Engineering Cooperation in an Anaerobic Coculture

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ABSTRACT Over the past century, microbiologists have studied organisms in pure culture, yet it is becoming increasingly apparent that the majority of biological processes rely on multispecies cooperation and interaction. While little is known about how such interactions permit cooperation, even less is known about how cooperation arises. To study the emergence of cooperation in the laboratory, we constructed both a commensal community and an obligate mutualism using the previously noninteracting bacteria *Shewanella oneidensis* and *Geobacter sulfurreducens*. Incorporation of a glycerol utilization plasmid (pGUT2) enabled *S. oneidensis* to metabolize glycerol and produce acetate as a carbon source for *G. sulfurreducens*, establishing a cross-feeding, commensal coculture. In the commensal coculture, both species coupled oxidative metabolism to the respiration of fumarate as the terminal electron acceptor. Deletion of the gene encoding fumarate reductase in the *S. oneidensis*/pGUT2 strain shifted the coculture with *G. sulfurreducens* to an obligate mutualism where neither species could grow in the absence of the other. A shift in metabolic strategy from glycerol catabolism to malate metabolism was associated with obligate coculture growth. Further targeted deletions in malate uptake and acetate generation pathways in *S. oneidensis* significantly inhibited coculture growth with *G. sulfurreducens*. The engineered coculture between *S. oneidensis* and *G. sulfurreducens* provides a model laboratory system to study the emergence of cooperation in bacterial communities, and the shift in metabolic strategy observed in the obligate coculture highlights the importance of genetic change in shaping microbial interactions in the environment.

IMPORTANCE Microbes seldom live alone in the environment, yet this scenario is approximated in the vast majority of pure-culture laboratory experiments. Here, we develop an anaerobic coculture system to begin understanding microbial physiology in a more complex setting but also to determine how anaerobic microbial communities can form. Using synthetic biology, we generated a coculture system where the facultative anaerobe *Shewanella oneidensis* consumes glycerol and provides acetate to the strict anaerobe *Geobacter sulfurreducens*. In the commensal system, growth of *G. sulfurreducens* is dependent on the presence of *S. oneidensis*. To generate an obligate coculture, where each organism requires the other, we eliminated the ability of *S. oneidensis* to respire fumarate. An unexpected shift in metabolic strategy from glycerol catabolism to malate metabolism was observed in the obligate coculture. Our work highlights how metabolic landscapes can be expanded in multispecies communities and provides a system to evaluate the evolution of cooperation under anaerobic conditions.

KEYWORDS *Shewanella*, *Geobacter*, metabolism, anaerobic respiration

Microorganisms are the major drivers of biochemical and nutrient cycling in environments ranging from ocean sediments to the human microbiome, yet factors that influence the behavior and function of multispecies ecosystems remain largely undefined (1–4). For the past century, microbiology has focused mainly on the isolation and study of individual species, leading to in-depth studies on genetic and physiological characterization, but the behavior of organisms as members of communities is often very different

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from behavior in pure laboratory cultures (5, 6). Sequencing and community-based omics studies have enabled the analysis of multispecies microbial communities answering questions such as “who is there” and “what functions are present,” yet they are limited in their ability to attribute key functions to individual species, identify active members, or provide an overall picture of how interaction and cooperation emerge in communities (3, 7).

In natural settings, microorganisms rarely act as individuals but instead act as inherently complex and dynamic communities that interact and communicate with one another. Interaction oftentimes leads to obligate interdependence or syntrophy in communities where two or more members combine metabolic strategies to gain energy and catabolize compounds that neither species alone could consume (8–11). In syntrophic systems, the metabolism of one species is directly influenced by actions of the other and vice versa (8–12). Interdependence between community members confounds their study because natural syntrophic communities oftentimes cannot be separated in the laboratory (13, 14). The inability to grow members separately also impedes studies on how syntrophy and obligate interactions emerge in multispecies ecosystems.

Synthetic ecology, the engineering of rationally designed communities in well-defined environments, provides an innovative and robust approach to reduce the complexity inherent in natural systems and mimic microbial interaction in a controlled framework (15–17). By distilling the salient features of communities into modular genetic and biochemical units, artificial consortia can be engineered from the bottom up to serve as model systems enabling investigation of conditions that prime the emergence of microbial interactions and higher-order community function (16, 17). The construction of simplified, artificial ecosystems using organisms with well-defined genetic backgrounds provides a context in which to identify interaction within community metabolic networks. Developed synthetic communities can also be used as a chassis for the continued engineering of more-complex ecosystems (16, 18).

Here, we report the construction and characterization of model synthetic anaerobic communities ranging from commensalism (one species benefits) to obligate mutualism (interdependence required for both species to benefit) using *Shewanella oneidensis* and *Geobacter sulfurreducens*, two bacteria chosen because (i) they have no naturally evolved metabolic interactions, enabling us to monitor the emergence of cooperation in a population, (ii) there is a breadth of genetic tools available for both, including sequenced and annotated genomes (19, 20), robust genetic systems (21–25), and well-described metabolic networks (26–28), and (iii) this coculture is potentially important in biotechnology applications (29, 30). *S. oneidensis* and *G. sulfurreducens* serve as the model organisms for extracellular electron transfer (EET), an ability to transfer electrons produced during oxidative metabolism across cellular membranes to insoluble terminal electron acceptors (31, 32). Through EET, *Shewanella* and *Geobacter* species respire a diverse array of substrates, including metals and electrodes, making these bacteria applicable—individually and potentially more in cooperative concert—to the remediation of environmental pollutants and to electricity production in microbial fuel cells (29, 30). A commensal coculture was first engineered through incorporation of a cross-feeding mechanism between *S. oneidensis* and *G. sulfurreducens*. A targeted deletion in the respiratory pathway of *S. oneidensis* shifted the commensal coculture to an obligate mutualism where neither species could grow alone in the presence of the supplied carbon source. Importantly, the obligate coculture also revealed a complete shift in metabolic strategy, highlighting that mutations in metabolic pathways may play a substantial role in shaping microbial interactions in the environment.

