Applied and Environmental Microbiology, Aug. 2007, p. 5347–5353 Vol. 73, No. 16
0099-2240/07/$08.00+0 doi:10.1128/AEM.00804-07

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 S^0 serving as the electron acceptor (26). *P. carbinolicus* and *P. acetylenicus* can also ferment ethanol to

* Corresponding author. Mailing address: Department of Microbiology, University of Massachusetts, Amherst, MA 01003. Phone: (413) 577-1251. Fax: (413) 577-4660. E-mail: hrichter@microbio.umass.edu.
▽ Published ahead of print on 15 June 2007.

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 (20.0 g/liter), $MgCl_2 \cdot 6H_2O$ (3.0 g/liter), $NaHCO_3$ (2.5 g/liter), NH_4Cl (0.25 g/liter), KH_2PO_4 (0.2 g/liter), KCl (0.5 g/liter), and $CaCl_2 \cdot 2H_2O$ (0.15 g/liter). Vitamins and trace minerals (23), acetoin (10 mM) and Na_2S (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 $NaHCO_3$ (2.5 g/liter), NH_4Cl (0.25 g/liter), NaH_2PO_4 (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 NaCl [180 g/liter], $MgCl_2 \cdot 6H_2O$ [54 g/liter], and $CaCl_2 \cdot 2H_2O$ [2.7 g/liter]) to establish a salt concentration at which both *P. carbinolicus* and *G. sulfurreducens* thrived.

Geobacter sulfurreducens 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 560Ω resistor over which the voltage was recorded hourly.

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 N_2-CO_2 (80:20), the medium in both chambers was replaced and the Tris-buffered medium in the cathode chamber was exchanged 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 chamber was continuously sparged with N_2-CO_2, 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 by 7.8 mm; Bio-Rad, Hercules, CA), with 8 mM H_2SO_4 eluent. Acetate was detected with an SPD-10AVP UV detector set at 210 nm. Ethanol was quantified with an RID-10A refractive index detector (Shimadzu, Kyoto, Japan).

Hydrogen partial pressures were measured using an RGD2 reduction gas analyzer (Trace Analytical, Menlo Park, CA) as described previously (25).

The protein content in fuel cell medium and on electrodes was determined using the bicinchoninic assay (K. P. Nevin, H. Richter, S. F. Covalla, J. P. Johnson, T. L. Woodard, H. Jia, M. Zhang, and D. R. Lovley, submitted for publication).

Calculations. Electron recovery from ethanol metabolism was calculated by the following formulas: moles of electrons recovered as electricity = amps produced × s/96,500 columbs per mole of electron, moles of electrons available from ethanol metabolism = (moles/liter of ethanol consumed × 12) − (moles/liter of acetate remaining × 8) − (moles/liter × hydrogen remaining × 2) × liters in anode chamber, and electron recovery = moles of electrons recovered as electricity/moles of electrons available from ethanol metabolism. The concentration of dissolved hydrogen was calculated from hydrogen partial pressure (22, 38) in the headspace of fuel cells.

16S rRNA gene clone libraries. The proportion of *P. carbinolicus* in the biofilm on anodes or in the fuel cell medium of cocultures was determined from the abundance of 16S rRNA genes. DNA was extracted from the biofilm on the anodes as described previously (13) or from an aliquot (2 ml) of the anode chamber medium with the Bio101 soil kit (Bio Systems, Carlsbad, CA). The DNA was purified, and 16S rRNA gene fragments were amplified with the primer 8 forward and 519 reverse (13). The PCR conditions were as described previously (16). PCR products were purified, and clone libraries were constructed with a TOPO TA cloning kit, version K2 (Invitrogen, Carlsbad, CA). 16S rRNA gene fragments were sequenced, and the proportion of *P. carbinolicus* sequences was determined as described previously (16).

