RESEARCH ARTICLE

Generation of High Current Densities by Pure Cultures of Anode-Respiring *Geoalkalibacter* spp. under Alkaline and Saline Conditions in Microbial Electrochemical Cells

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ABSTRACT **Anode-respiring bacteria (ARB) generate electric current in microbial electrochemical cells (MXCs) by channeling electrons from the oxidation of organic substrates to an electrode. Production of high current densities by monocultures in MXCs has resulted almost exclusively from the activity of *Geobacter sulfurreducens*, a neutrophilic freshwater Fe(III)-reducing bacterium and the highest-current-producing member documented for the *Geobacteraceae* family of the *Deltaproteobacteria*. Here we report high current densities generated by haloalkaliphilic *Geoalkalibacter* spp., thus broadening the capability for high anode respiration rates by including other genera within the *Geobacteraceae*. In this study, acetate-fed pure cultures of two related *Geoalkalibacter* spp. produced current densities of 5.0 to 8.3 and 2.4 to 3.3 A m^{-2} under alkaline (pH 9.3) and saline (1.7% NaCl) conditions, respectively. Chronoamperometric studies of halophilic *Glk. subterraneus* DSM 23483 and alkaliphilic *Glk. ferrihydriticus* DSM 17813 suggested that cells performed long-range electron transfer through electrode-attached biofilms and not through soluble electron shuttles. *Glk. ferrihydriticus* also oxidized ethanol directly to produce current, with maximum current densities of 5.7 to 7.1 A m^{-2} and coulombic efficiencies of 84 to 95%. Cyclic voltammetry (CV) elicited a sigmoidal response with characteristic onset, midpoint, and saturation potentials, while CV performed in the absence of an electron donor suggested the involvement of redox molecules in the biofilm that were limited by diffusion. These results matched those previously reported for actively respiring *Gb. sulfurreducens* biofilms producing similar current densities (~5 to 9 A m^{-2}).**

IMPORTANCE **This study establishes the highest current densities ever achieved by pure cultures of anode-respiring bacteria (ARB) under alkaline and saline conditions in microbial electrochemical cells (MXCs) and provides the first electrochemical characterization of the genus *Geoalkalibacter*. Production of high current densities among the *Geobacteraceae* is no longer exclusive to *Geobacter sulfurreducens*, suggesting greater versatility for this family in fundamental and applied microbial electrochemical cell (MXC) research than previously considered. Additionally, this work raises the possibility that different members of the *Geobacteraceae* have conserved molecular mechanisms governing respiratory extracellular electron transfer to electrodes. Thus, the capacity for high current generation may exist in other uncultivated members of this family. Advancement of MXC technology for practical uses must rely on an expanded suite of ARB capable of using different electron donors and producing high current densities under various conditions. *Geoalkalibacter* spp. can potentially broaden the practical capabilities of MXCs to include energy generation and waste treatment under expanded ranges of salinity and pH.**

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Anodes of microbial electrochemical cells (MXCs) exploit the anaerobic respiration capabilities of anode-respiring bacteria (ARB), which liberate electrons from the oxidation of organic compounds to solid electron acceptors located outside the cell. In nature, ARB perform extracellular electron transfer (EET) for respiration of insoluble Fe(III) and Mn(IV) oxides, and these bacteria are routinely enriched in current-generating anode biofilms in MXCs (1). Representatives span several phyla and are usually found in sediments and subsurface environments (2), where they harness energy available from oxidation of simple organic compounds coupled to reduction of solid electron acceptors (3). Members of the *Geobacteraceae* family of the *Deltaproteobacteria* have been well studied both for their omnipresence in high-current-producing MXCs (4, 5) and for their abundance in Fe(III)-reducing environments, which can vary greatly in temperature, salinity, and pH (6). However, *Geobacteraceae* members have shown poor conservation among the genes encoding outer membrane cytochromes that facilitate EET (7), in contrast to the conserved pathways for moving electrons past the outer membrane in metal-reducing *Shewanella* (8). This suggests that the

Geobacteraceae family has evolved multiple molecular strategies for carrying out metal reduction, thereby maintaining a competitive advantage under different environmental conditions. Amperometric techniques using microbial electrolysis cells (MECs) and a potentiostat (9) can help elucidate these EET mechanisms. A poised anode facilitates real-time measurement of current generation (10), bacterial growth and respiration kinetics (11), rate-limiting processes in electron and mass transport (12, 13), gene expression profiles (14), and redox properties of electron shuttles and whole biofilms (15).

