SYNTROPIC INTERSPECIES ELECTRON TRANSFER DRIVES CARBON FIXATION AND GROWTH BY *RHODOPSEUDOMONAS PALUSTRIS* UNDER DARK, ANOXIC CONDITIONS

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In natural anoxic environments, anoxygenic photosynthetic bacteria fix CO₂ by photoheterotrophy, photoautotrophy, or syntrophic anaerobic photosynthesis. Here, we describe electroautotrophy, a previously unidentified dark CO₂ fixation mode enabled by the electrosyntrophic interaction between *Geobacter metallireducens* and *Rhodopseudomonas palustris*. After an electrosyntrophic coculture is formed, electrons are transferred either directly or indirectly (via electron shuttles) from *G. metallireducens* to *R. palustris*, thereby providing reducing power and energy for the dark CO₂ fixation. Transcriptomic analyses demonstrated the high expression of genes encoding for the extracellular electron transfer pathway in *G. metallireducens* and the Calvin-Benson-Bassham carbon fixation cycle in *R. palustris*. Given that sediments constitute one of the most ubiquitous and abundant niches on Earth and that, at depth, most of the sedimentary niche is both anoxic and dark, dark carbon fixation provides a metabolic window for the survival of anoxygenic phototrophs, as well as an as-yet unappreciated contribution to the global carbon cycle.

INTRODUCTION

Microbial anoxygenic photosynthesis is hypothesized to play an important role in the global carbon cycle by providing a mechanism for light-driven carbon fixation in aquatic environments (1–3). Anoxygenic photosynthetic bacteria include a diverse group of bacteria that are metabolically versatile, with nutrition types ranging from autotrophy to heterotrophy, from organotrophy to lithotrophy, and, last, from chemotrophy to phototrophy (4, 5). Under illumination, anoxygenic photosynthetic bacteria fix CO₂ and grow autotrophically, and it is generally recognized that light and electron donors are the two key environmental components required for CO₂ fixation by these bacteria, with light serving as the energy source in governing adenosine 5’-triphosphate (ATP) synthesis and driving the formation of reducing power as NADPH [reduced form of nicotinamide adenine dinucleotide phosphate (NADP⁺)] for CO₂ reduction with extracellular electron donors providing electrons (6, 7). Inorganic compounds, such as H₂, S₂O₃²⁻, and S²⁻, are generally used as electron donors. In addition, some species can extract electrons from organic compounds, such as organic acids, while others have the ability to accept electrons from solid phases, such as ferrous sulfides and/or cathodic electrodes (1, 6, 8, 9).

Direct electric syntrophy between bacterial species involves a direct electron cross-feeding process (10, 11). Electrons are transported between species via outer-membrane multitheme cytchromes, conductive structures, and/or electron mediators (11–13). This process is in contrast to conventional syntrophy, which is driven by hydrogen or formate diffusion (14, 15). These electrons could also be electron sources to support anoxygenic photosynthesis. For example, under illumination, electrons released by *Geobacter sulfurreducens* could be used by *Prosthecochloris aestuarii* for photosynthesis (6). Therefore, a previously unidentified form of autotrophy by syntrophic anoxygenic photosynthesis was suggested (6). Notably, *P. aestuarii* are green sulfur bacteria that are able to perform noncyclic photophosphorylation with oxidation of various electron donors to replenish electrons in the photosynthetic reaction center in the presence of light, which is necessary to serve as an energy source. Therefore, it is not surprising that electrons produced by *G. sulfurreducens*, acting as electron sources but not energy sources, could directly enter the photosynthetic reaction center, thereby contributing to the phototrophic growth of *P. aestuarii*. Moreover, these electrons that transferred between species also can drive the energy generation of individual bacterial species (10–12) and can provide reducing power for CO₂ reduction in some species. For example, electrons generated by *Geobacter metallireducens* were demonstrated to drive CO₂ reduction for methanogenesis by *Methanoseta harundinacea* after forming a direct electric syntrophic coculture (16).