Fluorescent in situ hybridization. Biofilms growing on graphite electrodes were fixed for 1 h in paraformaldehyde–phosphate-buffered saline (PBS) (19) in plastic bags. Electrodes were then washed in PBS for 5 min and stored at −20°C in PBS buffer-50% ethanol until hybridization. For hybridization, samples were dehydrated in a graded series of 50, 80, and 95% ethanol solutions for 5 min each. Rectangular sections were drawn on one side of the electrode with a hydrophobic pen (Super PAP pen liquid blocker; Ted Pella, Inc., Redding, CA). Each section was for hybridization with a different set of probes. Electrodes were exposed for 20 min to 1 to 2 ml of an acetylation solution (19) and then washed gently with deionized water.

Hybridization solution (100 to 200 μl) (19), which contained 25 ng of fluorescent probe per microliter and helper oligonucleotides (Table 1) to increase the fluorescence signal (10), if required, was added to each rectangular section on the electrode. Autofluorescence controls were prepared without fluorescent probes, and nonspecific probes (37) were applied to rule out nonspecific hybridization. All fluorescent probes were coupled to Cy3, but another aliquot of GEO2 was also available with Cy5. Hybridization was carried out in a hybridization oven (Shake n Bake, model 136400; Boekel Scientific) for 3 h at 46°C. The humidity chamber was filled with towels soaked with 50 ml of hybridization buffer. Then electrodes were washed once for 20 min at 48°C in washing buffer (19), rinsed with deionized water, counterstained in the dark with 100 to 200 μl of 25 μM YOYO-1 (Molecular Probes, Eugene, OR) per section for 30 min, and rinsed for 5 min with deionized water. Electrodes were mounted with an antifade kit (ProLong; Molecular Probes).

Samples were examined on a Zeiss Axiovert LSM 510 Meta confocal system equipped with a 63× Zeiss Plan-Apochromat oil immersion objective (numerical aperture of 1.4) and a Meta detector (Carl Zeiss MicroImaging, Inc., Thornwood, NY). The confocal microscope was equipped with an Argon laser (lines at 458, 477, 488, and 514 nm; 25 mW total) and two HeNe lasers (lines at 543 nm, 1 mW, and 633 nm, 5 mW), a krypton-argon dual laser (488 and 568 nm) and a diode laser (638 nm). Representative three-dimensional scans of biofilm sections on the electrodes were taken and displayed as orthoviews. Images were averaged by Kalman filtration with eight running scans per image (28). The acquisition software was LSM 510 Meta, version 3.2 SP2.

RESULTS AND DISCUSSION

Current with *P. carbinolicus* alone and in culture with *G. sulfurreducens*. *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 N_2-CO_2 sparging in uninoculated fuel cells (Fig. 2).

However, current was produced when *Geobacter sulfurreducens* 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

TABLE 1. Fluorescent probes used in this study[a]