Alkaline Fe(III) reduction has been reported primarily for several low-G+C Gram-positive bacteria (16–18). Many of these microorganisms have been isolated from soda lakes (19), where they influence Fe biogeochemistry under elevated salinity and pH values sometimes exceeding pH 12. Respiratory Fe(III) reduction has been documented for the alkaliphilic isolates *Bacillus infernus*, *Alkaliphilus metalliredigens*, and *Anaerobacillus arseniciselenatis* (16, 17, 20) and for all genera belonging to the *Geobacteraceae*, with the exception of *Pelobacter* (21). The characterization of *Geoalkalibacter ferrihydriticus* DSM 17813, another soda lake isolate, expanded on the well-studied metal-reducing capabilities of the *Geobacteraceae* to include Fe(III) reduction up to pH 10 (22). More recently, isolation of the halophilic but neutrophilic *Geoalkalibacter subterraneus* strain DSM 23483 led to a broadening of the *Geoalkalibacter* genus description to include nonalkaliphiles (23). *Geoalkalibacter* spp. appear to form a distinct phylogenetic branch within the *Geobacteraceae* and are closely related to the halophilic *Desulfuromonas* clade, in agreement with *Geoalkalibacter* spp. having high salt tolerance (22, 23). However, the only physiological feature shared between *Glk. ferrihydriticus* and *Glk. subterraneus* is their ability to couple complete oxidation of simple organic molecules to reduction of Fe(III) and Mn(IV) oxides.

We previously identified *Geoalkalibacter* 16S rRNA gene sequences in enrichments from current-producing microbial electrolysis cells (5, 24). Therefore, we evaluated *Glk. ferrihydriticus* DSM 17813 and *Glk. subterraneus* DSM 23483 for current generation using poised electrodes under alkaline (pH 9.3) or saline (1.7% NaCl) conditions, respectively. Electrochemical characterizations established benchmarks for growth, anode respiration kinetics, and substrate utilization against other well-studied ARB. Production of high current densities, which we define as >1 A m^{-2} (25), now extends to haloalkaliphiles and provides further microbiological insight into the diversity of EET in the *Geobacteraceae* family. In addition, this work reinforces the potential for novel ARB in broadening the range of practical applications for MXCs.

RESULTS AND DISCUSSION

Chronoamperometry. Figure 1 shows *Glk. subterraneus* producing current using anodes poised at +0.04 V under saline conditions at pH ~7.2 within 3 days postinoculation. Cells initially grew with an estimated doubling time of 8.4 h (Fig. 1, inset), a value slightly lower than the ~6-h doubling time reported for *Geobacter sulfurreducens* respiring electrodes or soluble Fe(III) (11). After 6 days, the current density (based on anode surface area) reached 1.9 A m^{-2}, and visibly thick anode biofilms appeared. To investigate whether current production resulted from attached cells, we replaced the bulk medium on day 8. Anodic current resumed immediately after medium replacement (Fig. 1), indicating that anode biofilms, not planktonic cells or redox shuttles, were responsible for current production. The coulombic efficiency (CE) for acetate-fed *Glk. subterraneus* biofilms ranged from 55 to 119%, with lower values occurring during growth (days 0 to 7) and higher values during stable current production (days 20 to 30). These results suggest that *Glk. subterraneus* may accumulate intracellular storage polymers during growth and oxidize these reserves once biofilms are established and current remains steady (Fig. 1).

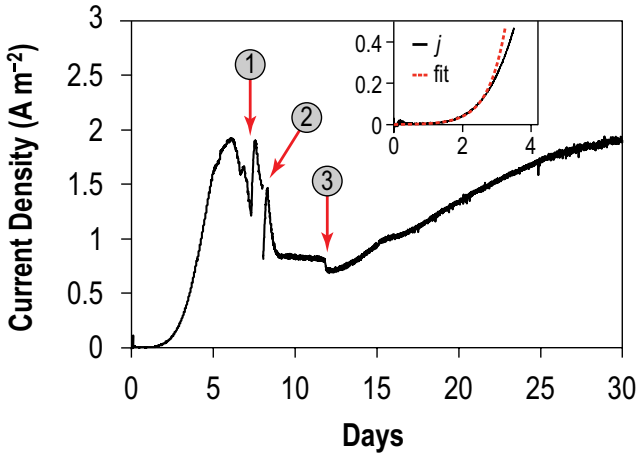

FIG 1 Chronoamperometry of acetate-fed *Glk. subterraneus* DSM 23483 poised at +0.04 V, following inoculation with cells grown on Fe(III) oxide. Numbered gray circles indicate the following: 1 and 3, generation of cyclic voltammograms (see Fig. 6); 2, medium replacement.

Although high salt concentrations can improve MXC performance by increasing conductivity and lowering internal resistance, recent work has demonstrated that NaCl concentrations of >1% led to inhibition of ARB and decreased CE (26). The fact that *Glk. subterraneus* prefers saline conditions and uses several organic electron donors (23) makes it an appealing candidate for energy generation from saline wastewaters. Replicate experiments with *Glk. subterraneus* produced stable current over 30 days and maximum current densities of 2.4 to 3.3 A m^{-2}, establishing the highest current densities reported for a pure culture of an ARB under saline conditions.