*Rhodopseudomonas palustris* is a model purple nonsulfur bacterial species that performs cyclic photophosphorylation, a process producing ATP and contributing to reducing power formation under illumination and requiring no exogenous electrons (17), and it reduces CO₂ through the Calvin-Benson-Bassham (CBB) cycle. In addition, in darkness, it is suggested that *R. palustris* can carry out chemoaotrophic growth with CO₂ fixation by coordinating H₂ oxidation coupled with O₂ reduction (17). However, in anoxic environments, such as fresh water and marine sediments, CO₂ fixation of *R. palustris* can only be observed under light through either photosynthesis or photoheterotrophy (1, 8, 18) but cannot be achieved via chemoaotrophy. Therefore, light is necessary as an energy source, providing ATP and reducing power, for autotrophic growth of *R. palustris* in the absence of oxygen. A recent study demonstrated that *R. palustris* can couple extracellular electron uptake from poised electrodes with the generation of reducing equivalents, NADPH, via reverse electron flow through the cyclic photosynthetic apparatus (7). Notably, this process is light independent, suggesting that when specific electron-donating sources are provided to induce the accumulation of reducing power, if *R. palustris* were to produce enough ATP simultaneously, it would also be able to grow autotrophically in the dark.

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Investigating this possibility, we have demonstrated a new type of autotrophy, electroautotrophy, a previously unrecognized mode of dark carbon fixation in *R. palustris*. Nitrate was chosen as an electron acceptor to induce anaerobic respiration for ATP generation, and either the natural electron donor $\text{S}_2\text{O}_3^{2-}$ and $\text{H}_2$ or the electron-donating strain of *G. metallireducens* was provided as an electron source to drive autotrophic CO$_2$ fixation of *R. palustris* in the dark. The results showed that neither $\text{S}_2\text{O}_3^{2-}$ nor $\text{H}_2$ oxidation could supply the energy requirement for dark carbon fixation. In contrast, electrons provided by *G. metallireducens* were shown to support autotrophic growth of *R. palustris* in darkness. Specifically, *G. metallireducens* and *R. palustris* formed a direct electric syntrophic coculture. The electrons were transferred either by direct physical contact or indirectly by electron shuttles and provided the reducing power for CO$_2$ reduction and ATP generation. The discovery of electroautotrophy in *R. palustris* highlights the possibility of anaerobic dark carbon fixation in conventional photosynthetic bacteria, broadening the ecological niche of anoxygenic photosynthetic bacteria and helping to elucidate global carbon cycling.

**RESULTS**

**Autotrophic growth of *R. palustris* in a dark and anoxic environment**

*Rhodopseudomonas* species can perform chemoautotrophy by oxidizing hydrogen and transferring electrons to oxygen for ATP generation, as previously suggested (17, 19). Nitrate was chosen as the anaerobic electron acceptor to induce anaerobic chemoautotrophy. However, initial attempts to induce chemoautotrophic growth of *R. palustris* with either hydrogen or thiosulfate as the electron and energy source under darkness failed unless light was reintroduced, thereby shifting the metabolism to photoautotrophy (fig. S1).

*G. metallireducens* is a heterotrophic bacterium and has been widely used as an electron donor strain to initiate electroautotrophic growth in concert with other species by direct contact after forming aggregates. It has been reported that *G. metallireducens* cannot continuously reduce nitrate when the iron concentration in the growth medium is low (<50 $\mu$M) (20). Similarly, *R. palustris* can take up electrons via extracellular electron transfer (7, 18) but cannot catabolize acetate under dark conditions. To test the possibility of electroautotrophic dark CO$_2$ fixation, we initiated a coculture of *G. metallireducens* and *R. palustris* in a coculture medium, with acetate serving as the electron donor and with/without nitrate serving as the electron acceptor. As shown in Fig. 1A, these two species gradually formed aggregates, similar to other electric syntrophic cocultures (10, 11). In contrast, these two species could not form cocultures in the absence of nitrate (fig. S2). These results demonstrated synergistic metabolism between these two species and suggested the possibility of electric syntrophic dark carbon fixation of *R. palustris*. Unexpectedly, *G. sulfurreducens*, another efficient electron-donating species that could oxidize acetate for electron generation but could not reduce nitrate, could not form cocultures with *R. palustris* (fig. S3), although it could serve as an electron donor to support anoxygenic phototrophy of *P. aestuarii* (6).

