Lack of Electricity Production by Pelobacter Carbinolicus Indicates that the Capacity for Fe(III) Oxide Reduction Does Not Necessarily Confer Electron Transfer Ability to Fuel Cell Anodes

Hanno Richter
Martin Lanthier
Kelly P Nevin, University of Massachusetts - Amherst
Derek Lovley, University of Massachusetts - Amherst

Available at: https://works.bepress.com/kelly_nevin/25/
Lack of Electricity Production by *Pelobacter carbinolicus* Indicates that the Capacity for Fe(III) Oxide Reduction Does Not Necessarily Confer Electron Transfer Ability to Fuel Cell Anodes

Hanno Richter, Martin Lanthier, Kelly P. Nevin and Derek R. Lovley

Published Ahead of Print 15 June 2007.

Updated information and services can be found at:
http://aem.asm.org/content/73/16/5347

These include:

This article cites 38 articles, 20 of which can be accessed free at: http://aem.asm.org/content/73/16/5347#ref-list-1

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more »

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
Lack of Electricity Production by *Pelobacter carbinolicus* Indicates that the Capacity for Fe(III) Oxide Reduction Does Not Necessarily Confer Electron Transfer Ability to Fuel Cell Anodes

Hanno Richter,* Martin Lanthier, Kelly P. Nevin, and Derek R. Lovley

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

Received 10 April 2007/Accepted 8 June 2007

The ability of *Pelobacter carbinolicus* to oxidize electron donors with electron transfer to the anodes of microbial fuel cells was evaluated because microorganisms closely related to *Pelobacter* species are generally abundant on the anodes of microbial fuel cells harvesting electricity from aquatic sediments. *P. carbinolicus* could not produce current in a microbial fuel cell with electron donors which support Fe(III) oxide reduction by this organism. Current was produced using a coculture of *P. carbinolicus* and *Geobacter sulfurreducens* with ethanol as the fuel. Ethanol consumption was associated with the transitory accumulation of acetate and hydrogen. *G. sulfurreducens* alone could not metabolize ethanol, suggesting that *P. carbinolicus* grew in the fuel cell by converting ethanol to hydrogen and acetate, which *G. sulfurreducens* oxidized with electron transfer to the anode. Up to 83% of the electrons available in ethanol were recovered as electricity and in the metabolic intermediate acetate. Hydrogen consumption by *G. sulfurreducens* was important for ethanol metabolism by *P. carbinolicus*. Confocal microscopy and analysis of 16S rRNA genes revealed that half of the cells growing on the anode surface were *P. carbinolicus*, but there was a nearly equal number of planktonic cells of *P. carbinolicus*. In contrast, *G. sulfurreducens* was primarily attached to the anode. *P. carbinolicus* represents the first Fe(III) oxide-reducing microorganism found to be unable to produce current in a microbial fuel cell, providing the first suggestion that the mechanisms for extracellular electron transfer to Fe(III) oxides and fuel cell anodes may be different.

It has been generally regarded that microorganisms which have the ability to use Fe(III) oxides as an electron acceptor are also able to transfer electrons to the anodes of microbial fuel cells without the requirement for an exogenous electron shuttle mediator (21, 35). Anodes and Fe(III) oxides both represent insoluble, extracellular electron acceptors, and it is reasonable to assume that the mechanisms for electron transfer to Fe(III) oxides and electrodes might be similar, because anodes are not natural electron acceptors (21). For example, *Geobacter sulfurreducens* produces current from the oxidation of acetate or hydrogen (3), and several outer-surface proteins known to be important in Fe(III) oxide reduction also appear to play a role in electricity production. Deletion of the gene for the outer-membrane c-type cytochrome OmcS specifically inhibits growth on Fe(III) oxides but not on soluble electron acceptors (29), and the OmcS-deficient strain is also severely inhibited in current production (14). The electrically conductive pili of *G. sulfurreducens* are also required for Fe(III) oxide reduction (31) as well as for maximum power production in fuel cells (32). Similar mechanisms for electron transfer to Fe(III) oxides and the anodes of microbial fuel cells would greatly facilitate the study of mechanisms for electricity production because studies with Fe(III) oxides as electron acceptors are often more technically tractable than studies with fuel cells.

