Microbial oxidation of elemental sulfur coupled to electron transfer to electrodes may be an important process contributing to the current production of marine benthic microbial fuel cells. Enrichments initiated with a marine sediment inoculum, with elemental sulfur as the electron donor and a positively poised (+300 mV versus Ag/AgCl) anode as the electron acceptor, yielded an anode biofilm with a diversity of micro-organisms, including *Thiobacillus*, *Sulfurimonas*, *Pseudomonas*, *Clostridium* and *Desulfuromonas* species. Further enrichment of the anode biofilm inoculum in medium with elemental sulfur as the electron donor and Fe(III) oxide as the electron acceptor, followed by isolation in solidified sulfur/Fe(III) medium yielded a strain of *Desulfuromonas*, designated strain TZ1. Strain TZ1 effectively oxidized elemental sulfur to sulfate with an anode serving as the sole electron acceptor, at rates faster than *Desulfobulbus propionicus*, the only other organism in pure culture previously shown to oxidize S\(^{2-}\) with current production. The abundance of *Desulfuromonas* species enriched on the anodes of marine benthic fuel cells has previously been interpreted as acetate oxidation driving current production, but the results presented here suggest that sulfur-driven current production is a likely alternative.

**INTRODUCTION**

Microbial oxidation of elemental sulfur coupled to electron transfer to electrodes may be an important process contributing to the current production of marine sediment microbial fuel cells (Holmes *et al.*, 2004a, 2004b; Nielsen *et al.*, 2009; Reimers *et al.*, 2007; Tender *et al.*, 2002) and is a key reaction in bioelectrical harvesting of electrons from sulfide wastes (Sun *et al.*, 2009, 2010). Effective conversion of sulfide to electricity requires microbial catalysis (Lovley, 2006). This is because although sulfide can abiotically donate electrons to electrodes (Rabaey *et al.*, 2006; Reimers *et al.*, 2001; Sun *et al.*, 2009), the abiotic reaction typically only yields two electrons per sulfide, with S\(^{2-}\) as the primary product (Lovley, 2006). The extraction of the remaining three-quarters of electrons potentially available for current production requires micro-organisms that can oxidize S\(^{2-}\) to sulfate with an electrode as an electron donor.

*Desulfobulbus propionicus* was previously shown to oxidize S\(^{2-}\) to sulfate coupled with electron transfer to an electrode (Holmes *et al.*, 2004a). This organism was chosen for initial studies on this reaction because micro-organisms closely related to *D. propionicus* were abundant on anodes harvesting electrical current from marine sediments with high sulfide content (Holmes *et al.*, 2004b; Tender *et al.*, 2002) and because *D. propionicus* was previously shown to oxidize S\(^{2-}\) to sulfate with the reduction of Mn(IV) oxide, another insoluble electron acceptor (Lovley & Phillips, 1994). However, other micro-organisms are often abundant on anodes harvesting electricity in sulfide-containing environments (Holmes *et al.*, 2004b; Ryckelynck *et al.*, 2005; Sun *et al.*, 2010; Tender *et al.*, 2002) and their potential role in current production is not completely understood. For example, *Desulfuromonas* species are often the most abundant micro-organisms colonizing anodes harvesting current from marine sediments and their presence has been attributed to their ability to oxidize acetate with current production (Bond *et al.*, 2002; Holmes *et al.*, 2004b). Here, we report the discovery of a pure culture of *Desulfuromonas* that is capable of elemental sulfur oxidation with current generation.

**METHODS**

**Organism and sediment sources.** *Desulfobulbus propionicus* (DSM 2032) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen and was anaerobically grown at 37 °C in a slightly modified NB basal medium with propionate (1.5 g l\(^{-1}\)) as the electron donor and sulfate (3 g l\(^{-1}\)) as the electron acceptor as previously described (Holmes *et al.*, 2004a).
Marine sediments were collected from Boston Harbor, MA and stored as previously described (Zhang et al., 2010). Seawater was collected from the same site and stored at 4°C in the dark. Freshwater medium (Lovley & Phillips, 1988), which was amended with additional salts (18 g l⁻¹ of NaCl, 5.4 g l⁻¹ of MgCl₂, and 0.27 g l⁻¹ of CaCl₂), was used for enrichment and isolation studies.

Studies with a graphite electrode as the electron acceptor. For electrode studies, anodes and cathodes were comprised of graphite stick (65 cm²; Mersen) and suspended in three-electrode, dual-chambered microbial fuel cells as previously described (Zhang et al., 2010). Seawater was collected from the same site and stored at 4°C in the dark. Freshwater medium (Lovley & Phillips, 1988), which was amended with additional salts (18 g l⁻¹ of NaCl, 5.4 g l⁻¹ of MgCl₂, and 0.27 g l⁻¹ of CaCl₂), was used for enrichment and isolation studies.

