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 alkalophilic *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* has no longer been exclusive to *Geobacter sulfurreducens*, suggesting greater versatility for this family in fundamental and applied microbial electrochemical cell (M XC) 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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A nodes 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 com-

pounds 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 mem-

brane in metal-reducing *Shewanella* (8). This suggests that the
**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 *Glbacter 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).

Although high salt concentrations can improve MEC 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* produces 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 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 reconstituted the exposed electrode surface (Fig. 2). Medium replacement did not disrupt current production by *Glk. ferrihydriticus*. Thus, cell-electrode attachment and biofilm formation govern
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). Cyclic voltammograms (CVs) of acetate-fed Geoalkalibacter (Fig. 4A; see the supplemental methods in Text S1). However, the catalytic wave of Glk. ferrihydriticus had a smaller slope due to cells

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 ($E^\text{0}$) of the electron donor (acetate, $E^\text{0}$ = −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
achieved 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. ferrirhydriticus* 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. ferrirhydriticus* 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. ferrirhydriticus* 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, indicating the presence of redox molecules limited by diffusion.

FIG 5 Nonturnover cyclic voltammograms at successively faster scan rates for *Glk. ferrirhydriticus* 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.

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. ferrirhydriticus* 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.
Scanning electron microscopy (SEM). Both *Glk. subterraneus* and *Glk. ferrirhydriticus* 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. ferrirhydriticus* 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. ferrirhydriticus* 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. ferrirhydriticus* 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. ferrirhydriticus* 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. ferrirhydriticus* 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. ferrirhydriticus* 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*.
Glk. subterraneus could potentially oxidize substrates not previously studied with pure cultures in MXCks, 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 ferriferriticus DSM 17813T and Geoalkalibacter subterraneus DSM 23483T 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 N2 or 80:20 N2:CO2 for Glk. ferriferriticus and Glk. subterraneus, respectively. Glk. ferriferriticus medium (pH 9.3) contained (per liter of deionized water) the following: 0.5 g NH4Cl, 0.2 g KCl, 0.1 g MgCl2 · 6H2O, 0.2 g KH2PO4, 1 g NaCl, 0.1 g yeast extract, 1 ml selenium-tungstate solution (49), 1 ml trace mineral solution (49), 3 g Na2CO3, 10 g NaHCO3, 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 MgCl2 · 6H2O, 0.35 g CaCl2 · 2H2O, 1 g NH4Cl, 0.08 g KH2PO4, 1 ml selenium-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. ferriferriticus and Glk. subterraneus, respectively. An isolate of Geoalkalibacter closely related to Glk. 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. ferriferriticus and Glk. subterraneus, respectively, as measured with a digital conductiv-

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. ferriferriticus 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 voltammo-

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 = i0 exp(μt), which simplifies to ln(i/i0) = μt. By plotting the natural logarithm of current density versus time and per-

Scanning electron microscopy. We fixed anodes at 4°C overnight in 2% glutaraldehyde, postfixed in 1% OsO4 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.
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