Although a substantial number of Fe(III) oxide-reducing microorganisms, such as *Aeromonas* (30), *Desulfobulbus* (12), *Desulfuromonas* (2), *Geobacter* (2, 3), *Geopsychrobacter* (17), *Geothrix* (4), *Rhodoferax* (6), and *Shewanella* (18; M. Lanthier, unpublished data) species, have been evaluated for the potential for current production, *Pelobacter* species have not. *Pelobacter* species are of interest, in part, because they are in the family *Geobacteraceae*, and *Geobacteraceae* are often specifically enriched on the surfaces of anodes harvesting electricity from aquatic sediments (2, 13, 36). *Geobacter* species are the most abundant *Geobacteraceae* on anodes of sediment fuel cells harvesting electricity from freshwater sediments, and *Desulfuromonas* species are the predominant members of this family on anodes from marine sediment fuel cells. However, microorganisms with 16S rRNA gene sequences most closely related to known *Pelobacter* species can account for ca. 20% of the *Geobacteraceae* sequences on both freshwater and marine anodes (13).

Like *Geobacter* and *Desulfuromonas* species, *Pelobacter* species are capable of Fe(III) reduction (11, 20, 26), but unlike these other members of the *Geobacteraceae*, which completely oxidize acetate and other organic compounds, *Pelobacter* species incompletely oxidize organic substrates and are typically cultured under fermentative conditions. For example, *Pelobacter carbinolicus*, which is the *Pelobacter* species that can be cultured most readily on Fe(III) oxides, ferments substrates such as 2,3-butanediol, acetoin, and ethylene glycol, to ethanol and acetate (33) and incompletely oxidizes ethanol to acetate with Fe(III) or S⁰ serving as the electron acceptor (26). *P. carbinolicus* and *P. acetylenicus* can also ferment ethanol to...
acetate with the production of hydrogen when grown with a hydrogen-consuming partner that maintains hydrogen concentrations low enough for this reaction to be thermodynamically favorable (33, 34).

In order to evaluate the potential for *Pelobacter* species to contribute to current production in sediment microbial fuel cells, current production in *P. carbinolicus* was investigated. The results of the investigation suggest that although this organism readily reduces Fe(III) oxides, it cannot effectively transfer electrons to the anode of a microbial fuel cell.

**MATERIALS AND METHODS**

Organisms, media, and growth conditions. For maintenance, *P. carbinolicus* (DSMZ 2380) was cultured at 30°C under strict anaerobic conditions in medium containing NaCl (2.00 g/liter), MgCl₂·6H₂O (3.0 g/liter), NaHCO₃ (25.5 g/liter), KH₂PO₄ (0.5 g/liter), KCl (0.5 g/liter), and CaCl₂·2H₂O (0.15 g/liter). Vitamins and trace minerals (23), acetoin (10 mM) and Na₂S (1.7 mM) were added from stock solutions (26). These cultures were also used to inoculate the fuel cells. The medium in the fuel cells was freshwater medium (24) containing NaHCO₃ (2.5 g/liter), NH₄Cl (0.25 g/liter), NaH₂PO₄ (0.6 g/liter), and KCl (0.1 g/liter). Vitamins and trace minerals (23) were added from stock solutions. The medium was additionally amended (5%, vol/vol) with a salt stock solution containing Fe(III) oxides, NaCl, MgCl₂·6H₂O, CaCl₂·2H₂O, and KCl to facilitate a salt concentration at which both *P. carbinolicus* and *G. sulfurireducens* thrived.

*Geo bacter sulfurireducens* strain PCA (ATCC 51573) was maintained in anoxic pressure tubes with acetate-fumarate (NBAF) medium containing 10 mM acetate and 40 mM fumarate, as described previously (7). Before inoculation in fuel cells, the bacteria were slowly adapted to the high salt concentration by sequentially transferring them into NBAF medium with increasing contents (1, 2, 3, 4, and 5% [vol/vol]) of the salt stock solution described above.

Fuel cells. Dual-chamber fuel cells (H type) were assembled and operated as described previously (3), with the exceptions that 5% (vol/vol) of the salt stock solution described above was added, the fuel cells had a liquid volume of 200 ml and a headspace of 100 ml, and each chamber was equipped with a glass screw thread aperture on top, which was sealed with a rubber stopper and screw cap. Ethanol (5 mM) or acetate (10 mM) served as the electron donor and carbon source in the anode chamber. The electrodes were connected via a 560 μm PTFE barrier, with high-pressure liquid chromatography using an LC-10ATVP high-pressure pump (Shimadzu, Kyoto, Japan). The working electrodes were modified by coating with a 5 nm gold film to improve their electrical conductivity.