RESULTS

To establish a coculture system, a cross-feeding mechanism was engineered between *S. oneidensis* and *G. sulfurreducens* (Fig. 1). Incorporation of a glycerol plasmid (pGUT2) enabled *S. oneidensis* to metabolize glycerol and secrete acetate (33). *G. sulfurreducens*, unable to utilize glycerol as a carbon source, was then dependent on acetate secreted by *S. oneidensis*/pGUT2 as a carbon source for growth (30). As engineered, the complete

FIG 1 Metabolic interactions enabling commensal coculture growth of *S. oneidensis*/pGUT2 and *G. sulfurreducens*. *S. oneidensis* oxidizes glycerol via an engineered glycerol utilization pathway (GlpF, glycerol facilitator; GlpK, glycerol kinase; GlpD, glycerol-3-P dehydrogenase; TpiA, triose phosphate isomerase) and secretes acetate that is used as a carbon source by *G. sulfurreducens*. Cross-feeding of acetate from *S. oneidensis*/pGUT2 to *G. sulfurreducens* is represented by the black arrow. Both organisms couple metabolic oxidation reactions to the respiration of fumarate (FccA and Frd represent fumarate reductase in *S. oneidensis* and *G. sulfurreducens*, respectively).

oxidation of glycerol to carbon dioxide required the metabolic activity of both *S. oneidensis*/pGUT2 and *G. sulfurreducens* and was coupled to fumarate respiration by both organisms (Fig. 1). The coculture outlined in Fig. 1 is described as commensal because coculture conditions are neither beneficial nor detrimental to *S. oneidensis*/pGUT2 whereas *G. sulfurreducens* gains a direct benefit from coculture growth with *S. oneidensis*/pGUT2.

We began by characterizing growth of *S. oneidensis*/pGUT2 and *G. sulfurreducens* in basal coculture medium containing glycerol as the carbon source and fumarate as the terminal electron acceptor (denoted as CMGF). As predicted, when cultured separately, CMGF medium supported growth of *S. oneidensis*/pGUT2 but not of *G. sulfurreducens* due to its reliance on *S. oneidensis*/pGUT2 for acetate (Fig. 2). Simultaneous growth of both species was only observed when *S. oneidensis*/pGUT2 and *G. sulfurreducens* were cultured together in medium containing both glycerol and fumarate, and the coculture reached a higher maximum optical density at 600 nm (OD₆₀₀) than did *S. oneidensis*/pGUT2 cultured alone (Fig. 2A). To determine growth rates for each species in the coculture, samples were removed periodically, and serial dilutions were plated on medium selective for either species. Resulting CFU were then determined and revealed an initial lag phase for *G. sulfurreducens* when cocultured with *S. oneidensis*/pGUT2, likely due to inhibition of *G. sulfurreducens* growth until secreted acetate concentrations were high enough to support growth (Fig. 2B). Growth of *S. oneidensis*/pGUT2 was similar when cultured alone or in coculture with *G. sulfurreducens* in CMGF medium (Fig. 2B).

To confirm that acetate was secreted by *S. oneidensis*/pGUT2 and served as the carbon source for *G. sulfurreducens* in coculture experiments, supernatant samples were analyzed by high-performance liquid chromatography (HPLC) (Fig. 3). Analysis of coculture metabolites over time by HPLC revealed that glycerol was completely oxidized and coupled to fumarate

FIG 2 Growth of *S. oneidensis*/pGUT2 and *G. sulfurreducens* alone and in coculture in basal coculture medium (CM) containing 7 mM glycerol (G) and/or 60 mM fumarate (F) as indicated. (A) Growth measured as OD₆₀₀ of the *S. oneidensis*/pGUT2-*G. sulfurreducens* coculture in CMGF (blue circles), CMG (black squares), or CMF (gray triangles) medium compared to that of *S. oneidensis*/pGUT2 (green triangles) or *G. sulfurreducens* (red diamonds) grown singly in CMGF. Growth was not observed for *S. oneidensis*/pGUT2 or *G. sulfurreducens* cultured alone in CMG or CMF, and data were removed for graph clarity. (B) Growth monitored as CFU in CMGF medium for *S. oneidensis*/pGUT2 (green circles) and *G. sulfurreducens* (red squares) grown alone (open symbols) or in coculture (closed symbols). Reported values are averages for triplicate experiments with error represented as standard error of the mean (SEM).

reduction as predicted (Fig. 3A; Table 1). Acetate concentration increased initially in coculture supernatants, but after reaching ~2 mM, it was completely consumed, and no acetate was detected at the final time point in agreement with the prediction of acetate consumption by *G. sulfurreducens* (Fig. 3A; Table 1). In cocultures, the concentration of fumarate utilized was slightly higher than predicted for the stoichiometric conversion of fumarate to succinate, indicating that some fumarate was taken up by the coculture in a process not linked to respiration (Table 1). Grown alone in CMGF medium, *S. oneidensis*/pGUT2 consumed glycerol and secreted acetate at nearly stoichiometric ratios and coupled this oxidation to the reduction of

FIG 3 Metabolite profiles in (A) *S. oneidensis*/pGUT2-*G. sulfurreducens* cocultures, (B) *S. oneidensis*/pGUT2 only, and (C) *G. sulfurreducens* only cultures in CMGF medium containing 7 mM glycerol and 60 mM fumarate. Metabolites include glycerol (green triangles), acetate (red squares), fumarate (blue circles), and succinate (purple inverted triangles). Reported values are for three independent experiments with error represented as SEM. Error bars associated with the above data were small and hence are masked due to the symbol size used.