Common probe name	Generic name	Target(s)	Sequence (5′–3′)	Position no.	Reference(s)
NON338	NA	None	ACTCCTACGGGAGGCAGC	NA	37
EUB338-I	S-D-Bact-0338-a-A-18	Most *Bacteria*	GCTGCCTCCCGTAGGAGT	338–355	1, 9
EUB338-II	S-*-BactP-0338-a-A-18	*Planctomycetales*	GCAGCCACCCGTAGGTGT	338–355	
EUB338-III	S-*-BactV-0338-a-A-18	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	338–355	
DELTA495A	S-C-dProt-0495-a-A-18	Most *Deltaproteobacteria*	AGTTAGCCGGTGCTTCCT	495–512	27
DELTA495B	S-*-dProt-0495-b-A-18	Some *Deltaproteobacteria*	AGTTAGCCGGCGCTTCCT	495–512	
DELTA495C	S-*-dProt-0495-c-A-18	Some *Deltaproteobacteria*	AATTAGCCGGTGCTTCCT	495–512	
GEO3-A	S-G-Geob-0818-a-A-21	*Geobacter* cluster	CCGCAACACCTAGTACTCATC	818–838	This study
GEO3-B	S-G-Geob-0818-b-A-21		CCGCAACACCTAGTTCTCATC	818–838	
GEO3-C	S-G-Geob-0818-c-A-21		CCGCAACACCTGGTTCTCATC	818–838	
DMONAS-A	S-G-Dmona-0433-a-A-21	*Desulfuromonas* cluster	TTTCTTCCCCTCTGACAGAGC	433–453	This study
DMONAS-B	S-G-Dmona-0433-b-A-21		TTTCTTCCCTCTTGACAGAGC	433–453	
DMONAS-C	S-G-Dmona-0433-c-A-21		TTTCTTTCCTCCTGACAGAGC	433–453	
DMONAS-D	S-G-Dmona-0433-d-A-21		GTTCTTCCCCTCTGACAGAGC	433–453	
PCARB1	S-S-Pcarb-0455-a-A-18	*P. carbinolicus*	GCCTATTCGACCACGATA	455–470	This study
GEO2	S-S-Gsulf-0207-a-A-19	*G. sulfurreducens*	GAAGACAGGAGGCCCGAAA	207–225	This study
HGEO2-1	S-S-Gsuh1-0114-(PCA)-a-A-22	Helper probes for GEO2	GTCCCCCCCTTTTCCCGCAAGA	114–135	This study
HGEO2-2	S-S-Gsuh2-0226-a-A-22	*G. sulfurreducens*	CTAATGGTACGCGGACTCATCC	226–243	
HGEO3-3	S-G-Geoh3-0798-a-A-20	Helper probes for GEO3	GTTTACGGCGGGTACTACC	798–817	This study
HGEO3-4	S-G-Geoh4-0839-a-A-18		CACTGCAGGGGTCAATAC	839–856	

[a] NA, not available.

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 S^0 (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

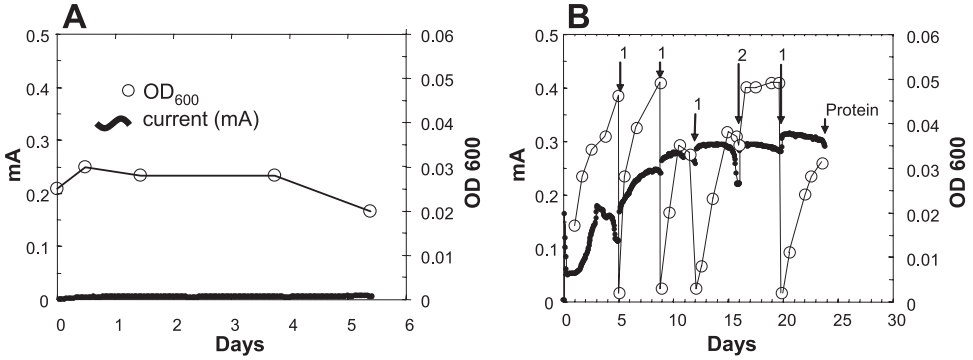

FIG. 1. Fuel cells with 5 mM ethanol, inoculated with *P. carbinolicus* (A) or a coculture of *G. sulfurreducens* and *P. carbinolicus* (B). In the coculture, the anode chamber medium was replaced several times and supplemented with 5 mM ethanol (1) or ethanol was added without medium replacement (2). OD_{600}, optical density at 600 nm.