Metabolic assessment provided further evidence of syntrophic autotrophic growth. As indicated, neither *R. palustris* nor *G. metallireducens* could grow in the coculture medium alone, because neither acetate nor nitrate was metabolized (Fig. 1B). In contrast, when *G. metallireducens* and *R. palustris* were both present in the coculture medium, after 8 days, approximately 15 mM acetate and approximately 20 mM nitrate were metabolized, indicating a syntrophic interaction between *G. metallireducens* and *R. palustris* (Fig. 1C). In addition, both species gradually accumulated in the coculture medium and achieved a final ratio of 33:17 (*G. metallireducens* to *R. palustris*) (Fig. 1D). As acetate cannot be assimilated by *R. palustris* under darkness and CO$_2$ is the only candidate carbon source in the coculture medium, this result indicated that *R. palustris* might perform autotrophic growth in coculture. To test this hypothesis, the coculture medium was amended with $^{13}$C-labeled NaHCO$_3$ and inoculated with the coculture. As controls, monocultures of *R. palustris* and *G. metallireducens* and a heat-killed coculture incubated under the same conditions were included. When arriving at the stationary phase, cells were harvested for stable isotope analysis. As shown in Fig. 1E, for the live coculture, the $\delta^{13}$C value of microbial biomass from $^{13}$C-labeled NaHCO$_3$ fixation reached 8860.27%. However, the $\delta^{13}$C values for the monocultures of *R. palustris* and *G. metallireducens* and heat-killed cocultures were almost equal to that of the coculture grown in $^{12}$C-NaHCO$_3$ medium. Therefore, these results indicated that *G. metallireducens* drove CO$_2$ fixation by *R. palustris* under dark and anoxic conditions by forming a “coculture,” or perhaps this phenomenon should be described as a long-distance electron transfer cometabolism.

**Electroautotrophy of *R. palustris* by forming cocultures with *G. metallireducens***

Both *G. metallireducens* and *R. palustris* can perform extracellular electron transfer (7, 11, 18, 21). To test the theoretical feasibility of
electric syntrophy via direct interspecies electron transfer (DIET) between *G. metallireducens* and *R. palustris*, linear sweep voltammetry (LSV) was performed, as previously described (22). As indicated in Fig. 2 (A and G), *metallireducens* could transfer electrons outward in a potential range of over −0.332 V (versus standard hydrogen electrode (SHE)), while *R. palustris* could accept electrons inward in a potential range of below 0.929 V. These results suggest that *G. metallireducens* could transfer electrons to *R. palustris* within a wide potential window ranging from −0.332 to 0.929 V if those two species were electrically connected. The possibility of DIET between these two species was further confirmed using a two-chamber microbial fuel cell (MFC), as previously described (Fig. 2B) (23). In the MFCs, a proton exchange membrane was used to separate each chamber. As shown in Fig. 2B, the voltage gradually increased with time, and cells in both chambers grew, forming metabolically active biofilms on both electrodes accordingly (fig. S4), which indicated effective direct electron transfer between those two species, because only protons can pass between those two chambers.

CO₂ fixation is an energy-consuming process that requires the investment of reducing power and chemical energy (e.g., ATP). It was previously determined that *R. palustris* was able to accept electrons from an electrode directly under illumination and subsequently generated reducing power for carbon fixation (7). The possibility that extracellular electrons could drive the reduction power and ATP generation in *R. palustris* under darkness was investigated by using the simulated electron source of a cathode to support autotrophic growth in *R. palustris* (fig. S5). After inoculation, *R. palustris* oxidized the cathode gradually (Fig. 2C), and reducing power accumulated correspondingly with the simultaneous generation of ATP (Fig. 2D). In particular, the log₂ ratio of NADPH/NADP⁺ (18.44 ± 0.23) was higher than that of NADH [reduced form of nicotinamide adenine dinucleotide (NAD⁻)]/NAD⁺ (2.02 ± 0.15) (Fig. 2D), suggesting that extracellular electrons could provide efficient reducing power to drive active carbon fixation in *R. palustris*.