TABLE 1 Concentration changes in metabolite profiles from initial to end time points for *S. oneidensis*/pGUT2 grown singly or in coculture with *G. sulfurreducens*^a

Metabolite	Change in concn	
	<i>S. oneidensis</i> /pGUT2 grown alone (metabolite concn [mM])	<i>S. oneidensis</i> /pGUT2 and <i>G. sulfurreducens</i> coculture (metabolite concn [mM])
Glycerol	−6.5 ± 0.0	−6.5 ± 0.0
Acetate	5.9 ± 0.1	ND
Fumarate	−25.0 ± 0.4	−55.4 ± 0.9
Succinate	21.6 ± 0.1	45.5 ± 0.2

^aMetabolite concentrations changed by <1% for *G. sulfurreducens* cultured alone and hence were not included in the table. Initial concentrations measured in NBGF medium by HPLC were 6.5 ± 0.0 mM glycerol and 58.5 ± 0.1 mM fumarate. Reported concentrations are averages ± SEM from three independent experiments. ND, not detected.

fumarate as the terminal electron acceptor subsequently producing succinate (Fig. 3B; Table 1). Fumarate uptake by *S. oneidensis*/pGUT2 grown alone was slightly higher than the concentration of succinate produced, which indicated some utilization of fumarate by *S. oneidensis* cultures not associated with respiration (Table 1). Glycerol and fumarate concentrations remained largely unchanged for *G. sulfurreducens* grown alone in CMGF over the time course analyzed (Fig. 3C).

An obligate interaction between *S. oneidensis*/pGUT2 and *G. sulfurreducens* was created by deleting the gene encoding fumarate reductase (*fccA*) in the chromosome of *S. oneidensis* (strain referred to as *S. oneidensis* $\Delta fccA$ /pGUT2), eliminating the ability of *S. oneidensis* to respire fumarate, thus requiring a condition or partner in the coculture to turn over reducing equivalents (Fig. 1). Growth in CMGF medium required the presence of both *S. oneidensis* $\Delta fccA$ /pGUT2 and *G. sulfurreducens* and occurred over a much longer timescale (~80 days) compared to that of the commensal *S. oneidensis*/pGUT2 and *G. sulfurreducens* coculture (~80 h) (Fig. 4A versus Fig. 2A). Interestingly, growth was also observed for *S. oneidensis* $\Delta fccA$ /pGUT2 and *G. sulfurreducens* cocultured in basal medium lacking glycerol (CMF). Selective plating was again utilized to

FIG 4 Obligate growth of *S. oneidensis* $\Delta fccA$ /pGUT2 and *G. sulfurreducens* in basal coculture medium (CM) containing 7 mM glycerol and 60 mM fumarate (CMGF, closed symbols) or coculture medium supplemented only with 60 mM fumarate (CMF, open symbols). (A) Growth measured as OD₆₀₀ for *S. oneidensis* $\Delta fccA$ /pGUT2 and *G. sulfurreducens* in coculture (blue) and for *S. oneidensis* $\Delta fccA$ /pGUT2 (green) and *G. sulfurreducens* (red) cultured alone. (B) Growth measured as CFU for *S. oneidensis* $\Delta fccA$ /pGUT2 (green) and *G. sulfurreducens* (red) in coculture in CMGF (closed symbols) and CMF (open symbols) media. Reported values are averages from triplicate experiments with error bars representing SEM.

FIG 5 Metabolite profiles in *S. oneidensis* $\Delta fccA$ /pGUT2-*G. sulfurreducens* cocultures in (A) CMGF medium or (B) CMF medium, *S. oneidensis* $\Delta fccA$ /pGUT2 cultured alone in (C) CMGF medium or (D) CMF medium, and *G. sulfurreducens* cultured alone in (E) CMGF medium or (F) CMF medium. Metabolites include glycerol (green), fumarate (blue), malate (orange), and succinate (purple). Media contained 7 mM glycerol and/or 60 mM fumarate as indicated. Reported values represent mean $\pm$ SEM metabolite concentrations (mM) measured for three independent experiments.

determine growth of each species in coculture, revealing that both *S. oneidensis* $\Delta fccA$ /pGUT2 and *G. sulfurreducens* grew similarly when cocultured in CMGF or CMF medium (Fig. 4B). *S. oneidensis* $\Delta fccA$ /pGUT2 populations experienced an initial drop in cell numbers followed by a moderate rise after approximately 40 days of coculture incubation with *G. sulfurreducens* both in CMGF and CMF media (Fig. 4B). In contrast, *G. sulfurreducens* CFU increased steadily in coculture, amounting to ~ 3.5 doublings in both CMGF and CMF media (Fig. 4B).