DNA extraction, PCR amplification of 16S rRNA and phylogenetic analysis. DNA was extracted using the FastDNA spin kit (Bio 101). The 16S rRNA genes were amplified by PCR using primers 8F (Eden et al., 1991) and 519R (Lane et al., 1985) for the mixed microbial community and primers 8F (Eden et al., 1991) and 1492R (Amann et al., 1990) for Desulfuromonas strain TZ1 as previously described (Holmes et al., 2004c; Nevin et al., 2005). PCR products were then purified with a gel extraction kit (Qiagen), and clone libraries were constructed with a TOPO TA cloning kit (Invitrogen) according to the manufacturer’s instructions. For each clone library, 96 clones were sequenced with the M13F primer at the University of Massachusetts Sequencing Facility. Completed gene sequences were aligned to the greengenes database and classified using the NCBI database and BLAST search matches for percentage identity. Nucleotide sequences were aligned using CLC Sequence Viewer (CLCBio) and phylogenetic trees were inferred using SeaView (Gouy et al., 2010). Branching order and distances were determined using parsimony and neighbour-joining algorithms. Bootstrap values were calculated for 100 replicates.

Electron microscopy. The Gram type was determined using Gram staining and bright-field microscopy as previously described (Nevin et al., 2005). For transmission electron microscopy, strain TZ1 cultures were placed on 300-mesh carbon-coated copper grids, incubated for 15 min and then stained with 2% aqueous uranyl acetate. Cells were observed using a JEOL 100S transmission electron microscope at an accelerating voltage of 80 kV. Images were taken.
In order to search for organisms capable of oxidizing S⁻, digitally using the MaxIm-DL software and analysed using Image J (http://rsbweb.nih.gov/ij/index.html).

RESULTS AND DISCUSSION

Microbial communities associated with anaerobic S⁻ oxidation coupled to electrode reduction

In order to search for organisms capable of oxidizing S⁻ with an electrode as the electron acceptor, marine sediments collected from Boston Harbor were slurried with seawater under anaerobic conditions and added to the anode chambers of bioelectrical systems, comparable to those previously described with a graphite anode poised at +300 mV (vs Ag/AgCl) as the sole electron acceptor (Zhang et al., 2010). In sulfide-based microbial fuel cells, the abiotic electrocatalysis of H₂S into oxyanions of sulfur might allow the organisms to immediately reuse these to produce more sulfide by electron abstraction from organic substrate as a cyclic process within the electrode biofilms. However, by setting the anode potential at +300 mV vs Ag/AgCl, the abstraction of electrons from sulfur by sulfur-oxidizing species forms sulfate that cannot be reduced again by the micro-organisms of the anode biofilm. The sediment slurries were amended with 0.32 g l⁻¹ of colloidal sulfur as an electron donor. Current was generated, accompanied with concurrent production of sulfate (Fig. 1a, b). When current production declined, the medium in the anode chamber was successively replaced with S⁻-containing medium which was the previously described (Lovley & Phillips, 1988) freshwater medium amended with extra salts. Current production continued. With successive enrichment the stoichiometry approached the production of 1 mol of sulfate produced per 6 moles of electrons harvested for S⁻ oxidation to sulfate with an electrode serving as the sole electron acceptor (Fig. 1), but was always less, presumably because some electrons from S⁻ oxidation were required to fix carbon dioxide for cell growth.

After the fourth medium exchange, the biofilm on the anode was scrapped off and 16S rRNA gene sequences analysed as previously described (Holmes et al., 2004b). The sequences recovered were predominantly Proteobacteria with β-Proteobacteria and ε-Proteobacteria each accounting for more than 25% of the total sequences (Fig. 1c). Within the β-Proteobacteria, the majority of the clones were most similar to Thiobacillus (Fig. 1c). Thiobacillus species are well known as chemolithotrophs that can anaerobically oxidize sulfur with the reduction of Fe(III) or nitrate (Friedrich et al., 2005; Garcia-de-Lomas et al., 2007; Pronk et al., 1992; Schedel & Trüper, 1980; Sugio et al., 1985; Waksman & Joffe, 1922). Within the ε-Proteobacteria, the majority of the clones were most similar to Sulfurimonas (Fig. 1c). Sulfurimonas species have been shown to anaerobically oxidize sulfur with nitrate as the electron acceptor (Sievert et al., 2007, 2008; Takai et al., 2006; Zhang et al., 2009). Pseudomonas (Friedrich et al., 2001; Sun et al., 2009, 2010) and Clostridia (Michaelidou et al., 2011; Sun et al., 2009, 2010; Zhao et al., 2008, 2009), which have previously
been suggested to be responsible for the oxidation of sulfur in microbial fuel cells, were also detected (Fig. 1c). \(\delta\)-Proteobacteria including close relatives to known sulfate-, Fe(III)-, and \(S^2\)-reducing bacteria were in low abundance (Fig. 1c). Three per cent of the total sequences found in the anode enrichment were members of the