In addition to aggregates, some planktonic cells were observed in the coculture medium (Fig. 1A), indicating the possibility of mediated IET (MIET) between *G. metallireducens* and *R. palustris*, as previously reported (12). To test this possibility, the *G. metallireducens* mutant strain G.m-ΔGmet2896, deficient in Gmet_2896, was recruited to coculture with *R. palustris*. Gmet_2896 cytochrome was thought to be the key electron outlet of *G. metallireducens* to transfer electrons directly to other species after forming DIET coculture, and its mutant could not perform direct electric syntrophy (11, 12). Expectedly, both species formed a syntrophic coculture with nitrate metabolism (1.91 ± 0.07 mM day⁻¹) more slowly than the wild-type coculture (2.47 ± 0.11 mM day⁻¹) (Fig. 3A) due to the fact that DIET is more efficient than MIET (14, 24). To further identify MIET, cultures of *G. metallireducens* and *R. palustris* were physically separated by using a dialysis membrane with a molecular weight cutoff of 1 kDa (fig. S6) to prevent direct contact between those two species but not to affect the exchange of culture medium (including supposed electron mediators). As shown in Fig. 3B, those two physically separated species still performed syntrophic growth by steadily catabolizing acetate for the reduction of nitrate, indicating the presence of electron shuttles to facilitate electron transfer between those two species. It has been reported that hydrogen and formate can mediate IET (25, 26) but neither was detected in the culture medium, and it has been reported that hydrogen cannot support the formation of coculture between *G. metallireducens* and other bacterial species (16, 27). In addition, hydrogen could not support the growth

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Fig. 2. Direct electric syntrophy between *G. metallireducens* and *R. palustris*. (A) LSV analysis of *G. metallireducens* and *R. palustris*. (B) Voltage response of the two-chamber MFCs with an external resistance of 1 megohm. The inset shows the setup of two-chamber MFCs. (C) Extracellular electron uptake from the electrode poised at a potential of 0.1 V (versus SHE) by *R. palustris* under darkness. The electrolyte was NB medium supplied with 30 mM nitrate. (D) NADH/NAD⁺, NADPH/NADP⁺ ratio, and ATP concentrations of *R. palustris* when the cathode was poised at 0.1 V or unpolarized at open circuit. **P < 0.01, n = 3 for each group.
of *R. palustris* by synchronizing nitrate reduction in darkness (fig. S1A). The possibility of the presence of redox cofactors was verified by performing differential pulse voltammetry (DPV) on the spent culture medium from the physically separated culture. The differential pulse voltammogram showed that there were at least three redox active species present (Fig. 3C) with redox peak potentials of $-0.45$, $-0.2$, and $-0.08$ V sequentially, which were absent in the blank culture medium (fig. S7A). In particular, the peak around $-0.2$ V was the most prominent and should represent the mediator primarily participating in the IET, because it is in the potential window (from $-0.332$ to $0.9$ V) allowing IET between *G. metallireducens* and *R. palustris* and is low enough to drive reducing power generation. Furthermore, the peak current at $-0.2$ V increased with culture time (fig. S8). Previous studies indicate that riboflavin also has a redox potential of approximately $-0.2$ V and can accumulate in the coculture medium and mediate electron transfer between *Geobacter* species (12). Coincidently, fluorescence spectrometry analysis of the cell-free culture medium showed an emission at $525$ nm under excitation at $370$ and $440$ nm, a characteristic signal of flavins (Fig. 2D) (12, 28). Furthermore, further mass spectrometry analysis detected riboflavin in the spent culture medium (fig. S9). Actually, a concentration of approximately $30$ nM riboflavin was calculated from the fluorescence excitation spectrum in the spent culture medium, and supplementation with $30$ nM riboflavin accelerated the syntrophic growth of the two species (fig. S10). Meanwhile, riboflavin was also detected in the spent coculture medium (figs. S8 and S11). These results suggested that riboflavin was probably involved in MIET in *G. metallireducens* and *R. palustris* coculture.

**Syntrophic electroautotrophy between *G. metallireducens* and *R. palustris***

Triplicate *G. metallireducens* and *R. palustris* cocultures were collected for gene expression analysis to further study electrosyntrophic electroautotrophy. As shown in Fig. 4 and data S1, genes encoding enzymes involved in acetate oxidation were highly expressed in *G. metallireducens*; in contrast, genes involved in acetate oxidation in *R. palustris* were not highly expressed. Similarly, genes encoding functions related to nitrate reduction in *R. palustris* were highly expressed, and genes encoding enzymes for nitrate reduction in *G. metallireducens* were not highly expressed. These results are consistent with the hypothesis that *G. metallireducens* was responsible for acetate oxidation, *R. palustris* was in charge of nitrate reduction in the coculture, and these two species formed syntrophic cocultures. In addition, the complete CO$_2$ fixation pathway of the CBB cycle in *R. palustris*, especially genes encoding RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) form II, was highly expressed, although not for the acetate assimilation pathway (Fig. 4B), indicating autotrophic growth of *R. palustris* in the coculture. Notably, RuBisCo form I was not highly expressed, in contrast to the previous finding that RuBisCo form I was highly expressed and was the primary enzyme used to assimilate CO$_2$ during photoautotrophic growth (6, 18).