Glk. ferrihydriticus produced electricity in MECs operated under alkaline conditions (pH 9.3), emerging as the first example of an alkaliphilic ARB within the *Geobacteraceae* (Fig. 2). *Glk. ferrihydriticus* generated maximum current densities of 5.0 to 8.3 A m^{-2}, values similar to the those reported for *Gb. sulfurreducens* at neutral pH (5 to 9 A m^{-2}) (27, 28) and much higher than those observed in alkaline microbial fuel cells with mixed communities (29, 30). *Glk. ferrihydriticus* channeled 85 to 95% of the electrons from acetate to current, thereby achieving a CE similar to that of acetate-fed *Gb. sulfurreducens* biofilms under strictly anoxic conditions (31). Figure 2 (inset) shows exponential growth of *Glk. ferrihydriticus* at a poised potential of +0.07 V, with an estimated doubling time (7.5 h) similar to that for *Glk. subterraneus*, suggesting that utilization of the anode as a respiratory electron acceptor was directly coupled to cell growth. The current density decreased in response to scraping off a section of attached *Glk. ferrihydriticus* cells and increased when remaining cells recolonized the exposed electrode surface (Fig. 2). Medium replacement did not disrupt current production by *Glk. ferrihydriticus*. Thus, cell-electrode attachment and biofilm formation govern

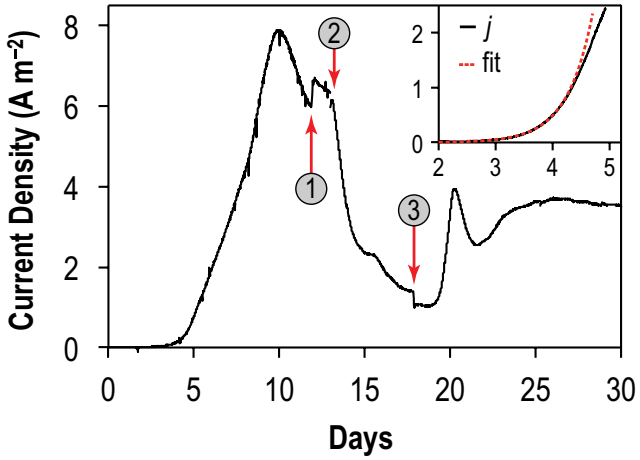

FIG 2 Chronoamperometry of acetate-fed *Glk. ferrihydriticus* DSM 17813 poised at +0.07 V, following inoculation with cells grown on Fe(III) oxide. Numbered gray circles indicate the following: 1, medium replacement; 2, generation of cyclic voltammograms (see Fig. 4); 3, scraping a section of the anode biofilm.

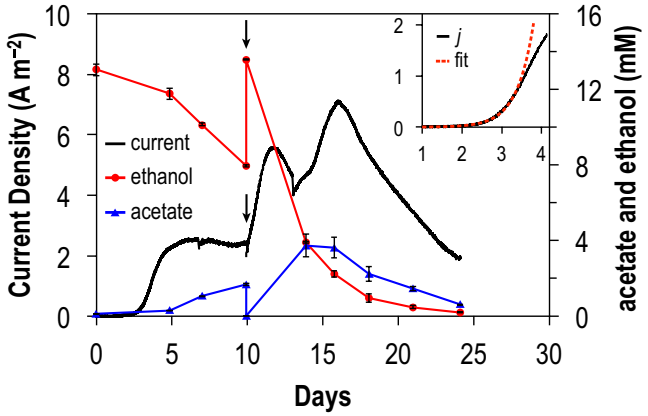

FIG 3 Current production and substrate consumption by *Glk. ferrihydriticus* with ethanol fed as the sole electron donor. Error bars may be smaller than data points and indicate standard deviations of triplicate HPLC measurements. The black arrows indicate medium replacement.

EET to anodes for *Geoalkalibacter* spp. in a fashion similar to that of the anode respiration scheme employed by *Gb. sulfurreducens*. Comparison of current: biomass ratios for the determination of biofilm development as a function of respiration rates (11) will be the subject of future investigations.

The fact that *Glk. ferrihydriticus* produced higher current densities and CE than *Glk. subterraneus* is perhaps not surprising given the wide range of electron acceptors used by *Glk. subterraneus* (23). In contrast, *Glk. ferrihydriticus* has been reported primarily to reduce insoluble electron acceptors, suggesting its metabolic machinery is finely tuned for obtaining energy available from coupling oxidation of simple organic molecules to reduction of extracellular Fe(III). The diversity of outer membrane cytochromes among the *Geobacteraceae* (7) supports the hypothesis that differences in EET machinery associated with utilization of insoluble electron acceptors may be the ultimate factors in determining anode respiration capabilities.