When fuel cells were switched to potentiostat mode, the anode became the working electrode, and the setup was adjusted as described previously (3). Briefly, while the chambers were sparged with N₂-CO₂ (80/20), the medium in both chambers was replaced and the Tris-buffered medium in the cathode chamber was replaced with bicarbonate-buffered FW medium. An Ag/AgCl reference electrode was placed in the working electrode chamber, and the working electrode was poised at +300 mV with a potentiostat. The counter electrode was continuously sparged with N₂-CO₂, while sparging of the working electrode was stopped after adding ethanol (5 mM), to avoid further losses of ethanol by evaporation.

Analytical techniques. Concentrations of ethanol and acetate were determined with high-pressure liquid chromatography using an LC-10ATVP high-pressure liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (300 mm by 7.8 mm; Bio-Rad, Hercules, CA), with 8 mM H₂SO₄ as the mobile phase. Analytical techniques included hydrolysis of the sample by heating at 100°C for 60 min with 0.5% heptfluorobutyric acid and filtration through a 0.2 μm filter before injection into the chromatograph.

Calculations. Electron recovery from ethanol metabolism was calculated by the following formulas: moles of electrons recovered as electricity = amperes × seconds × volts / (moles of ethanol × 2 × 96,485 C/mole of electron). The yield of electricity generated from ethanol was calculated from the anode chamber current (I) and the potential of the working electrode (E) using the formula:

\[ \text{yield (mole/liter) = } \left( \frac{E - I \cdot R}{n \cdot z \cdot F} \right) \times \text{Current} \times \text{Volume} \]

where E is the cell potential, I is the current, R is the resistance, n is the number of electrons transferred, z is the number of moles of charge carriers, F is the Faraday constant, and Volume is the volume of the cell.

**RESULTS AND DISCUSSION**

Current with *P. carbinolicus* alone and in culture with *G. sulfurireducens*. *P. carbinolicus* did not produce current in fuel cells operated either in true fuel cell mode (Fig. 1) or when the anode was artificially poised at +300 mV with a potentiostat (data not shown). If acetoin, which supports fermentative growth, was provided, the cells grew via fermentation in the fuel cell chamber. There was no growth when ethanol was the potential electron donor (Fig. 1A). The loss of ethanol over time in fuel cells inoculated with *P. carbinolicus* was similar to the evaporative loss of ethanol due to the N₂-CO₂ sparging in uninoculated fuel cells (Fig. 2).

However, current was produced when *Geo bacter sulfurireducens* was inoculated into an ethanol-amended anode chamber along with *P. carbinolicus* (Fig. 1B). Current production was associated with an increase in culture density. When the current began to decline, the medium in the anode chamber was replaced. This replacement resulted in increased current production and continued growth of planktonic cells (Fig. 1B). With continued medium replacements, the current production
increased to a maximum of ca. 0.3 mA, which is comparable to the power output previously observed for other microorganisms incubated in similar fuel cells (2, 3; data not shown).

Fuel cells inoculated with just *G. sulfurreducens* did not produce current with ethanol as the electron donor (data not shown). In order to further evaluate whether ethanol supported electricity production by *G. sulfurreducens*, a fuel cell was established with just *G. sulfurreducens* and acetate as the electron donor. As expected from previous studies (3) *G. sulfurreducens* produced current under these conditions (Fig. 2C). However, when the medium was switched to one with ethanol as the electron donor, current production rapidly declined. Current resumed when acetate was reintroduced (Fig. 2C). These results are consistent with a previous report that *G. sulfurreducens* does not use ethanol as an electron donor for the reduction of Fe(III) (5).

Ethanol metabolism and current production in the coculture fuel cells were associated with the production of acetate (Fig. 2D). Acetate accumulation was less than ethanol removal (Fig. 2D), suggesting that *G. sulfurreducens* was oxidizing some of the acetate with electron transfer to the anode. Acetate production from ethanol requires that four electrons be transferred to an electron acceptor. When *P. carbinolicus* lacks electron acceptors, such as Fe(III) or S⁰ (26), it can transfer electrons to protons, producing hydrogen as long as hydrogen concentrations are maintained at sufficiently low levels (33, 34). The fact that ethanol was not significantly metabolized in fuel cells containing just *P. carbinolicus* suggests that *G. sulfurreducens* was required to consume hydrogen as well as acetate to make ethanol metabolism thermodynamically favorable.