Electrons were not transferred from *G. metallireducens* to *R. palustris* via hydrogen or formate in the coculture, as indicated by gene expression profiles showing that those genes encoding functions responsible for hydrogen and formate production were not highly expressed (data S1). *G. metallireducens* has been demonstrated to transfer electrons to other species via structures of c-type cytochromes and conductive nanowires (11, 12, 29). In addition, flagella could...
also facilitate DIET (29, 30). All of the genes encoding these determinants were actively expressed in the coculture, further indicating DIET. Genes encoding flavin biosynthesis and flavin exporters were also highly expressed in G. metallireducens, suggesting that flavins facilitated IET in the coculture (12).

Previous studies indicated that the PioABC complex was directly involved in taking up extracellular electrons for photoferrotrophic and photoautotrophic growth of R. palustris (7, 18, 31, 32). However, this pathway was neither actively expressed in the coculture (Fig. 4B) nor highly expressed in the cathode biofilm grown in darkness (fig. S12). These results suggested that R. palustris was able to recruit different electron uptake pathways to accept extracellular electrons, depending on the presence of dark or light conditions, and were corresponding to previous reports showing that the expression of the PioABC complex was stimulated by light (7, 18). In addition, the data also suggested that the cycling of Fe³⁺/Fe²⁺ (minimum iron contained in culture medium) should not contribute to the electron exchange between G. metallireducens and R. palustris, as PioABC was required for ferrous iron oxidation by R. palustris (31).

Similar to PioABC, it was reported that Mtr-like proteins, including c-type cytochromes and porins, usually constitute the electron transfer pathway on the outer membrane to exchange electrons with extracellular electron acceptors/donors in electroactive bacteria (33, 34). However, aside from PioABC, no other Mtr complex–like analogs could be identified in R. palustris strain CGMCC 1.2180. In contrast, nanofilaments of pili and flagella were highly expressed (Fig. 4A). It was reported that the filaments of R. palustris are conductive (35), and pili and flagella are important in IET (14, 29). Therefore, nanofilaments of R. palustris are likely to participate in the efficiency of direct electron uptake from G. metallireducens in electroautotrophic coculture.

Cytochrome c2 was highly expressed in R. palustris (Fig. 4). Cytochrome c2 is a periplasmic protein and can enable electron transport from complex III to the photosystem under illumination (36, 37). Considering that complex III can catalyze ubiquinone cycling and was highly expressed, the primary electron uptake process performing electroautotrophy by R. palustris is suggested to be cytochrome c2 transporting electrons inward through the periplasm to complex III, thereby driving the generation of ubiquinol (Fig. 4B) (7). Meanwhile, nitrate reductase catalyzed the oxidation of ubiquinol for the reduction of nitrate, as indicated by the high expression of nitrate reductase, generating a transmembrane gradient.

Moreover, genes encoding energy metabolism functions were highly expressed, including complex I and adenosine triphosphatase (ATPase), consistent with the autotrophic metabolism that requires energy investment. Therefore, the proton motive force not only drove the oxidation of ubiquinol for the reduction of NAD(P)⁺ to generate reducing equivalents (38, 39) but also energized ATPase to produce ATP. Both NADPH and ATP entered the CBB cycle, thereby providing reducing power and energy for the autotrophic growth of R. palustris. In support of this mechanism, blocking the electron transport pathway by addition of the inhibitor antimycin A, carbonyl cyanide m-chlorophenyl hydrazine (CCCP), or rotenone inhibited the electron uptake of R. palustris (fig. S13).