Current generation from ethanol. *Glk. ferrihydriticus* can couple ethanol oxidation to Fe(III) reduction (22). To evaluate utilization of ethanol as an electron donor in MECs, *Glk. ferrihydriticus* cells were cultured twice on 10 mM ethanol and 60 mM Fe(III) oxide and inoculated into MECs poised at +0.07 V. Figure 3 shows current generation under alkaline conditions by *Glk. ferrihydriticus* fed 10 mM ethanol as the sole electron donor. Cells grew on electrodes with an initial doubling time slightly faster (6.9 h) than when fed acetate (7.5 h) and produced 2.6 A m^{-2} before acetate was detected as a by-product of ethanol oxidation (Fig. 3). We confirmed using terminal restriction length fragment polymorphism (T-RFLP) that the appearance of acetate was not a result of contamination (data not shown). Medium replacement had a positive effect on current generation, providing further evidence that accumulation of acetate was not a requirement for cells to perform anode respiration and that cells rapidly metabolized ethanol to produce current. Maximum current densities in two independent growth experiments reached 5.7 to 7.1 A m^{-2} when both ethanol and acetate were present, and *Glk. ferrihydriticus* eventually channeled 84 to 95% of electrons from ethanol to current by also consuming any acetate produced (Fig. 3; see also Fig. S1 in the supplemental material). *Glk. ferrihydriticus* cells did not grow fermentatively in culture tubes lacking an electron acceptor when fed 10 mM ethanol, and acetate was not detected (data not shown). Other *Geobacteraceae* genomes (32) suggest that ethanol is oxidized to acetate via acetaldehyde, raising the possibility that a conserved pathway may exist in *Glk. ferrihydriticus*.

These results establish the highest current densities and CEs reported for an ethanol-fed pure culture of MXCs. In mixed communities, fermentation of ethanol to acetate and H_2 can result in low CE (33) unless competing electron sinks, such as methanogenesis, are inhibited (34). Ethanol-fed current densities similar to those produced by *Glk. ferrihydriticus* have been reported for defined cocultures (35) and mixed consortia (36). However, these resulted from efficient acetate oxidation by bacteria closely related to *Gb. sulfurreducens* and not from direct ethanol oxidation by ARB. In addition, none of the described Gram-positive alkaliphilic respiratory Fe(III) reducers can utilize ethanol as an electron donor. Thus, *Geoalkalibacter* spp. could emerge as model anaerobes for understanding extracellular respiratory pathways for obtaining energy from fermentable substrates under alkaline or saline conditions.

Anode respiration kinetics. Electrochemical measurements of living anode biofilms are indispensable in elucidating respiration kinetics of ARB (10). Low-scan-rate cyclic voltammetry (LSCV) applies gradual changes in anode potential in the presence of a substrate such that biofilms achieve steady-state current production at each potential (37). Figure 4A shows classic sigmoidal behavior for *Gb. sulfurreducens* biofilms scanned by LSCV under substrate turnover conditions (38), with anodic current appearing slightly above the half-reaction potential ($E^{\circ\prime}$) of the electron donor (acetate, $E^{\circ\prime} = -0.28$ V), inflecting at a half-saturation potential of −0.15 V, and saturating at maximum current density around 0 V.

Cyclic voltammograms (CVs) of acetate-fed *Glk. ferrihydriticus* biofilms after 12 days of growth at pH 9.3 showed a sigmoidal curve reaching a saturation potential (~0 V) similar to that for a laboratory *Geobacter* isolate highly similar to *Gb. sulfurreducens* (Fig. 4A; see the supplemental methods in Text S1). However, the catalytic wave of *Glk. ferrihydriticus* had a smaller slope due to cells

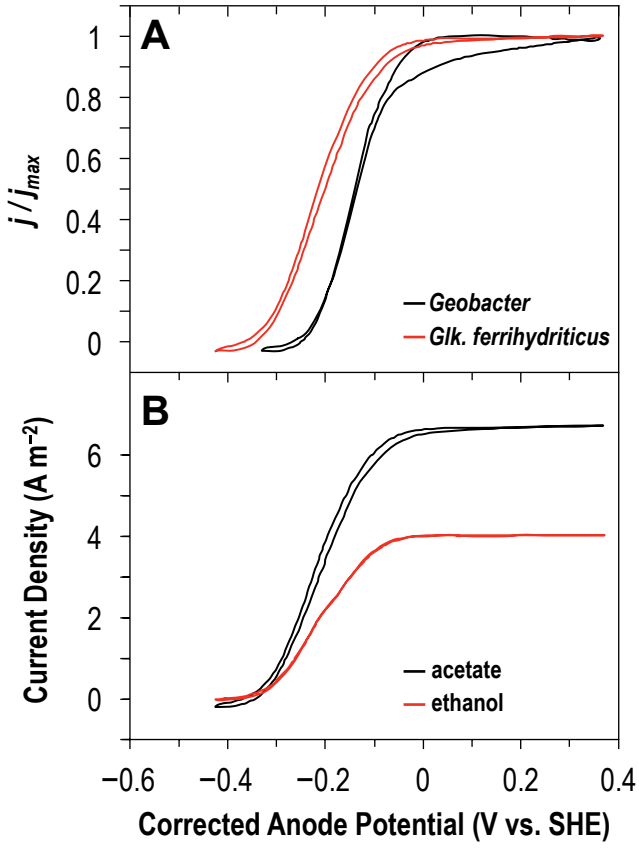

FIG 4 Cyclic voltammograms (CVs) recorded at 1 mV s^{-1} for *Glk. ferrihydriticus*. Cells were grown with 20 mM acetate or 10 mM ethanol at pH 9.3 after inoculation with a small section of scraped biofilm. (A) Comparison with a *Geobacter* isolate at pH 7 (see the supplemental methods in Text S1) of open circuit and half-saturation potentials under substrate turnover conditions at pH 9.3. (B) Kinetics of acetate and ethanol utilization by *Glk. ferrihydriticus*. When normalized to the same maximum current density as for panel A, the acetate and ethanol CVs are indistinguishable.