### Significance of hydrogen consumption by *G. sulfurreducens*

In order to determine the significance of hydrogen consumption by *G. sulfurreducens* in the coculture fuel cells, wild-type *G. sulfurreducens* was replaced with a strain in which the gene *hybL* was deleted. This gene encodes the large subunit of the uptake hydrogenase, HyB. The *hybL*-deficient strain is capable of metabolizing acetate, but does not grow with hydrogen as an electron donor (data not shown).
electron donor (8). In fuel cells, it produced current from acetate, but not hydrogen (data not shown). In order to establish *P. carbinolicus* with the hydrogenase-deficient mutant in a fuel cell coculture, it was necessary to vigorously sparge the anode chamber with N₂-CO₂ to strip hydrogen from the system. Once the coculture was established, the media in both chambers were exchanged as described in Materials and Methods, ethanol (5 mM) was added, and the anode was poised at $-100$ to $-300$ mV. Sparging was stopped 1 day after this ethanol addition. Controls were treated similarly, but grown with wild-type *G. sulfurreducens*.

The nonsparged coculture with the hydrogenase-deficient *G. sulfurreducens* mutant initially produced current with the consumption of the remaining acetate that had been produced from the metabolism of ethanol during the sparging phase, when hydrogen was able to escape the fuel cell (Fig. 3A). The ethanol concentration declined at a rate which was an order of magnitude less than that in the coculture with wild-type *G. sulfurreducens*, and hydrogen accumulated. Once the acetate was consumed, current declined to very low levels and hydrogen concentrations stabilized, indicating that even though *P. carbinolicus* can oxidize hydrogen with the reduction of Fe(III) (26), it did not significantly oxidize hydrogen with electron transfer to the fuel cell anode. In contrast, ethanol was rapidly degraded in the coculture with wild-type *G. sulfurreducens* (Fig. 3B). Hydrogen initially accumulated, but to lower levels than that in the coculture with the hydrogenase-deficient mutant, and in the presence of wild-type cells, the hydrogen levels subsequently declined. These results suggest that hydrogen uptake by *G. sulfurreducens* plays an important role in promoting the ethanol metabolism of *P. carbinolicus* and, thus, current production by the coculture.

**Electron recovery.** The stoichiometry of ethanol consumption and recovery of electrons as current with wild-type *G. sulfurreducens* and *P. carbinolicus* were determined in fuel cell mode because this mode most closely represents the conversion of fuels to current for practical applications. However, when oxygen was used as the oxidant for the cathode, the anode chamber had to be bubbled with N₂-CO₂ in order to remove oxygen diffusing into the anode chamber. As noted above, this caused evaporative losses of ethanol, resulting in low values of electron recovery (37 to 49%). To alleviate the need for sparging the anode chamber, 50 mM K₃Fe(CN)₆ was added as the oxidant in the cathode chamber and the fuel cells were placed in an anaerobic glove bag to prevent oxygen diffusion into the system (Fig. 4). Under these conditions, 74 to 83% of the electrons that were present in the ethanol consumed were recovered as current and in the accumulated acetate.

**Biomass distribution.** The measurable optical density in the *P. carbinolicus*-*G. sulfurreducens* fuel cells (Fig. 1B) contrasts with that of previously described *G. sulfurreducens* fuel cells in which the anode chamber typically exhibits little or no turbidity upon repeated exchanges of the medium (3). The amounts of planktonic protein, 1.00 to 1.44 mg, and protein on the anode, 1.03 to 1.25 mg, were comparable. An analysis of 16S rRNA gene clone libraries constructed from planktonic cells demonstrated that 98% of the planktonic cells were *P. carbinolicus*. In
contrast, 16S rRNA gene clone libraries constructed from cells scraped from the anode surface indicated that *P. carbinolicus* and *G. sulfurreducens* each accounted for 48 to 52% of the cells on the anode. The fact that ca. two-thirds of the *P. carbinolicus* biomass was not associated with the anode is consistent with the concept that *P. carbinolicus* is important for ethanol metabolism, but not for current production, and that *G. sulfurreducens* associated with the anode is responsible for electron transfer to the anode. The average current generated per milligram of cell protein attached to the anode surface in the coculture fuel cells was 0.27 mA/mg. This value is low compared to the 0.34 to 1.93 mA/mg protein from *G. sulfurreducens* pure cultures (3). This can be attributed to the inability of *P. carbinolicus* to participate in current production.