**Fig. 3.** Sulfur oxidation by Desulfuromonas strain TZ1 and D. propionicus with a graphite anode poised at +300 mV (vs Ag/AgCl) as the sole electron acceptor. Current production (a, c) and rate of electron transfer to anode and sulfate generation rate (b, d). (e) Confocal laser scanning microscopy of Desulfuromonas strain TZ1 grown on the graphite anode surface. The cells were stained with a LIVE/DEAD BacLight Viability kit and imaged with confocal laser microscopy as previously described (Reguera et al., 2006). The arrows indicate when medium was replaced and additional sulfur was provided. The mean standard errors of the current and sulfate measurements were 14\% and 6\% for Desulfuromonas strain TZ1 and 18\% and 4\% for D. propionicus, respectively.
Desulfuromonadales order, which includes the genus Desulfuromonas.

**Enrichment and isolation of a S\(^-\)-oxidizing electrode reducer**

In an attempt to further enrich for a S\(^-\)-oxidizing organism, an inoculum of the anode biofilm was added to the defined medium with 0.16 g l\(^{-1}\) of colloidal sulfur as the sole electron donor and 100 mM poorly crystalline Fe(III) oxide (Lovley & Phillips, 1988) as the electron acceptor. Enrichments were incubated at 30 °C under anoxic conditions as previously described (Lovley & Phillips, 1988). When Fe(II) sufficient to account for oxidation of the added colloidal sulfur accumulated, the culture was transferred (10 %) to fresh medium. After five successive transfers in liquid medium, the culture was inoculated (10 %, v/v) into an anaerobic dilution series of the same medium amended with 2 % noble agar to form roll tubes (Nevin et al., 2005; Zhang et al., 2012). A single isolated colony was selected and further purified by repeating serial dilution in roll tubes and restreaking of single colonies on solidified medium (Nevin et al., 2005). A colony from this extended purification, designated strain TZ1, was selected for further study.

Analysis of 96 sequences of a clone library of 16S rRNA gene sequences indicated that the culture was pure, with a sequence most closely related (94.8 % similarity) to Desulfuromonas palmitatis (Fig. 2b), a marine isolate capable of oxidizing a wide range of fatty acids with Fe(III) or S\(^-\) as the electron acceptor (Coates et al., 1995).

Cells of strain TZ1 were Gram-negative rods, 1–3 mm \(\times\) 0.5–0.8 mm (Fig. 2a).

**Anaerobic oxidation of S\(^-\) coupled to electron transfer to an electrode by Desulfuromonas strain TZ1 compared to D. propionicus**

When strain TZ1 was inoculated into an anode chamber with S\(^-\) as the electron donor, current was produced with concurrent production of sulfate (Fig. 3a, b). Confocal scanning laser microscopy combined with live/dead staining revealed a thin layer of metabolically active cells on the
anode surface (Fig. 3e). Current and sulfate production rates were consistently higher for strain TZ1 than *D. propionicus* (Fig. 3), the only other organism in pure culture previously shown to oxidize S²⁻ to sulfate coupled with electron transfer to an electrode (Holmes et al., 2004a). Addition of a carbon source (100 μM acetate or propionate for strain TZ1 and *D. propionicus*, respectively) significantly increased rates of current and sulfate generation for both species (Fig. 4), probably due to the fact that these two organisms are not effective in fixing carbon dioxide.

**Implications**

The finding that a *Desulfuromonas* species is capable of S²⁻ oxidation coupled to electron transfer to electrodes not only doubles the number of pure cultures known to carry out this reaction, but also has important implications for the interpretation of the function of microbial communities colonizing anodes harvesting electricity from marine sediments. *Desulfuromonas* species are among the most abundant species in the biofilms of marine sediment fuel cells (Bond et al., 2002; Holmes et al., 2004b; Lovley, 2006; Tender et al., 2002). It was previously assumed that the primary role of these organisms was the oxidation of acetate coupled to current production, because acetate is a key intermediate in anaerobic oxidation of organic matter and *Desulfuromonas* and closely related *Geobacter* species are highly effective in acetate oxidation coupled to current production (Lovley et al., 2011). However, sulfide may be a more abundant electron donor for current production in marine sediment fuel cells than acetate. This is because acetate is a transient metabolic intermediate, maintained at low (approximately 1 μM) concentrations, whereas sulfide is an end product that builds up in sediments. Therefore, sulfide produced at substantial (>40 mm) distances from anodes may contribute to current production (Reimers et al., 2001, 2006). *Desulfuromonas* species that can utilize both acetate produced from neighbouring fermentative micro-organisms, as well as the sulfur accumulating on anodes from the abiotic oxidation of sulfide, are likely to have a competitive advantage over other micro-organisms attempting to colonize marine sediment fuel cell anodes.

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