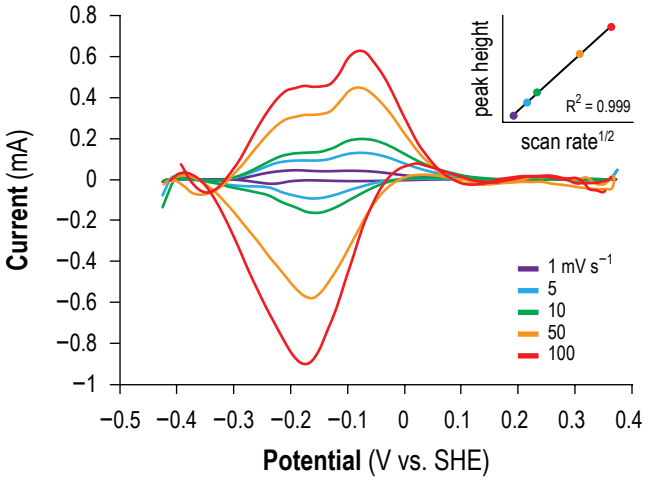

FIG 5 Nonturnover cyclic voltammograms at successively faster scan rates for *Glk. ferrihydriticus* inoculated with a small section of scraped biofilm and fed 10 mM acetate. CV scans were performed at pH 9.3 in electron donor-free medium. The inset shows linear dependence of the peak height with the square root of the scan rate, indicating the presence of redox molecules limited by diffusion.

achieving a significantly lower open circuit potential (−0.37 V versus −0.25 V for *Geobacter*; $E = -0.42$ V for acetate at pH 9). The negative shift of ~120 mV in open circuit potential closely matches the dependence of redox potentials on pH, according to the Nernst equation; half-reaction potentials should become 59 mV more negative with each pH unit increase at room temperature. As a consequence of the lower open circuit potential, the half-saturation potential for *Glk. ferrihydriticus* was −0.21 V, 60 mV more negative than that of *Gb. sulfurreducens* (10) and the lowest half-saturation potential reported to date for any ARB. CVs generated when ethanol was the substrate showed a sigmoidal catalytic wave nearly identical to that of acetate-fed cells (Fig. 4B). However, the open circuit potential with ethanol (−0.41 V) was ~40 mV more negative than that with acetate, in agreement with ethanol being a more reduced substrate ($E = -0.46$ V at pH 9). These results suggest that the open circuit potential of ARB is achieved through equilibrium with the electron donor and not with intracellular redox cofactors, such as NADH. In its apparent ability to capture more energy from respiration by generating current over a wider potential window, *Glk. ferrihydriticus* may enjoy a competitive advantage in a natural Fe(III)-reducing environment at pH 9 in which the donor half-reaction potentials shift ~120 mV more negative but reduction potentials of insoluble Fe(III) phases could remain variable (39).

CV performed on *Glk. ferrihydriticus* biofilms in the absence of substrates (nonturnover CV) produced peaks whose intensity increased at higher scan rates (Fig. 5). The height of reversible peaks near −0.08 V in the forward scan showed a linear dependence on the square root of the scan rate. Such a relationship is a hallmark of films containing bound redox molecules exchanging electrons via collisions or interprotein transfer reactions, creating diffusion-like behavior (37). The fact that similar behavior has been observed for *Gb. sulfurreducens* biofilms (11) raises the possibility that similar mechanisms of electron transfer within biofilms exist in different members of the *Geobacteraceae*.

CVs of acetate-fed *Glk. subterraneus* biofilms started from more-positive open circuit potentials (−0.27 to −0.28 V) and exhibited different behavior depending on the growth stage (Fig. 6). After 7 days, forward and reverse voltammograms were asymmetrical and nonsigmoidal, and multiple redox peaks appeared across the 0.7-V potential window scanned (Fig. 6A). These peaks began to gradually disappear 4 days later, when only a single prominent peak near −0.13 V remained and biofilms sustained a more-stable saturation current above −0.1 V (Fig. 6B). After prolonged incubation (36 days), peaks were no longer visible, and mature biofilms (Fig. 6C) eventually yielded a classic sigmoidal catalytic wave with a half-saturation potential of −0.19 V, roughly ~40 mV more negative than that of *Gb. sulfurreducens*. Similar maturation of CVs over time was confirmed in subsequent *Glk. subterraneus* growth experiments (data not shown). Therefore, at least some of the molecular machinery used by halophilic *Glk. subterraneus* for establishing electron transfer to the anode during early stages of attachment and growth seems to be different from that employed not only by mature biofilms but also by *Glk. ferrihydriticus* and *Gb. sulfurreducens*. These results provide further evidence for the *Geobacteraceae* possessing a broad range of functionally redundant strategies for accomplishing extracellular respiration under diverse environmental conditions.