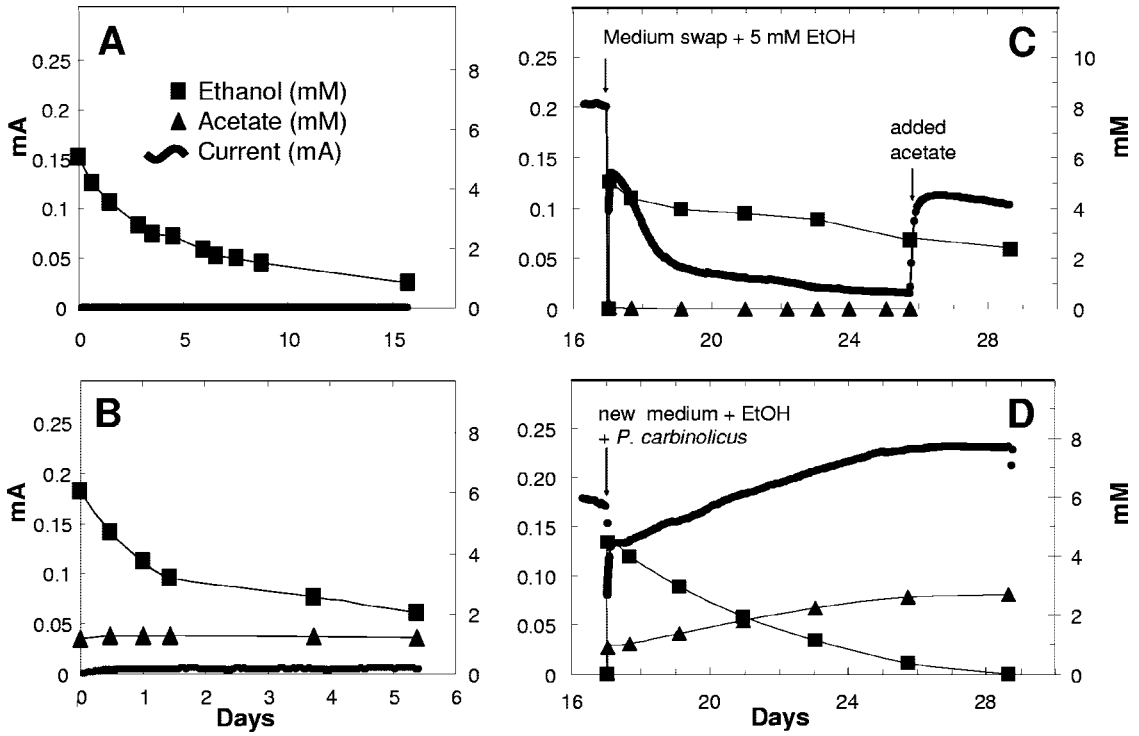

FIG. 2. Comparison of current generation, ethanol consumption, and acetate production in fuel cells with sterile control plus ethanol (A), *P. carbinolicus* plus ethanol (B), *G. sulfurreducens* grown with acetate until day 16 and then fed with ethanol (EtOH) (C), and a coculture of *P. carbinolicus* and *G. sulfurreducens* fed with ethanol (D). In panels C and D, *G. sulfurreducens* was grown with acetate in the fuel cell until current production was stable and then media were exchanged (acetate was omitted, and ethanol or ethanol and *P. carbinolicus* were added).

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 N_2-CO_2 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 +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 N_2/CO_2 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 $K_3Fe(CN)_6$ 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