Growth of *S. oneidensis* $\Delta fccA$ /pGUT2 and *G. sulfurreducens* cocultures in medium lacking glycerol (CMF) indicated a shift in metabolic strategy and usage of a carbon source other than glycerol. To determine carbon source utilization by the obligate *S. oneidensis* $\Delta fccA$ /pGUT2-*G. sulfurreducens* coculture, supernatant samples were analyzed by HPLC. Indeed, glycerol concentration decreased <0.5 mM in cocultures of *S. oneidensis* $\Delta fccA$ /pGUT2 and *G. sulfurreducens* in CMGF medium over 100 days (Fig. 5A). Instead of using glycerol as the carbon source, the *S. oneidensis* $\Delta fccA$ /pGUT2-*G. sulfurreducens* coculture converted fumarate to malate, and malate was then consumed after concentrations reached ~ 40 mM (Fig. 5A). Malate was also produced and subsequently consumed by the *S. oneidensis* $\Delta fccA$ /pGUT2-*G. sulfurreducens* coculture in CMF medium, highlighting the

FIG 6 Obligate growth of *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ and *G. sulfurreducens* in basal coculture medium (CM) containing 7 mM glycerol and 60 mM fumarate (CMGF, closed symbols) or coculture medium supplemented only with 60 mM fumarate (CMF, open symbols). (A) Growth measured as OD_{600} for the *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ -*G. sulfurreducens* coculture (blue) and for *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ (green) and *G. sulfurreducens* (red) cultured singly. (B) Growth measured as CFU/ml media for *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ (green) and *G. sulfurreducens* (red) cocultures. Reported values are averages for triplicate experiments with error bars representing SEM.

metabolic shift to malate catabolism (Fig. 5B). Succinate concentration rose in cocultures in both CMGF and CMF media as malate was consumed (Fig. 5A and B). Growth related to malate metabolism required *S. oneidensis* $\Delta fccA/pGUT2$ and *G. sulfurreducens* to be cultured together, as neither species was able to grow alone in CMGF or CMF medium (Fig. 4A). Supernatant from *S. oneidensis* $\Delta fccA/pGUT2$ and *G. sulfurreducens* cultured alone showed that both organisms were able to convert fumarate to malate, likely catalyzed by fumarase, yet at rates lower than those that occurred in the coculture (Fig. 5C to F). *S. oneidensis* $\Delta fccA/pGUT2$ cultured alone was not able to consume malate and only produced trace amounts of succinate in CMGF medium (Fig. 5C and D). Low concentrations of succinate were also produced by *G. sulfurreducens* cultured alone in CMGF and CMF media but were produced at a significantly lower rate than those produced by the coculture (Fig. 5E and F). Fumarate has previously been shown to serve as an additional carbon source in the presence of acetate by *G. sulfurreducens* (34).

To determine if the obligate *S. oneidensis* $\Delta fccA/pGUT2$ -*G. sulfurreducens* coculture was dependent on acetate transfer from *Shewanella* to *Geobacter*, as was determined for the commensal coculture, targeted deletions were made in the metabolic pathway linked to acetate production by *Shewanella*. *S. oneidensis* generates acetate from a variety of carbon sources via the acetate kinase/phosphotransacetylase (Ack/Pta) pathway (26, 35), so to significantly diminish acetate secretion by *S. oneidensis* $\Delta fccA/pGUT2$, we deleted *pta* from the chromosome (strain referred to as *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$). Growth yield, in CMGF and more so in CMF medium, was severely diminished for cocultures initiated with *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ and *G. sulfurreducens* compared to that in cocultures containing *S. oneidensis* $\Delta fccA/pGUT2$ and *G. sulfurreducens* after 100 days of incubation (Fig. 6 versus Fig. 4). Interestingly, increase in OD_{600} of the *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ -*G. sulfurreducens* coculture occurred following a sharp increase in *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ in the coculture (after an initial population decline) (Fig. 6). CFU counts for *G. sulfurreducens* increased slightly following the CFU increase for *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ (Fig. 6B).

Conversion of fumarate to malate was significantly slowed for the *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ -*G. sulfurreducens* coculture compared to that of the original obligate *S. oneidensis* $\Delta fccA/pGUT2$ -*G. sulfurreducens* cocultures, and malate concentration did not decline

FIG 7 Metabolite profiles in *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ -*G. sulfurreducens* cocultures in (A) CMGF medium or (B) CMF medium and in *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ cultured alone in (C) CMGF medium or (D) CMF medium. Metabolites include glycerol (green), fumarate (blue), malate (orange), and succinate (purple). Media contained 7 mM glycerol and/or 60 mM fumarate as indicated. Reported values represent mean $\pm$ SEM metabolite concentrations (mM) measured for three independent experiments.

until ~60 days, mirroring growth measured by OD_{600} . Subsequent succinate production was also greatly diminished in the *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ -*G. sulfurreducens* coculture compared to that in *S. oneidensis* $\Delta fccA/pGUT2$ -*G. sulfurreducens* cocultures over the time period monitored both in CMGF and CMF media (Fig. 7A and B). Taken together, results from the *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ -*G. sulfurreducens* coculture (Fig. 6A) compared to those from the *S. oneidensis* $\Delta fccA/pGUT2$ -*G. sulfurreducens* coculture (Fig. 4A) indicated that deletion of *pta* inhibited coculture growth. HPLC analysis of supernatant from cultures with *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ cultured alone indicated that fumarate conversion to malate occurred at a lower rate in CMF medium than in CMGF medium, though glycerol levels remained largely unchanged in CMGF medium (Fig. 7C and D). It is possible that the presence of 7 mM glycerol acts as a cell protectant in CMGF and may explain why growth of the *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ and *G. sulfurreducens* cocultures was also inhibited more in CMF than in CMGF media (Fig. 6A).