**DISCUSSION**

Our findings identify a previously unknown type of autotrophic metabolism in anoxygenic photosynthetic bacteria that enables them to survive in dark and anoxic environments after forming an electric syntrophic coculture with *Geobacter* species. It is generally recognized that under anoxic conditions, light is necessary to provide energy to drive CO₂ fixation either phototrophically or photoautotrophically (Fig. 5). In this study, we demonstrated in dark-grown *R. palustris* a third CO₂ fixation mode, which we call syntropic electroautotrophy. In addition, both DIET via cytochromes and/or conductive filaments and riboflavin-mediated IET were indicated to achieve extracellular electron-driven syntropic electroautotrophy of *R. palustris*.

These results demonstrate that the species of *G. metallireducens*, acting as electron donor, was able to provide both reducing power and energy for CO₂ fixation of *R. palustris* under dark and anaerobic conditions. This result is consistent with the fact that *G. metallireducens* is widely used as an electron-donating partner that is able to form
electric syntrophic cocultures with other species (10, 16, 40) and to drive CO$_2$ reduction (16, 40), suggesting that _G. metallireducens_ could act as a model species to find new electric syntrophic cocultures. Although some anoxygenic photosynthetic bacterial species can perform chemoelectroautotrophic growth after respiring oxygen under dark conditions (19, 41), their chemoelectroautotrophy was inhibited in an anaerobic environment, even in the presence of oxidized electron acceptors. However, our study suggests that either poised electrodes or specific electron-donating species could drive carbon fixation in dark, anaerobic environments. The reason for this result might be the lower energy barrier to direct uptake of electrons from electrodes or other bacterial species compared with electron uptake via metabolic oxidation of reduced substrates. Unexpectedly, _G. sulfurreducens_ could not support electroautotrophy of _R. palustris_. It could be attributed to the much lower redox potential of extracellular electron transfer pathway from _G. metallireducens_ than that from _G. sulfurreducens_, and is consistent with the fact that _G. sulfurreducens_ also could not act as electron-donating species to transfer electrons inversely to _G. metallireducens_ to form electroautotrophic coculture (fig. S14). Anoxic and dark sediments constitute one of the most ubiquitous and abundant niches on Earth and are abundant in organic acids and oxidized inorganic substances. Considering the coexistence and domination of _Geobacter_ species and anoxygenic photosynthesis bacteria in dark sediment (42), the anaerobic electroautotrophy of _R. palustris_ suggests a survival strategy of anoxygenic photosynthetic bacteria in dark and anoxic environments and provides a new insight to understand the global carbon cycle.

**MATERIALS AND METHODS**

**Microorganisms and growth medium**

_G. metallireducens_ wild-type strain GS15 (American Type Culture Collection 33774) and mutant strain GS15_AGmet2896 deficient in Gmet_2896 cytochrome (a tetraheme c-type cytochrome important in extracellular electron transfer) were both obtained from frozen stocks in our laboratory (11). _R. palustris_ strain CGMCC 1.2180 was purchased from the China General Microbiological Culture Collection Center. Pure cultures of _G. metallireducens_ wild-type or mutant strain were pregrown at 30°C in FCA medium, as previously described (12, 21). _R. palustris_ was precultured at 30°C in 0.259 medium containing KH$_2$PO$_4$ (1 g liter$^{-1}$), CaCl$_2$ (0.1 g liter$^{-1}$), NaHCO$_3$ (3 g liter$^{-1}$), MgCl$_2$ (0.5 g liter$^{-1}$), NH$_4$Cl (1 g liter$^{-1}$), NaCl (1 g liter$^{-1}$), acetate (1 g liter$^{-1}$), succinate (1 g liter$^{-1}$), yeast (0.5 g liter$^{-1}$), peptone (0.5 g liter$^{-1}$), trace element solution (1 ml liter$^{-1}$) (43), and vitamin solution (1 ml liter$^{-1}$) (43) under illumination by a 15-W light bulb with an optical power density of 20 W m$^{-2}$.

Cocultures of _G. metallireducens_ and _R. palustris_ were initiated by inoculating 5% (relative to the volume of coculture medium) of each pure culture in the stationary phase and anoxically incubating at 30°C in nutrient broth (NB) medium (including NaHCO$_3$) (11), with 20 mM acetate serving as the only electron donor and 30 mM nitrate serving as the electron acceptor under dark conditions. The coculture was transferred at least four times before all tests were performed. All experiments were performed in triplicate.