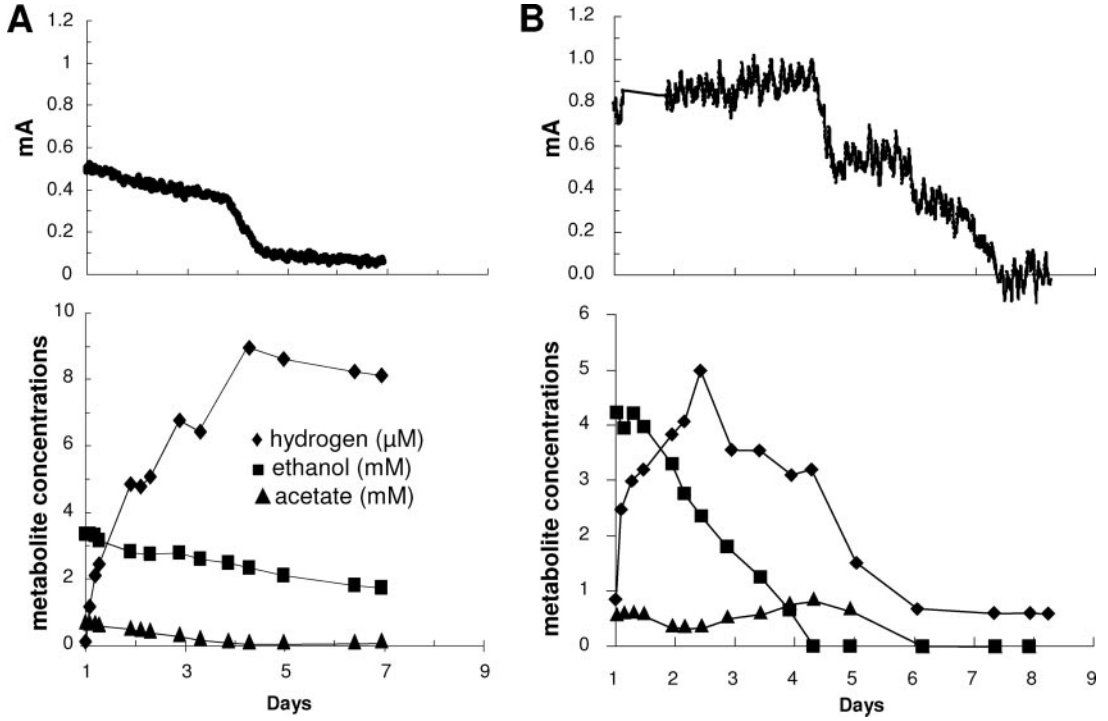

FIG. 3. Current generation and metabolite concentrations in cocultures of *P. carbinolicus* and a *G. sulfurreducens* strain with the uptake hydrogenase gene deleted (A) or wild-type *G. sulfurreducens* (B). The anode was poised at +300 mV with a potentiostat. Cultures were initiated with vigorous sparging in order to remove hydrogen produced as outlined in the text. Time courses are shown after the sparging was stopped on day 1.

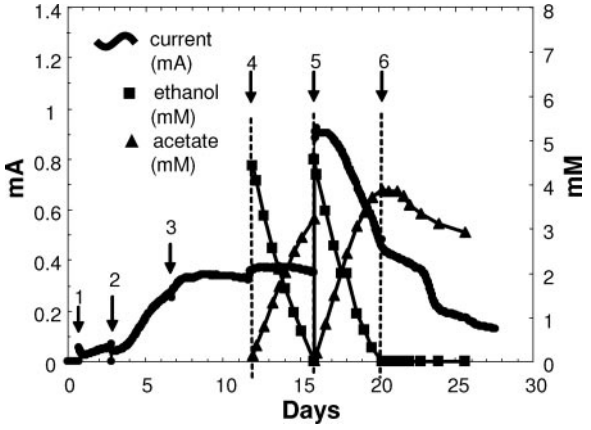

FIG. 4. Current and concentrations of ethanol and acetate in a coculture fuel cell in an anaerobic glove bag. First the fuel cell was set up to sparge the cathode (air) and anode (N_2-CO_2). It was then inoculated with *G. sulfurreducens* plus 10 mM acetate (arrow 1). After initial growth, the anode medium was exchanged (*P. carbinolicus* and 5 mM ethanol were added) (arrow 2). Medium plus ethanol was replaced two more times until stable current was achieved (arrows 3 and 4). Ethanol, acetate, and current were measured in "sparging mode" until ethanol was completely consumed (arrows 4 and 5). Then oxygen was sparged out of the cathode chamber with N_2-CO_2, while the cathode medium was supplemented with $K_3Fe(III)(CN)_6$. Sparging of both chambers was stopped, and the fuel cell was put into an anaerobic glove bag (arrow 5). Electron recovery was then determined without evaporative loss of metabolites (arrows 5 and 6).