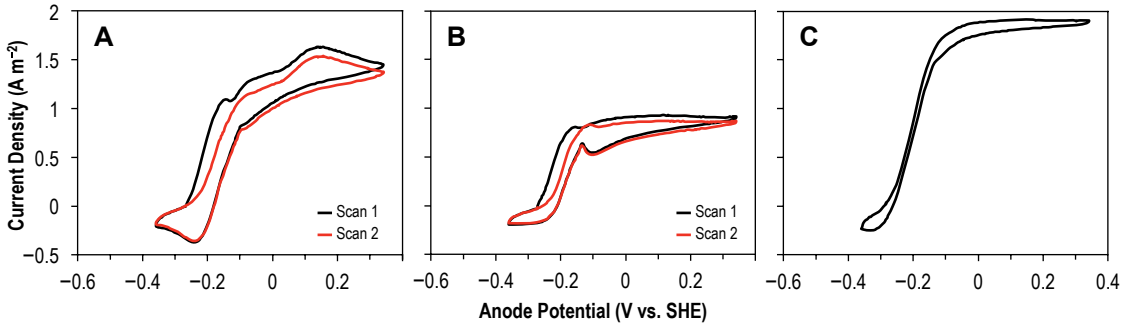

FIG 6 Cyclic voltammograms of *Glk. subterraneus*, inoculated from Fe(III)-grown cultures, recorded at 1 mV s^{-1} at early (A) (day 7), middle (B) (day 11), and late (C) (day 36) stages of biofilm growth.

Scanning electron microscopy (SEM). Both *Glk. subterraneus* and *Glk. ferrihydriticus* formed anode biofilms several micrometers thick whose ultrastructure resembled that of *Gb. sulfurreducens* (Fig. 7) (40). Throughout the *Glk. subterraneus* biofilm, cells were surrounded by web-like extracellular material, some of which appeared to condense into long filaments (see Fig. S2a and b in the supplemental material), possibly as a result of sample preparation (41). In contrast, components of the extracellular matrix, such as proteins and polysaccharides, which have been shown to assist in anchoring *c*-type cytochromes in *Gb. sulfurreducens* biofilms (42), were not abundant within *Glk. ferrihydriticus* biofilms but rather seemed to accumulate as an amorphous layer of densely packed globular material encapsulating the entire electrode surface (see Fig. S2c to e). Cells closest to this layer appeared to establish a physical connection with extracellular filaments resembling those observed in *Gb. sulfurreducens* (43). However, much more information about the physiology of *Glk. ferrihydriticus* is needed before specific components of the EET pathway can be identified.

Physiological and practical implications for *Geoalkalibacter*. *Geoalkalibacter* spp., along with most other genera within the *Geobacteraceae*, share the ability to respire insoluble, extracellular electron acceptors using electrons derived from the complete oxidation of simple organic substrates. Phylogenetic clustering indicates that distinct lineages evolved to perform this metabolism across a range of salinity, since *Geobacter* spp. prefer freshwater environments while *Desulfuromonas* and *Desulfuromusa* exist in marine habitats (6). The discovery of *Glk. ferrihydriticus* suggested that other members of the *Geobacteraceae* also evolved to take advantage of alkaline environments, such as soda lakes, which can vary in their salinity (19). The fact that both *Glk. ferrihydriticus* and *Glk. subterraneus* can grow in saline environments agrees with their close relationship to *Desulfuromonas* (23). It is possible that the capacity for alkaline extracellular respiration by *Glk. ferrihydriticus* may have evolved as a specialized extension of a halotolerant lifestyle, but characterization of other alkaliphilic representatives of the *Geobacteraceae* is needed before such evolutionary patterns can be discerned.

Metal-reducing *Geobacteraceae* face several bioenergetic challenges in extracellular respiration given the small amounts of free energy available and the charge imbalance generated from passing electrons outside the cell (44). These hurdles are potentially magnified under alkaline and saline conditions, since cells must also invest energy in maintaining intracellular pH and osmolarity (45). CE can serve as an extracellular indicator of intracellular energy demands, since directing a high fraction of electrons to respiration maintains sufficient proton motive force to drive energy-intensive processes such as ion pumping. Differences in CE values for *Glk. ferrihydriticus* and *Glk. subterraneus* suggest that pH and salinity might manifest differently in terms of the energetic burden borne by cells performing anode respiration. More likely, however, these values arise from differences in molecular machinery for conducting electrons from cells to the anode surface. Genome sequences of *Geoalkalibacter* spp. are needed to further explore the genetic elements shared across the *Geobacteraceae* and those unique to *Geoalkalibacter*, such as genes for high pH and salt tolerance and utilization of higher substrates. Such a comparative genomics approach could reveal whether a set of core genes is in fact conserved in members of the *Geobacteraceae* capable of producing high current, since this capability is no longer exclusive to *Gb. sulfurreducens*.