**Analytical techniques**

Acetate consumption by monocultures and coculture of _G. metallireducens_ and _R. palustris_ was monitored using ultrahigh-performance liquid chromatography (U-3000, Thermo Fisher Scientific, Massachusetts, USA), as described previously with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) and detected at 210 nm by an ultraviolet detector (11). Nitrate was measured with an ion chromatograph (ICS 900, Thermo Fisher Scientific), as previously described (44).

**Stable isotope analysis**

To verify dark CO$_2$ fixation, $^{13}$C-labeled NaHCO$_3$ was used as the source of CO$_2$ in the coculture medium. When _G. metallireducens_ and _R. palustris_ cocultures were grown at their stationary phase, coculture cells were collected by centrifugation at 6000g for 10 min and then dehydrated using a vacuum freeze dryer (ALPHA 1-4 LDplus, Osterode, Germany). Dry cells (4 mg) were wrapped with tinfoil and placed into stable isotope ratio mass spectrometers (Isoprime 100, Elementar, Langenselbold, Germany) for dark $^{13}$CO$_2$ fixation analysis following the manufacturers’ instructions.

**Quantification of bacterial biomass and gene expression**

Cells were quantified by quantitative polymerase chain reaction (PCR) targeting species-specific genes and absolute quantification. Target genes were cloned into the pMD19-T vector (Takara, Beijing, China) for standard curve preparation. Ten milliliters of cocultures was collected at 0, 4, 7, 10, and 12 days, and genomic DNA was extracted using a Rapid Bacterial Genomic DNA Isolation kit (Sangon Biotech, Shanghai, China) according to the manufacturer’s directions. _G. metallireducens_ cells in coculture were quantified using primer pairs Gmet_F (ATGGCCCACATCTTCATCTC) and Gmet_R (TGCArGTTTTTCACTCCAGAT). _R. palustris_ cells in coculture were quantified using the following primer pairs: RpFor: 5’-CTG-TGCATGTTTTCATCCACGAT). _R. palustris_ cells growing either in darkness or under illumination using an RNeasy Mini kit (Qiagen, Germany). The FastKing One Step RT-QPCR Kit (Tiangen, China) was used for reverse transcription with random primers. Primers used for the assays were listed in table S1. recA was used as the internal standard as described previously (18). Quantitative PCR was performed using the LightCycler 480 System (Roche Applied Science, Penzberg, Germany).

**Setup of two-chamber MFCs**

Direct electron transfer from _G. metallireducens_ to _R. palustris_ under darkness was monitored with two-chamber MFCs with a
proton exchange membrane (Nafion 117, DuPont de Nemours Inc., Delaware, USA) separating each chamber as described previously (23). The proton exchange membrane was activated by treatment with 5% hydrogen peroxide at 80°C for 1 hour and washed by soaking in deionized water for 10 min following the manufacturer’s instructions. The volume of each chamber was 30 ml, with a 25-ml liquid volume and a 5-ml mixed gas filled volume. Graphite electrodes (1.5 cm × 1 cm × 0.5 cm) were used as both the anodic and cathodic electrodes. Hg|Hg2Cl2 (sat. KCl) was used as the reference electrode. The electrolyte of the MFCs was NB medium supplied with 20 mM acetate as the electron donor and 30 mM nitrate as the electron acceptor. For the initiation of MFCs, G. metallireducens and R. palustris cells were inoculated into each chamber of MFCs with a final OD600 of 0.2. An external resistance of 1 meqohm was applied in the external circuit, and the voltage was recorded with a data acquisition system (model 2700, Keithley Instruments). Biofilms on both electrodes were treated with a LIVE/DEAD BacLight Bacterial Viability kit (Thermo Fisher Scientific) and examined by a confocal laser scanning microscope (Carl Zeiss LSM 880, Zeiss, Oberkochen, Germany).

Setup of physically separated cells
A physically separated setup was used to test the possibility of redox shuttle-mediated IET between G. metallireducens and R. palustris. The physically separated setup was a modification of the two-chamber MFCs generated by replacing a proton exchange membrane with a semipermeable membrane with a molecular weight cutoff of 1 kDa to inhibit direct connection between those two bacterial species, as previously described (12), and removing both electrodes to impede IET via an external circuit. Washed G. metallireducens or R. palustris cells were inoculated into each chamber with a final OD600 of 0.2 to initiate growth. Acetate oxidation and nitrate reduction were measured as described above.