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

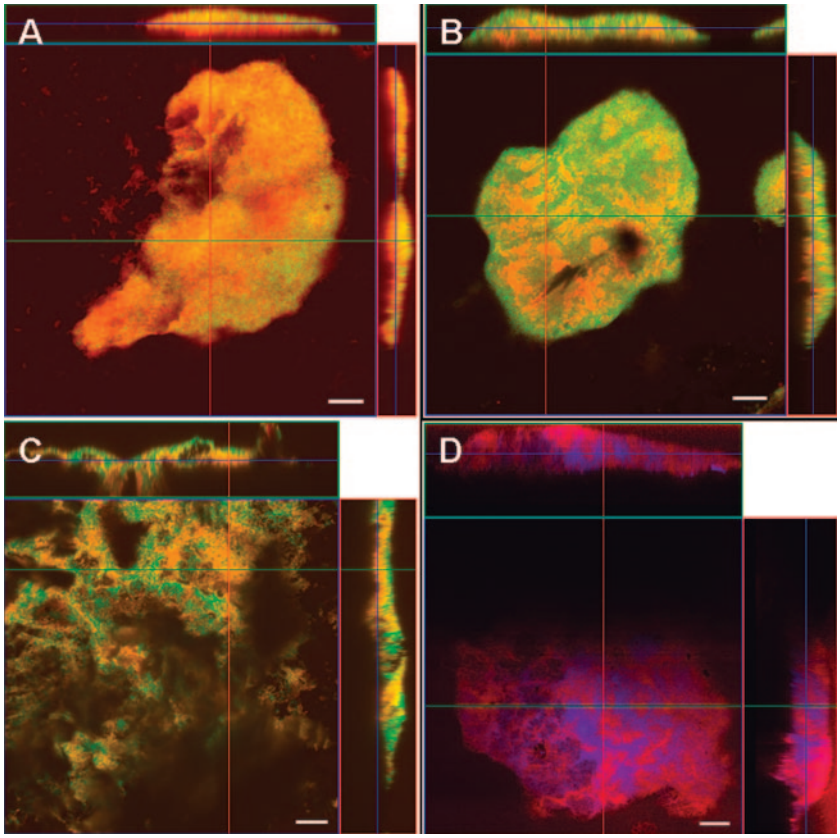

FIG. 5. Top views (large squares) and orthogonal side views (top and side rectangles) of *P. carbinolicus* and *G. sulfurreducens* biofilm growing on an anode and hybridized with probes targeting *Deltaproteobacteria* (probe DELTA495, red) (A), *Desulfuromonas* cluster (probe DMONAS, red) (B), *G. sulfurreducens* (probe GEO2, red) (C), and *P. carbinolicus* (probe PCARB1, red) and *G. sulfurreducens* (GEO2, blue) (D). All biofilm sections are shown with YOYO-1 counterstain (green), except in panel D. Helper probes HGEO2-1 and HGEO2-2 were used in panels C and D. Bar = 10 μm.

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 able 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* grew 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 BBS8714235 to the University of Massachusetts—Amherst Microscopy Facility.