Due to inhibited coculture growth by deletion of *pta* in *Shewanella*, we hypothesized that growth in the original *S. oneidensis* $\Delta fccA/pGUT2$ -*G. sulfurreducens* obligate coculture was due to uptake of malate and production of acetate by *Shewanella*. *Shewanella* encodes pathways to convert malate to pyruvate via malic enzymes MaeB and SfcA or phosphoenolpyruvate (PEP) through concerted malate dehydrogenase and PEP carboxykinase activity. PEP and pyruvate could then be catabolized via central metabolic pathways producing acetate. Growth was inhibited in cocultures containing *S. oneidensis* $\Delta fccA$ $\Delta maeB$ $\Delta sfcA$ $\Delta pckA/pGUT2$ and *G. sulfurreducens*, similar to that in cocultures containing the *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ strain (Fig. 8A and Fig. 6A).

FIG 8 Obligate growth of *S. oneidensis* $\Delta fccA$ $\Delta maeB$ $\Delta sfca$ $\Delta pckA/pGUT2$ and *G. sulfurreducens* in basal coculture medium (CM) containing 7 mM glycerol and 60 mM fumarate (CMGF, closed symbols) or coculture medium supplemented with only 60 mM fumarate (CMF, open symbols). (A) Growth measured as OD_{600} for the *S. oneidensis* $\Delta fccA$ $\Delta maeB$ $\Delta sfca$ $\Delta pckA/pGUT2$ -*G. sulfurreducens* coculture (blue) and for *S. oneidensis* $\Delta fccA$ $\Delta maeB$ $\Delta sfca$ $\Delta pckA/pGUT2$ (green) and *G. sulfurreducens* (red) cultured singly. (B) Growth measured as CFU/ml media for *S. oneidensis* $\Delta fccA/pGUT2$ (green) and *G. sulfurreducens* (red) cocultures. Reported values are averages for triplicate experiments with error bars representing SEM.

The *S. oneidensis* $\Delta fccA$ $\Delta maeB$ $\Delta sfca$ $\Delta pckA/pGUT2$ population initially decreased in coculture with *G. sulfurreducens* followed by an increase in CFU at ~40 days (Fig. 8B), similar to the growth pattern observed for *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ (Fig. 6B). CFU counts for *G. sulfurreducens* increased slightly following CFU increase by *S. oneidensis* $\Delta fccA$ $\Delta maeB$ $\Delta sfca$ $\Delta pckA/pGUT2$ (Fig. 8B). Malate consumption and succinate production were also significantly slower for the *S. oneidensis* $\Delta fccA$ $\Delta maeB$ $\Delta sfca$ $\Delta pckA/pGUT2$ -*G. sulfurreducens* cocultures compared to those for the original *S. oneidensis* $\Delta fccA/pGUT2$ -*G. sulfurreducens* coculture in CMGF and CMF media (Fig. 9A and B). Cocultures were also initiated between *G. sulfurreducens* and *S. oneidensis* containing deletions in pathways both for malate consumption and for acetate production (*S. oneidensis* $\Delta fccA$ Δpta $\Delta maeB$ $\Delta sfca$ $\Delta pckA/pGUT2$), and growth and metabolite profiles were not significantly different for cocultures containing either *S. oneidensis* $\Delta fccA$ $\Delta pta/pGUT2$ or *S. oneidensis* $\Delta fccA$ $\Delta maeB$ $\Delta sfca$ $\Delta pckA/pGUT2$ (data not shown).

Finally, because growth rates of all cocultures were slightly higher in CMGF medium than in CMF medium (Fig. 4A, Fig. 6A, and Fig. 8A), an obligate coculture was initiated between *S. oneidensis*/ $\Delta fccA$ without the pGUT2 plasmid and *G. sulfurreducens* to determine if higher growth rates in CMGF medium were associated with activity encoded by pGUT2. Growth rate and yield were similar between cocultures containing *S. oneidensis*/ $\Delta fccA$ with or without the pGUT2 plasmid, and both had slightly higher growth rates in CMGF medium than in CMF (Fig. 4A versus Fig. 10A). Metabolite profiles were also similar for cocultures of *G. sulfurreducens* with *S. oneidensis*/ $\Delta fccA$ with or without the pGUT2 plasmid (Fig. 5A and B versus Fig. 11A and B). Results from cocultures containing *S. oneidensis*/ $\Delta fccA$ lacking the pGUT2 plasmid indicated that coculture growth in CMGF and CMF media was not due to activity mediated by pGUT2.

DISCUSSION

Cooperative interactions sustaining diverse species in microbial populations are common in the environment and are continuously influenced by changing biological, chemical, and physical factors (36). The dynamics of natural communities combined with their enormous diversity and the fact that many microorganisms cannot be grown in the laboratory have confounded the study of multispecies ecosystems (36, 37).

FIG 9 Metabolite profiles in *S. oneidensis* $\Delta fccA$ $\Delta maeB$ $\Delta sfcA$ $\Delta pckA$ /pGUT2-*G. sulfurreducens* cocultures in (A) CMGF medium or (B) CMF medium and in *S. oneidensis* $\Delta fccA$ $\Delta maeB$ $\Delta sfcA$ $\Delta pckA$ /pGUT2 cultured alone in (C) CMGF medium or (D) CMF medium. Metabolites include glycerol (green), fumarate (blue), malate (orange), and succinate (purple). Media contained 7 mM glycerol and/or 60 mM fumarate as indicated. Reported values represent mean $\pm$ SEM metabolite concentrations (mM) measured for three independent experiments.