When the physically separated cultures were in their stationary phases, culture supernatants were collected by centrifuging at 6000 g for 10 min and filtered through a 0.2-μm membrane. These supernatants were used for DPV analysis running at a pulse height of 50 mV, a pulse width of 250 ms, a step height of 2 mV, a step time of 500 ms, and a scan rate of 5 mV s⁻¹ using a CHI600 electrochemical workstation (CH Instruments, Texas, USA). The redox state of the supernatant was determined using a CHI600 electrochemical workstation at a scan rate of 1 mV s⁻¹.

Electrochemical analysis
LSV analysis was performed as previously described using a single-chamber microbial electrolysis cell with indium tin oxide coated glass as the working electrode applied at the bottom, a graphite electrode as the counter electrode, and Hg|Hg2Cl2 (sat. KCl) as the reference electrode (22). The electrolyte was NB medium supplied with 20 mM acetate for G. metallireducens or 30 mM nitrate for R. palustris. LSV was conducted with a CHI600 electrochemical workstation at a scan rate of 1 mV s⁻¹. Ethanol for 30 min at 4°C (11). Next, the samples were incubated with fluorescence probes in hybridization buffer for 2 hours at 46°C. Last, the samples were washed for 30 min in washing buffer at 48°C and rinsed briefly with Milli-Q water. The probe for G. metallireducens is 5’-(cy3)AGAAATCAGGACTCCGT-3’ and that for R. palustris is 5’-(cy5)CTTCCTAAACAGGCCAGTCTC-3’. Samples were imaged with a confocal laser scanning microscope (Carl Zeiss LSM 880).

Determination of cellular redox state and ATP concentration
Two-chamber microbial electrolysis cells with a volume of 100 ml of each chamber were constructed as previously described (45). Graphite plates (1 cm × 3 cm × 4 cm) served as the anode and cathode, and Hg|Hg2Cl2 (sat. KCl) electrode acting as the reference electrode was applied in the cathode chamber, and the electrolyte was NB medium with 30 mM nitrate supplied in the catholyte. To start electroautotrophic growth, 8 ml of R. palustris cells grown in 0.529 medium was collected and washed using 0.9% NaCl solution and subsequently inoculated into the cathode chamber with the cathode poised at 0.1 V (versus SHE) using a multichannel potentiostat (1000C, CH Instruments Inc., USA). Cells were run at 30°C under darkness, and the current was monitored accordingly.

For measurements of NAD+/NADH, NADP+/NADPH, and ATP, cells were harvested from the cathode and treated with appropriate buffers as indicated by the providers. In particular, NAD+/NADH and NADP+/NADPH ratios were determined using an NAD+/NADH Quantification kit (Millipore Sigma, Missouri, USA) and a NADP+/NADPH Assay kit (Solarbio, Beijing, China) and were detected using an absorbance spectrophotometer for a microplate reader (Eon, BioTek Instruments Inc., Vermont, USA) with detection wavelengths of 450 and 570 nm, respectively. The ATP concentration was calculated using an enhanced ATP assay kit (Beyotime Biotechnology, Shanghai, China) by measuring chemiluminescence with a luminometer plate reader (Promega Biotech Co. Ltd., Beijing, China).

Transcriptomic analysis
Triplicate cocultured cells were collected by centrifugation and stored at −80°C. Total RNA was extracted using TRIzol reagent (Invitrogen, California, USA), as described previously (29). Directional libraries were prepared with the NEBNext Ultra II Directional RNA Library Prep Kit. Coculture mRNA was subsequently sequenced with paired ends on the Illumina HiSeq/MiSeq platformsOpen. All the raw sequencing data were quality-checked and filtered. Sequence reads matching 16S and 23S ribosomal RNA genes were removed, and the remaining reads were subsequently used to map against the published genome of G. metallireducens GS15 (NC_0075171.1) and R. palustris CGMCC 1.2180 (NZ_CP058907.1). Mapped reads were normalized with FPKM (fragments per kilobase per million mapped reads) (fig. S15).

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/27/eabh1852/DC1

View/request a protocol for this paper from Bio-protocol.

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