REFERENCES

1. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
2. **Bond, D. R., D. E. Holmes, L. M. Tender, and D. R. Lovley.** 2002. Electrode-reducing microorganisms that harvest energy from marine sediments. Science **295:**483–485.
3. **Bond, D. R., and D. R. Lovley.** 2003. Electricity production by *Geobacter sulfurreducens* attached to electrodes. Appl. Environ. Microbiol. **69:**1548–1555.
4. **Bond, D. R., and D. R. Lovley.** 2005. Evidence for involvement of an electron shuttle in electricity generation by *Geothrix fermentans*. Appl. Environ. Microbiol. **71:**2186–2189.
5. **Caccavo, F., Jr., D. J. Lonergan, D. R. Lovley, M. Davis, J. F. Stolz, and M. J. McInerney.** 1994. *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl. Environ. Microbiol. **60:**3752–3759.
6. **Chaudhuri, S. K., and D. R. Lovley.** 2003. Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells. Nat. Biotechnol. **21:**1229–1232.
7. **Coppi, M. V., C. Leang, S. J. Sandler, and D. R. Lovley.** 2001. Development of a genetic system for *Geobacter sulfurreducens*. Appl. Environ. Microbiol. **67:**3180–3187.
8. **Coppi, M. V., R. A. O'Neil, and D. R. Lovley.** 2004. Identification of an uptake hydrogenase required for hydrogen-dependent reduction of Fe(III) and other electron acceptors by *Geobacter sulfurreducens*. J. Bacteriol. **186:**3022–3028.
9. **Daims, H., A. Bruhl, R. Amann, K. H. Schleifer, and M. Wagner.** 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22:**434–444.
10. **Fuchs, B. M., F. O. Glockner, J. Wulf, and R. Amann.** 2000. Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. **66:**3603–3607.
11. **Haveman, S. A., D. E. Holmes, Y. H. Ding, J. E. Ward, R. J. Didonato, Jr., and D. R. Lovley.** 2006. *c*-Type cytochromes in *Pelobacter carbinolicus*. Appl. Environ. Microbiol. **72:**6980–6985.
12. **Holmes, D. E., D. R. Bond, and D. R. Lovley.** 2004. Electron transfer by *Desulfobulbus propionicus* to Fe(III) and graphite electrodes. Appl. Environ. Microbiol. **70:**1234–1237.
13. **Holmes, D. E., D. R. Bond, R. A. O'Neil, C. E. Reimers, L. R. Tender, and D. R. Lovley.** 2004. Microbial communities associated with electrodes harvesting electricity from a variety of aquatic sediments. Microb. Ecol. **48:**178–190.
14. **Holmes, D. E., S. K. Chaudhuri, K. P. Nevin, T. Mehta, B. A. Methe, A. Liu, J. E. Ward, T. L. Woodard, J. Webster, and D. R. Lovley.** 2006. Microarray and genetic analysis of electron transfer to electrodes in *Geobacter sulfurreducens*. Environ. Microbiol. **8:**1805–1815.
15. **Holmes, D. E., K. P. Nevin, and D. R. Lovley.** 2004. Comparison of 16S rRNA, nifD, recA, gyrB, rpoB and fusA genes within the family Geobacteraceae fam. nov. Int. J. Syst. Evol. Microbiol. **54:**1591–1599.
16. **Holmes, D. E., K. P. Nevin, R. A. O'Neil, J. E. Ward, L. A. Adams, T. L. Woodard, H. A. Vrionis, and D. R. Lovley.** 2005. Potential for quantifying expression of the *Geobacteraceae* citrate synthase gene to assess the activity of *Geobacteraceae* in the subsurface and on current-harvesting electrodes. Appl. Environ. Microbiol. **71:**6870–6877.
17. **Holmes, D. E., J. S. Nicoll, D. R. Bond, and D. R. Lovley.** 2004. Potential role of a novel psychrotolerant member of the family *Geobacteraceae*, *Geopsychrobacter electrodiphilus* gen. nov., sp. nov., in electricity production by a marine sediment fuel cell. Appl. Environ. Microbiol. **70:**6023–6030.
18. **Kim, H. J., H. S. Park, M. S. Hyun, I. S. Chang, M. Kim, and B. H. Kim.** 2002. A mediator-less microbial fuel cell using a metal reducing bacterium, Shewanella putrefaciens. Enzyme Microb. Technol. **30:**145–152.
19. **Lanthier, M., P. Juteau, F. Lepine, R. Beaudet, and R. Villemur.** 2005. *Desulfitobacterium hafniense* is present in a high proportion within the biofilms of a high-performance pentachlorophenol-degrading, methanogenic fixed-film reactor. Appl. Environ. Microbiol. **71:**1058–1065.
