* and *glpK*, genomic DNA of *E. coli* K-12 was used as a PCR template with primers J3 and J4 to clone the native *glpFK* operon. PCR products were cloned into modified pUC19, previously described (52), creating JF4. JF4 was used as a PCR template with primers J9 and J10, designed to incorporate the previously cloned genes in addition to the lac promoter preceding *glpF*. PCR products were cloned into JF3, creating JF5. To clone *tpiA*, genomic DNA of K-12 was used as a PCR template with primers J5 and J6. PCR products were cloned into JF5, creating pGUT2. Plasmid pLOI297 (53) obtained from the ATCC (ATCC 68239), which contains *pdc* and *adhB* cloned from *Z. mobilis*, was digested with BamHI and EcoRI. The fragment containing these genes was cloned into pBBR1MCS-2, creating pPET. Plasmid pPET was used as a template for a PCR with primer J11 and the standard M13 reverse primer. PCR products were A-tailed and then cloned into the TOPO TA vector, creating JF7. JF7 was digested with XhoI (at a site introduced by the J11 primer), yielding a 3.3-kb band containing a lac promoter, *pdc*, and *adhB*, and then cloned into pGUT2, creating pGUT2PET (Fig. 2A). In every case, vector inserts were sequenced to verify accuracy and orientation.

Growth on glycerol. Strains were grown overnight aerobically in LB medium supplemented with Km, washed twice with SBM, and resuspended in SBM. For measuring the conversion of lactate to ethanol, cells were then inoculated to an OD600 of ~0.8 into a culture containing 50 mM lactate and 50 mM fumarate and made anaerobic. For measuring the conversion of glycerol to ethanol, washed cells were inoculated to an OD600 of ~0.8 into an anaerobic culture tube containing 40 mM glycerol and 60 mM fumarate. Periodically, 0.2-ml aliquots were removed and centrifuged, and supernatants were immediately frozen at −80°C for high-performance liquid chromatography (HPLC) analysis.

HPLC analysis. Metabolites were quantified by HPLC (all components were from Shimadzu Scientific) equipped with a UV–visible-light (Vis) detector and refractive index detector. The system consisted of an SCL-10A system controller, LC-10AT liquid chromatograph, SIL-10AF autoinjector, RID-10A refractive index detector, SPD-10A UV-Vis detector, and CTO-10A column oven. Separation of compounds was performed as described previously (37) with an Aminex HPX-87H guard column and an HPX-87H cation-exchange column (Bio-Rad [Hercules, CA]). The mobile phase consisted of 0.005 N H2SO4, set at a flow rate of 0.4 ml/min. The column was maintained at 42°C, and the injection volume was 50 μl.

Resting cell assays. Strains were grown overnight aerobi
cally in LB medium supplemented with Km, washed twice with SBM, and resuspended in SBM. For measuring the conversion of lactate to ethanol, cells were then inoculated to an OD600 of ~0.8 into a culture containing 50 mM lactate and 50 mM fumarate and made anaerobic. For measuring the conversion of glycerol to ethanol, washed cells were inoculated to an OD600 of ~0.8 into an anaerobic culture tube containing 40 mM glycerol and 60 mM fumarate. Periodically, 0.2-ml aliquots were removed and centrifuged, and supernatants were immediately frozen at −80°C for high-performance liquid chromatography (HPLC) analysis.

HPLC analysis. Metabolites were quantified by HPLC (all components were from Shimadzu Scientific) equipped with a UV–visible-light (Vis) detector and refractive index detector. The system consisted of an SCL-10A system controller, LC-10AT liquid chromatograph, SIL-10AF autoinjector, RID-10A refractive index detector, SPD-10A UV-Vis detector, and CTO-10A column oven. Separation of compounds was performed as described previously (37) with an Aminex HPX-87H guard column and an HPX-87H cation-exchange column (Bio-Rad [Hercules, CA]). The mobile phase consisted of 0.005 N H2SO4, set at a flow rate of 0.4 ml/min. The column was maintained at 42°C, and the injection volume was 50 μl.

Bioreactor analysis. Bioreactors were constructed as previously described, with modifications (54). The counter electrode, housed in a glass capillary tube with dialysis tubing at one end, facilitated ion movement but inhibited gas transfer to avoid any utilization of stray H2 produced at the counter electrode and allow precise accounting of electron recovery.
Isolation of the counter electrode was not necessary for routine glycerol conversion to ethanol. Strains were grown overnight in SBM supplemented with 50 mM glycerol and resuspended in 1 ml of SBM containing 50 mM glycerol and 4 μM riboflavin. The cell suspension was added to 11 ml of the same anaerobic medium in the bioreactor, which was continuously flushed at the counter electrode with nitrogen gas. The electrodes were maintained at an oxidizing potential (+0.44 V versus the standard hydrogen electrode [SHE]) using a 16-channel VMP1 potentiostat (Bio-Logic SA [Knoxville, TN]). Current production was monitored over time; 0.2-ml samples were taken periodically for HPLC analysis.

Enzyme assays. The activity assay for glycerol-3-phosphate dehydrogenase was performed as previously described (55). Fifty-milliliter cultures of cells to be tested were grown in LB medium supplemented with 50 μg/ml Kno overnight, shaken, and incubated (37°C for E. coli and 30°C for S. oneidensis). The cultures were centrifuged at 6,000 × g for 15 minutes, and the cells were resuspended in 1 ml of 0.1 M potassium phosphate buffered to pH 7.5 (PBS). Cells were then sonicated for 1 minute on ice. Sonicated samples were centrifuged at 15,000 × g for 10 minutes. Glycerol kinase (56), alcohol dehydrogenase (23) and pyruvate decarboxylase (24) activities in cell lysates were determined as previously described, and cell lysates were prepared as described above.

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