In order to further evaluate the association of *P. carbinolicus* and *G. sulfurreducens*, the biofilm on the anode surface was examined with confocal laser scanning microscopy and fluorescent in situ hybridization. There was no autofluorescence when no probe was added to the hybridization buffer, or with a nonspecific probe (NON338), which does not hybridize with the rRNA. All cells in the biofilm on the anode hybridized with a set of probes targeting all *Bacteria* (EUB338-I, -II, and -III) (data not shown) or all *Deltaproteobacteria* (DELTA495A, -B, and -C) (Fig. 5A). Probes targeting the *Geobacter* cluster (15) of the *Geobacteraceae* (GEO3A, -B, and -C) (data not shown), which includes *G. sulfurreducens*, or the Desulfuromonas cluster, which includes *P. carbinolicus* (15) (DMONAS-A, -B, -C, and -D) (Fig. 5B), revealed that both organisms colonized the anode in near-equal numbers, in accordance with the results.
from the clone libraries. This result was further confirmed with species-specific probes targeting *G. sulfurreducens* (GEO2) or *P. carbinolicus* (PCARB1) (Fig. 5C and D). Although there appeared to be species-specific clusters of cells within the biofilm, neither organism appeared to have a preference for growth near the anode surface or the outer surface of the biofilm.

**Implications.** These results demonstrate that *P. carbinolicus* has little, if any, capacity for electron transfer to electrodes. This result is surprising, because *P. carbinolicus* readily grows with insoluble Fe(III) oxides as the electron acceptor (26), and, typically, microorganisms capable of dissimilatory Fe(III) oxide reduction are also known to use anodes as an electron acceptor (21, 35). The lack of current production by *P. carbinolicus* was not due to improper culture conditions in the fuel cell, because *P. carbinolicus* grows readily during fermentation of acetoin or ethanol in coculture with *G. sulfurreducens* in the same system. The current produced from the coculture resulted from *G. sulfurreducens* oxidizing acetate and hydrogen not removed by sparging, with the anode serving as the electron acceptor. Both acetate and hydrogen oxidation can serve as electron donors for current production by *G. sulfurreducens* (3).

An apparent difference in extracellular electron transfer strategies in *P. carbinolicus* and *G. sulfurreducens* is that *P. carbinolicus* contains far fewer c-type cytochromes than *G. sulfurreducens* does (11). Most notably, *P. carbinolicus* lacks the outer-membrane cytochromes that are thought to serve as an electrical contact between the cell and the anode in *G. sulfurreducens* cells closely associated with the anode surface (14). *P. carbinolicus* does contain genes for pili, which in *G. sulfurreducens* are considered to be electrically conductive (31) and provide long-range electron transfer through the biofilm on fuel cell anodes (32). Reverse transcriptase PCR and gel electrophoresis demonstrated that in *P. carbinolicus*, the putative *pilA* gene (Pcar_2144, VIMSS 586177) coding for the pilin subunit of type IV pili is expressed during growth on the electrode in coculture with *G. sulfurreducens* (unpublished results). However, whether the pili in *G. sulfurreducens* are sufficient for electron transfer to anodes in the absence of outer-membrane c-type cytochromes has not been determined. Attempts to determine whether the pili of *P. carbinolicus* are conductive have been inconclusive due to technical difficulties in obtaining sufficient pili for evaluation. Therefore, it is not clear whether the inability of *P. carbinolicus* to generate current is due to the lack of required c-type cytochromes or due to pili which are nonconductive.

Although the possibility for current production in other *Pelobacter* species has yet to be determined, the difficulties in growing the other available pure cultures on Fe(III) oxides...
suggest that it is unlikely that any of these strains are able to directly generate current as a pure culture in a microbial fuel cell. Although Pelobacter species clearly colonize the anodes of sediment fuel cells (13), it seems likely that their primary role in current production is indirect, converting organic substrates to acetate and hydrogen, which Geobacter or Desulfuromonas species can oxidize with electron transfer to the anode.

ACKNOWLEDGMENTS

This research was supported by the Office of Science (BER), U.S. Department of Energy, cooperative agreement number DE-FC02-02ER63446, and the Office of Naval Research, award number N00014-06-1-0802. Confocal laser scanning microscopy was supported by National Science Foundation grant BBS714235 to the University of Massachusetts—Amherst Microscopy Facility.

REFERENCES