Obligate interactions among community members have been posed as one reason for recalcitrance to laboratory culture by many microorganisms (5, 38). Interaction in multispecies communities often involves reciprocity between members, and synthetic model laboratory communities have provided a powerful tool to begin to study how interaction and overall cooperation can emerge in multispecies ecosystems (39–45).

Here, we describe construction of synthetic model communities ranging from commensalism to obligate mutualism using the bacteria *S. oneidensis* and *G. sulfurreducens*. *S. oneidensis* has been cultured previously with *Klebsiella pneumoniae*, which oxidized glycerol into unknown products that *S. oneidensis* could use to reduce poised electrodes (46). Several studies have also been done with *S. oneidensis* and *G. sulfurreducens* incubated together in bioelectrochemical reactors (47–49) without integration of a metabolic cascade. Because *S. oneidensis* and *G. sulfurreducens* were originally isolated from different environments (50, 51), their coculture enabled us to monitor the emergence of cooperation from the initial point of contact. We show here also that a targeted deletion in the respiratory pathway of *S. oneidensis* altered interaction requiring obligate cooperation with *G. sulfurreducens* and completely shifted the metabolic strategy of the community. It is known that gene loss can lead to obligate interaction (43) and that population bottlenecks can lead to mutation or loss of essential genes (52). The obligate coculture between *S. oneidensis* $\Delta fccA$ /pGUT2 and *G. sulfurreducens* provides a model laboratory system to monitor how metabolic strategy may shift in environments due to gene loss and provide a possible explanation for the emergence of

FIG 10 Obligate growth of *S. oneidensis* $\Delta fccA$ and *G. sulfurreducens* in basal coculture medium (CM) containing 7 mM glycerol and 60 mM fumarate (CMGF, closed symbols) or coculture medium supplemented only with 60 mM fumarate (CMF, open symbols). (A) Growth measured as OD_{600} for the *S. oneidensis* $\Delta fccA$ -*G. sulfurreducens* coculture (blue) and for *S. oneidensis* $\Delta fccA$ (green) and *G. sulfurreducens* (red) cultured singly. (B) Growth measured as CFU/ml media for *S. oneidensis* $\Delta fccA$ (green) and *G. sulfurreducens* (red) cocultures. Reported values are averages for triplicate experiments with error bars representing SEM.

obligate interaction in multispecies communities. While little is known about how cooperation emerges in natural environments, adaptive genetic loss or mutation may lead to metabolic synergy and context-dependent obligate interaction (40, 43, 53, 54).

To engineer cooperation between *Shewanella* and *Geobacter*, we began by constructing a commensal, one-way cross-feeding mechanism into the community. Incorporation of the pGUT2 plasmid enabled *S. oneidensis* to metabolize glycerol and secrete acetate as a carbon source for *G. sulfurreducens* (Fig. 1). Because *G. sulfurreducens* could not utilize glycerol, it was reliant on *S. oneidensis* to produce its carbon source. Both species coupled oxidative metabolic reactions to the respiration of fumarate (Fig. 1 and 3). Complete oxidation of glycerol required the presence of both species, and highest growth yield per mole glycerol used was achieved when both *S. oneidensis*/pGUT2 and *G. sulfurreducens* were cultured together (Fig. 2A). Selective plating enabled monitoring of individual species within cocultures and revealed, as predicted, that growth of *G. sulfurreducens* in basal coculture medium containing glycerol and fumarate required the presence and metabolic activity of *S. oneidensis*, as *G. sulfurreducens* was unable to grow in this medium alone (Fig. 2B). *S. oneidensis*/pGUT2 growth was similar when cultured alone or in coculture with *G. sulfurreducens*, indicating that *S. oneidensis* did not receive an appreciable benefit from consumption of its waste product acetate by *G. sulfurreducens* (Fig. 2B).

A deletion of the fumarate reductase (*fccA*) in *S. oneidensis*/pGUT2, leaving it unable to respire fumarate, shifted the engineered coculture from a commensal to an obligate interaction, as neither *S. oneidensis* $\Delta fccA$ /pGUT2 nor *G. sulfurreducens* was able to grow in basal coculture medium containing glycerol and fumarate alone (Fig. 4A). Surprisingly, growth was also seen for the obligate coculture in medium containing only fumarate (Fig. 4A). Analysis of *S. oneidensis* $\Delta fccA$ /pGUT2-*G. sulfurreducens* coculture supernatants revealed that glycerol was not utilized (Fig. 5A) and activity mediated by the pGUT2 plasmid was not required for obligate coculture growth (Fig. 10A). Instead, fumarate was converted to malate, and malate was subsequently consumed by the *S. oneidensis* $\Delta fccA$ /pGUT2-*G. sulfurreducens* coculture, highlighting a complete shift in metabolic strategy between the commensal and obligate cocultures (Fig. 5A and B). Mutations inhibiting acetate production (Δpta) or malate uptake into central

FIG 11 Metabolite profiles in *S. oneidensis* $\Delta fccA$ -*G. sulfurreducens* cocultures in (A) CMGF medium or (B) CMF medium or in *S. oneidensis* $\Delta fccA$ cultured alone in (C) CMGF medium or (D) CMF medium. Metabolites include glycerol (green), fumarate (blue), malate (orange), and succinate (purple). Media contained 7 mM glycerol and/or 60 mM fumarate as indicated. Reported values represent mean $\pm$ SEM metabolite concentrations (mM) measured for three independent experiments.