The production of high current densities using *Geoalkalibacter* spp. broadens the potential applications for MXCs to include direct current generation from ethanol and from treatment of saline (26) and alkaline wastewaters, such as those produced in the textile and brewing industries (46, 47). Cathodic processes in MXCs lead to elevated pH and thus a thermodynamic decrease in cathode potential (48); a possible advantage for alkaline MXCs is that some of this potential loss can be recovered by ARB capable of current generation at lower anode potentials. While breakdown of complex wastes might best be accomplished by mixed cultures of other halophilic and alkaliphilic bacteria, *Geoalkalibacter* spp. already possess greater metabolic versatility than *Gb. sulfurreducens*.

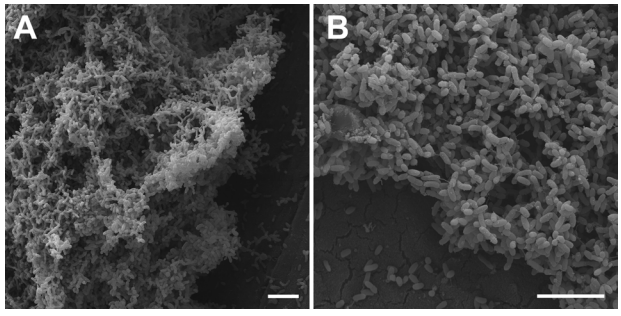

FIG 7 Scanning electron micrographs of biofilm of *Glk. subterraneus* (A) or *Glk. ferrihydriticus* (B). Samples were fixed, dehydrated, critical point dried, and sputter coated as described in Materials and Methods. Scale bar, 5 μm.

Glk. subterraneus could potentially oxidize substrates not previously studied with pure cultures in MXCs, such as propionate, butyrate, and higher fatty acids, directly to current without the need for fermentative partners. Further investigation is justified in order to affirm the potential benefits for *Geoalkalibacter* spp. in fundamental and applied MXC research.

MATERIALS AND METHODS

Strains and culture conditions. We obtained *Geoalkalibacter ferrihydriticus* DSM 17813^T and *Geoalkalibacter subterraneus* DSM 23483^T from the German Collection of Microorganisms and Cell Cultures (DSMZ). The cultures (10 ml; 1:10 inoculum) were maintained in butyl rubber-stoppered Hungate tubes containing the recommended anoxic mineral medium for each organism. We flushed the headspaces with either ultra-high-purity N_2 or 80:20 N_2-CO_2 for *Glk. ferrihydriticus* and *Glk. subterraneus*, respectively. *Glk. ferrihydriticus* medium (pH 9.3) contained (per liter of deionized water) the following: 0.5 g NH_4Cl, 0.2 g KCl, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.2 g KH_2PO_4, 1 g NaCl, 0.1 g yeast extract, 1 ml selenite-tungstate solution (49), 1 ml trace mineral solution (49), 3 g Na_2CO_3, 10 g $NaHCO_3$, and 1.36 g sodium acetate trihydrate (22). *Glk. subterraneus* medium (pH 7.2) contained (per liter of deionized water) the following: 17 g NaCl, 4.5 g $MgCl_2 \cdot 6H_2O$, 0.35 g $CaCl_2 \cdot 2H_2O$, 1 g NH_4Cl, 0.08 g KH_2PO_4, 1 ml selenite-tungstate solution, 1 ml trace mineral solution, 3.5 g sodium bicarbonate, 1.36 g sodium acetate trihydrate, and 3 g yeast extract (23). Sterile, anoxic stock solutions of carbonates and yeast extract were added aseptically to autoclaved, cooled media. Cultures of both *Geoalkalibacter* spp. were amended with 50 mM Fe(III) oxide as the electron acceptor from a sterile, anoxic stock solution prepared as previously described (50). Incubation temperatures were 30 and 40°C in all experiments for *Glk. ferrihydriticus* and *Glk. subterraneus*, respectively. An isolate of *Geobacter* closely related to *Gb. sulfurreducens* was grown on electrodes as described in the supplemental methods in Text S1.

For MEC experiments, we used the same medium compositions as above with the following modifications: acetate was increased to 20 mM, all electron acceptors were omitted, and yeast extract was replaced with 5 ml/liter Wolfe's vitamin solution (ATCC, Manassas, VA). Media had conductivities of 14.9 mS cm^{-1} and 38.9 mS cm^{-1} for *Glk. ferrihydriticus* and *Glk. subterraneus*, respectively, as measured with a digital conductivity meter (Oakton Instruments, Vernon Hills, IL). We performed two independent growth tests with *Glk. ferrihydriticus* using ethanol as the electron donor in medium containing 10 mM absolute ethanol in lieu of acetate.