20. **Lonergan, D. J., H. L. Jenter, J. D. Coates, E. J. Phillips, T. M. Schmidt, and D. R. Lovley.** 1996. Phylogenetic analysis of dissimilatory Fe(III)-reducing bacteria. J. Bacteriol. **178:**2402–2408.
21. **Lovley, D. R.** 2006. Bug juice: harvesting electricity with microorganisms. Nat. Rev. Microbiol. **4:**497–508.
22. **Lovley, D. R., F. H. Chapelle, and J. C. Woodward.** 1994. Use of dissolved H2 concentrations to determine distribution of microbially catalyzed redox reactions in anoxic groundwater. Environ. Sci. Technol. **28:**1205–1210.
23. **Lovley, D. R., R. C. Greening, and J. G. Ferry.** 1984. Rapidly growing rumen methanogenic organism that synthesizes coenzyme M and has a high affinity for formate. Appl. Environ. Microbiol. **48:**81–87.
24. **Lovley, D. R., and E. J. Phillips.** 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl. Environ. Microbiol. **54:**1472–1480.
25. **Lovley, D. R., E. J. Phillips, and D. J. Lonergan.** 1989. Hydrogen and formate oxidation coupled to dissimilatory reduction of iron or manganese by *Alteromonas putrefaciens*. Appl. Environ. Microbiol. **55:**700–706.
26. **Lovley, D. R., E. J. Phillips, D. J. Lonergan, and P. K. Widman.** 1995. Fe(III) and S^0 reduction by *Pelobacter carbinolicus*. Appl. Environ. Microbiol. **61:**2132–2138.
27. **Loy, A., A. Lehner, N. Lee, J. Adamczyk, H. Meier, J. Ernst, K. H. Schleifer, and M. Wagner.** 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. **68:**5064–5081.
28. **Manz, W., K. Wendt-Potthoff, T. R. Neu, U. Szewzyk, and J. R. Lawrence.** 1999. Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorescent in situ hybridization and confocal laser scanning microscopy. Microb. Ecol. **37:**225–237.
29. **Mehta, T., M. V. Coppi, S. E. Childers, and D. R. Lovley.** 2005. Outer membrane *c*-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. Appl. Environ. Microbiol. **71:**8634–8641.
30. **Pham, C. A., S. J. Jung, N. T. Phung, J. Lee, I. S. Chang, B. H. Kim, H. Yi, and J. Chun.** 2003. A novel electrochemically active and Fe(III)-reducing bacterium phylogenetically related to *Aeromonas hydrophila*, isolated from a microbial fuel cell. FEMS Microbiol. Lett. **223:**129–134.
31. **Reguera, G., K. D. McCarthy, T. Mehta, J. S. Nicoll, M. T. Tuominen, and D. R. Lovley.** 2005. Extracellular electron transfer via microbial nanowires. Nature **435:**1098–1101.
32. **Reguera, G., K. P. Nevin, J. S. Nicoll, S. F. Covalla, T. L. Woodard, and D. R. Lovley.** 2006. Biofilm and nanowire production leads to increased current in *Geobacter sulfurreducens* fuel cells. Appl. Environ. Microbiol. **72:**7345–7348.
33. **Schink, B.** 1984. Fermentation of 2,3-butanediol by *Pelobacter carbinolicus* sp. nov., and *Pelobacter propionicus* sp. nov., and evidence for propionate formation from C2 compounds. Arch. Microbiol. **137:**33–41.
34. **Seitz, H. J., B. Schink, and R. Conrad.** 1988. Thermodynamics of hydrogen metabolism in methanogenic cocultures degrading ethanol or lactate. FEMS Microbiol. Lett. **55:**119–124.
35. **Seop, C. I., H. Moon, O. Bretschger, J. K. Jang, H. I. Park, K. H. Nealson, and B. H. Kim.** 2006. Electrochemically active bacteria (EAB) and mediatorless microbial fuel cells. J. Microbiol. Biotechnol. **16:**163–177.
36. **Tender, L. M., C. E. Reimers, H. A. Stecher III, D. E. Holmes, D. R. Bond, D. A. Lowy, K. Pilobello, S. J. Fertig, and D. R. Lovley.** 2002. Harnessing microbially generated power on the seafloor. Nat. Biotechnol. **20:**821–825.
37. **Wallner, G., R. Amann, and W. Beisker.** 1993. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry **14:**136–143.
38. **Wilhelm, E., R. Battino, and R. J. Wilcox.** 1977. Low-pressure solubility of gases in liquid water. Chem. Rev. **77:**219–262.