metabolic intermediates ($\Delta maeB$ $\Delta sfcA$ $\Delta pckA$) in the *S. oneidensis* $\Delta fccA$ /pGUT2 background significantly inhibited growth with *G. sulfurreducens* (Fig. 6A and Fig. 8A). Inhibition of growth by cocultures containing *S. oneidensis* $\Delta fccA$ /pGUT2 with additional deletion of *pta*, or combined *maeB/sfcA/pckA* deletions, indicates that obligate coculture growth likely involved malate uptake by *Shewanella* leading to production of either pyruvate or PEP subsequently metabolized to acetate, which then served as the carbon source for *G. sulfurreducens*. Interestingly, malate uptake and catabolism by *Shewanella* is not redox balanced as outlined above, and the presence of *G. sulfurreducens* to accept reducing equivalents from *Shewanella* strains may explain coculture growth. Transfer of reducing equivalents between species enabling the catabolic breakdown of compounds that neither species alone can digest is a hallmark of many syntrophic communities (9–11, 55).

Here, we have generated a commensal coculture using a strain of *S. oneidensis* engineered to utilize glycerol and provide acetate to *G. sulfurreducens*. Both strains used

TABLE 2 Strains used in this study

Strain	Description	Reference
<i>Escherichia coli</i>		
UQ950	DH5 α host for cloning: <i>F</i> -(<i>argF-lac</i>)169 80 <i>dlacZ</i> 58(M15) <i>glnV44</i> (AS) <i>rfdD1</i> <i>gyrA</i> 96(NalR) <i>recA1</i> <i>endA1</i> <i>spoT1</i> <i>thi-1</i> <i>hsdR17</i> <i>deoR</i> <i>pir</i> +	23
WM3064	DAP auxotroph used for conjugation: <i>thrB1004</i> <i>pro</i> <i>thi</i> <i>rpsL</i> <i>hsdS</i> <i>lacZ</i> M15 RP4-1360 (<i>araBAD</i>)567 <i>dapA1341::[erm</i> <i>pir</i> (wt)]	23
<i>Shewanella oneidensis</i>		
MR-1	Wild type	51
pGUT2	Wild type <i>Shewanella</i> containing the glycerol utilization plasmid	33
$\Delta fccA$ /pGUT2	<i>Shewanella</i> containing the glycerol utilization plasmid; in-frame deletion of the fumarate reductase <i>fccA</i>	This study
$\Delta fccA \Delta pta$ /pGUT2	<i>Shewanella</i> containing the glycerol utilization plasmid; in-frame deletions of the fumarate reductase <i>fccA</i> and phosphotransacetylase <i>pta</i>	This study
$\Delta fccA \Delta maeB \Delta sfcA \Delta pckA$ /pGUT2	<i>Shewanella</i> containing the glycerol utilization plasmid; in-frame deletions of genes encoding the fumarate reductase <i>fccA</i> , malic enzymes <i>maeB</i> and <i>sfcA</i> , and phosphoenolpyruvate carboxykinase <i>pckA</i>	This study
<i>Geobacter sulfurreducens</i>		
PCA	Wild type	50

fumarate in the commensal coculture, but cooperation between the species was demanded when we introduced a respiratory mutation in the engineered *S. oneidensis* strain. The metabolic network for growth shifted dramatically in the obligate coculture, illustrating how metabolic pathways play a key role in driving microbial interactions in the environment.

MATERIALS AND METHODS

Reagents. Enzymes were purchased from New England Biolabs (Ipswich, MA). Kits for PCR cleanup, gel purification, and plasmid preparations were purchased from Invitrogen (Carlsbad, CA). Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), with the exception of glycerol, which was purchased from Fisher Chemical (Pittsburgh, PA). Medium components were purchased from Becton, Dickinson and Company (Sparks, MD).

Bacterial strains and culture conditions. Strains and plasmids used in this study are listed in Table 2. Plasmid pGUT2 has been described previously (33). *S. oneidensis* and *E. coli* strains were maintained at 30°C and 37°C, respectively, on lysogeny broth (LB) medium solidified with 1.5% agar and supplemented with 50 μ g/ml kanamycin when required. *G. sulfurreducens* was maintained at 30°C under strict anaerobic conditions using an anaerobic chamber with a 5% H₂/75% N₂/20% CO₂ atmosphere (Coy Lab Products; Grass Lake, MI) on basal medium (NB) plates containing the following per liter: 0.38 g KCl, 0.2 g NH₄Cl, 0.069 g NaH₂PO₄·H₂O, 0.04 g CaCl₂·2H₂O, 0.2 g MgSO₄·7H₂O, 2.0 g NaHCO₃ (pH 6.8). The plates also contained 10 ml/liter of a mineral mix (56), were supplemented with 0.1% Trypticase (wt/vol), 1 mM cysteine, 20 mM acetate, and 40 mM fumarate (NBFA+TC), and were solidified with 1.5% agar.

Generation of deletion mutants. Oligonucleotide primers used to amplify portions of the *S. oneidensis* chromosome for deletion constructs are listed in Table 3. PCR products were cloned using standard laboratory molecular biology protocols. Regions flanking deletion targets were amplified using PCR and cloned into the pSMV3 suicide vector (57). In-frame gene deletions were generated using homologous recombination as previously described (58). Deletion constructs were moved into *S. oneidensis* by conjugal transfer from *E. coli* donor strain WM3064 (23). All plasmid constructs and gene deletions were verified by sequencing (University of Minnesota Genomics Center).