MEC construction, inoculation, and operation. To maintain strict control over the anode, we constructed dual-chamber H-type microbial electrolysis cells (350-ml anode volume) with two graphite rod anodes (5.3-cm^2 total area) as previously described (34) and sterilized them by autoclaving. We polished anodes to a smooth surface by sanding with 600-grit followed by 1,500-grit aluminum oxide-silicon carbide sandpaper (McMaster-Carr). Pieces of anion exchange membrane (AMI 7001; Membranes International, Inc.) were autoclaved while submerged in 3 M NaCl and inserted after autoclaving. An Ag/AgCl reference electrode (BASi, West Lafayette, IN) was dipped in 70% (vol/vol) ethanol, flicked dry, and inserted into anode chambers. The cathode contained a sterile, anoxic NaOH solution (pH 12.5). We filled the reactors in a biosafety cabinet by flowing pressurized media through sterile Norprene tubing (Cole Parmer, Vernon Hills, IL). Before inoculation, we flushed the cathodes with N_2 and anodes with filtered (0.22-μm-pore-size) gas appropriate for the organism as described above. For *Glk. ferrihydriticus* experiments, NH_4Cl was added to anode chambers after gas flushing to prevent loss as NH_3 gas ($pK_a = 9.26$). Anode chambers were agitated constantly at 150 rpm by magnetic stirring.

To promote utilization of anodes as electron acceptors, we cultured *Geoalkalibacter* cells in medium containing a limiting concentration of 50 mM Fe(III) oxide, and 6 ml of stationary-phase cultures were used to inoculate two independent MECs (~1:60 inoculum). To inoculate two subsequent independent growth experiments, we scraped a small biofilm section (~2 mm^2) using a needle into 1 ml sterile medium in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) and used 0.1 ml of this cell suspension as the inoculum. We performed bulk medium replacement in the anaerobic chamber by transferring the anode and reference electrode to a clean MEC containing sterile medium.

Chronoamperometry and cyclic voltammetry. Anodes were poised using a VMP3 digital potentiostat (Bio-Logic USA, Knoxville, TN) at −0.2 V versus Ag/AgCl (+0.07 V and +0.04 V versus a standard hydrogen electrode [SHE] for *Glk. ferrihydriticus* and *Glk. subterraneus*, respectively), making the conversion from Ag/AgCl to SHE based on the ionic strength of each medium as described previously (24). Cyclic voltammograms (CVs) were collected after anodes reached open circuit potential, and two consecutive forward and reverse voltage sweeps at a scan rate of 1 mV s^{-1} were recorded over a potential window of −0.6 to +0.1 V (for *Glk. subterraneus*) or −0.7 to +0.1 V versus Ag/AgCl (for *Glk. ferrihydriticus*) with the second sweep shown. We corrected the CVs by measuring Ohmic loss (iR drop) between the working and reference electrodes (27) by electrochemical impedance spectroscopy (EIS) immediately after performing the CV. Unless noted, all potentials are reported versus SHE.

We performed CV in the absence of electron donors (nonturnover CV) (37) by transferring established *Glk. ferrihydriticus* biofilms grown to maximum current density (>6 A m^{-2}) to substrate-free medium and incubating until current decayed to baseline levels. CVs for two independent experiments were recorded as described above, with biofilms scanned at successively higher rates (1, 5, 10, 50, and 100 mV s^{-1}).

Estimation of specific growth rate. Exponential growth of bacteria in terms of current density (i), assuming that doubling time is constant during this period (10), can be expressed as a function of specific growth rate (μ) and time (t), $i = i_0 \exp(\mu t)$, which simplifies to $\ln(i/i_0) = \mu t$. By plotting the natural logarithm of current density versus time and performing linear regression for the initial growth period, we estimated the values for μ from the slope of the regression line.

Chemical and molecular analyses. pH was measured with a microelectrode pH probe (Cole Parmer) and digital pH meter (Thermo Scientific). Concentrations of acetate and ethanol were determined by high-pressure liquid chromatography (HPLC) as previously described (34). We calculated coulombic efficiency, defined as the percentage of electrons present in the substrate that are recovered as current (34), by comparing HPLC measurements at different time points with net charge recorded by the potentiostat over the same time interval. For ethanol-fed experiments, we calculated the coulombic efficiency based on electrons recovered over the entire time course.

We confirmed culture purity at the beginning and end of experiments by light microscopy using a 100× oil immersion objective and differential interference contrast (DIC) optics. In addition, we extracted DNA from scraped biofilm samples (see above), and 16S rRNA genes were amplified and analyzed using terminal restriction fragment length polymorphism (T-RFLP) as previously described in detail (51).

Scanning electron microscopy. We fixed anodes at 4°C overnight in 2% glutaraldehyde, postfixed in 1% OsO_4 for 1 h at room temperature, and dehydrated in a graded acetone series. Critical point-dried samples were mounted on aluminum stubs, sputter coated with Au/Pd, and imaged on an XL-30 environmental SEM (Philips) with an accelerating voltage of 10 kV and a working distance of 7 to 8 mm.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00144-13/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, EPS file, 1.2 MB.
Figure S2, TIF file, 1.4 MB.

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