Coculture growth assays. All experiments were initiated with single colonies picked from freshly streaked –80°C 15% glycerol or 10% dimethyl sulfoxide (DMSO) stocks on LB containing 50 μ g/ml kanamycin or NBFA+TC plates for *S. oneidensis* and *G. sulfurreducens*, respectively. In preparation for growth assays, single colonies of *S. oneidensis* were grown shaken at 30°C in LB containing 50 μ g/ml kanamycin for 16 h and were then subcultured into *Shewanella* basal medium (SBM) containing 7 mM glycerol for 6 h [pH 7.2; containing per 1 liter of medium: 0.225 g K₂HPO₄, 0.225 g KH₂PO₄, 0.46 g NaCl, 0.225 g (NH₄)₂SO₄, 0.117 g MgSO₄·7H₂O, 10 mM HEPES buffer, supplemented with 5 ml/liter vitamin mix and 5 ml/liter mineral mix (56)]. Single colonies of *G. sulfurreducens* were grown in an anaerobic chamber (5%:75%:20% H₂:N₂:CO₂) in 1 ml of basal medium (NB) supplemented with 20 mM acetate and 40 mM fumarate (NBFA) for 48 h at 30°C. Following this initial growth, 1 ml of *G. sulfurreducens* was subcultured into 10 ml of NBFA medium in anaerobic culture tubes under an 80%:20% N₂:CO₂ headspace 24 h prior to coculture experiments.

For coculture growth assays, *S. oneidensis* and *G. sulfurreducens* cultures were washed in their respective basal media, normalized to an optical density measured at 600 nm (OD₆₀₀) of 1, and subcultured in 15 ml of coculture medium (CM) which contained per liter 0.38 g KCl, 0.2 g NH₄Cl, 0.069 g NaH₂PO₄·H₂O, 0.04 g CaCl₂·2H₂O, 0.2 g MgSO₄·7H₂O, and 2.0 g NaHCO₃ as buffer (pH 7.0), supplemented with 10 ml/liter of mineral mix, 10 ml/liter vitamin mix (56), 0.05% (wt/vol) Casamino Acids, 7 mM glycerol (+G), and 55 mM fumarate (+F) where indicated (CMGF, CMG, CMF). Cultures were grown stationary under a 20% CO₂/80% N₂ headspace using butyl rubber-stoppered glass tubes (Bellco; Vineland, NJ). Culture OD₆₀₀ was monitored using a spectrophotometer (Spectronic 20D+; Thermo Fisher Corporation, Waltham, MA).

TABLE 3 Primers used in this study

Primer name	DNA sequence (5' to 3') or reference ^a
Primers used to delete <i>fccA</i>	
FccA_1	NNactagtTGCAGCGGTGCTATTAAG
FccA_2	NNgaattcATTGCGCCAGAGATCAG
FccA_3	NNgaattcATCGCGGTGCATCTGCC
FccA_4	NNgagctcATGGCAGGCTGATAGGC
Primers used to delete <i>pta</i>	
	35
Primers used to delete <i>maeB</i>	
maeB_Up_F	CGATggatccGTGGTGCATGTACCCATTCTACAC
maeB_Up_R	CGATactagtGTGGCTCGTTTCAGCGGGTTTAG
maeB_Dn_F	CGATactagtGAAGCGGTACCTGCAAGTGTC
maeB_Dn_R	CGATgagctcGTCATACTGAGGCCCGACTGAG
Primers used to delete <i>sfcA</i>	
sfcA_Up_F	CGATggatccGGCGGTAATCAATGGCAA
sfcA_Up_R	CGATactagtGGGCAAATTCGACCAAGTT
sfcA_Dn_F	CGATactagtAAAAGTGGCCGAGCTTT
afca_Dn_R	CGATgagctcTGTGCCATCGAGTCTT
Primers used to delete <i>pckA</i>	
pckA_Up_F	CGATggatccGGCGGTAATCAATGGCAA
pckA_Up_R	CGATactagtGGGCAAATTCGACCAAGTT
pckA_Dn_F	CGATactagtAAAAGTGGCCGAGCTTT
pckA_Dn_R	CGATgagctcAAGACTCGATGGCACA

^aLowercase letters indicate restriction sites.

CFU to monitor growth. Enumeration of cells in growth experiments was accomplished by plating for CFU. At the time points indicated, 100- μ l culture samples were removed using degassed syringes, and serial dilutions were performed in LB or CMF medium to prevent cell lysis for *S. oneidensis* and *G. sulfurreducens*, respectively. Several dilutions were plated onto solid medium as described below. Plates with readily countable colonies (~50 to 200/plate) were analyzed. To specifically enumerate *S. oneidensis*, LB dilutions were plated and grown aerobically on LB plates. To specifically enumerate *G. sulfurreducens*, CMF dilutions were plated and grown anaerobically on NBFA+TC plates (75% N₂/20% CO₂/5% H₂) at 30°C (22).

High-performance liquid chromatography. Metabolites were quantified by HPLC using Shimadzu Scientific equipment, including an SCL-10A system controller, LC-10AT pump, SIL-10AF auto-injector, CTO-10A column oven, RID-10A refractive index detector (cell maintained at 40°C), and SPD-10A UV-Vis detector (210 nm). Mobile phase consisted of 0.015 N H₂SO₄ set at a flow rate of 0.400 ml min⁻¹. Injection volumes of 50 μ l were separated on an Aminex HPX-87H column maintained at 46°C. Metabolite concentrations were determined by comparison with standard curves for each compound ($R^2 > 0.99$). Metabolite standards were run before and after unknown samples to ensure accuracy.

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