**Geobacter** sp. SD-1 with enhanced electrochemical activity in high-salt concentration solutions

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**Summary**

An isolate, designated strain SD-1, was obtained from a biofilm dominated by *Geobacter sulfur-reducens* in a microbial fuel cell. The electrochemical activity of strain SD-1 was compared with type strains, *G. sulfurreducens* PCA and *Geobacter metallireducens* GS-15, and a mixed culture in microbial electrolysis cells. SD-1 produced a maximum current density of $290 \pm 29 \text{Am}^{-3}$ in a high-concentration phosphate buffer solution (PBS-H, 200 mM). This current density was significantly higher than that produced by the mixed culture ($189 \pm 44 \text{Am}^{-3}$) or the type strains ($< 70 \text{Am}^{-3}$). In a highly saline water (SW; 50 mM PBS and 650 mM NaCl), current by SD-1 ($158 \pm 4 \text{Am}^{-3}$) was reduced by 28% compared with 50 mM PBS ($220 \pm 4 \text{Am}^{-3}$), but it was still higher than that of the mixed culture ($147 \pm 19 \text{Am}^{-3}$), and strains PCA and GS-15 did not produce any current. Electrochemical tests showed that the improved performance of SD-1 was due to its lower charge transfer resistance and more negative potentials produced at higher current densities.

These results show that the electrochemical activity of SD-1 was significantly different than other *Geobacter* strains and mixed cultures in terms of its salt tolerance.

**Introduction**

Bioelectrochemical systems (BESs) which include microbial fuel cells (MFCs), microbial electrolysis cells (MECs) as well as other systems, are technologies in which microorganisms can drive oxidation reactions at the anode and reduction reactions at the cathode (Logan and Rabaey, 2012). The microbes that can generate an electrical current on the anode, called exoelectrogens, have been extensively researched in the past decade. Current densities generated by pure cultures have usually been lower than those produced by mixed cultures under otherwise identical conditions (Kim *et al*., 1999; Rabaey *et al*., 2004; Zuo *et al*., 2008). Only *Geobacter sulfurreducens* has so far been shown to be able to produce a current comparable to those of mixed cultures, and microorganisms most similar to this species are commonly found to predominate the biofilms of mixed cultures (Bond and Lovley, 2003; Nevin *et al*., 2008; Call *et al*., 2009; Torres *et al*., 2009; Sun *et al*., 2012). The type strain *G. sulfurreducens* PCA was first isolated as a dissimilatory metal-reducing bacteria (DMRB) from sediments, and later used in BESs (Caccavo *et al*., 1994).

Recent research has shown that not all DMRB are exoelectrogenic, and there are different characteristics needed for optimal growth on dissolving minerals compared with electrodes (Lovley, 2012). Some bacteria capable of iron reduction, such as *Pelobacter carbinolicus* and certain mutants of *Shewanella oneidensis* MR-1 (SO4144 and SO4572), are incapable of current generation without addition of exogenous mediators (Bretschger *et al*., 2007; Richter *et al*., 2007). Conversely, certain exoelectrogenic bacteria that produce current in BESs, such as *Ochrobactrum anthropi* YZ-1, are unable to reduce insoluble iron oxides (Zuo *et al*., 2008). This suggests that different electron transport proteins or mediators may be involved in electron transfer to electrodes compared with metal oxides, and that there may be exoelectrogens that have more optimal growth in BESs than strains isolated on the basis of metal oxide...
reduction. The importance of an evolutionary pressure in BESs has previously been shown based on current production using acetate. A variant of *G. sulfurreducens*, strain KN400, was isolated from the anode biofilm originally inoculated with *G. sulfurreducens* DL-1. Strain KN400 was more effective in current production than the original strain (Yi et al., 2009). However, this isolate was not selected from a natural population based on electrochemical pressure.

In order to obtain an exoelectrogen based on electrochemical pressure from a natural exoelectrogenic population, we enriched microorganisms on an anode biofilm in mini-MECs and obtained isolates through dilution to extinction (Sun et al., 2012). A previous study had shown that microbes most similar to *G. sulfurreducens* (>50% of clones) were dominant member of the anode community in MFCs fed formic acid. Current generation was sustained by acetate generated by *Acetobacterium* from conversion of formate to acetate. Even though the acetate concentration in the formic acid fed MFCs was very low (maximum of < 50 mg l⁻¹), the power density (697 mW m⁻²) was only slightly less than that obtained in these MFCs fed a much higher concentration of acetate (1 g l⁻¹ acetate, 835 mW m⁻²). This high current density suggested that *Geobacter* spp. in this biofilm had high electrochemical activity even under comparatively oligotrophic conditions. Therefore, it was decided to use the previously studied MFCs that were fed formic acid as the inoculum for isolation studies here.

**Results and discussion**

**Isolation of strain SD-1**

After inoculation for 12 days, 10⁻⁶ and the higher dilutions failed to produce current in formate-fed MECs (Supporting Information Fig. S1A), while only the lowest (10⁻⁷) dilution in acetate-fed MECs failed to generate current (Supporting Information Fig. S1B). This suggested that microbes in the 10⁻⁶–10⁻⁷ samples could not directly use formate to generate current, and that they required syntrophic partners that were absent at higher dilution levels. Based on this assumption, only the highest dilution sample (10⁻⁷) showing current generation with acetate was used in the subsequent isolation cycles.

After five repeated dilution and growth cycles, a single bacterial morphology was observed by microscopic examination of the anode bacteria, and all clones in the clone library of the isolate were identical. These results indicated that a pure culture was obtained, and this isolate was designated strain SD-1. Based on 16S rRNA sequences (1457 bp, GenBank accession number: KF006333), the isolate was classified as strain SD-1, in the genus *Geobacter*, with the closest known relative *G. sulfurreducens*, based on a similarity of 98% to *G. sulfurreducens* PCA.

**Current generation**

Strain SD-1 produced a stable maximum current of 220 ± 4 A m⁻³ (7.32 ± 0.13 mA m⁻²) with acetate over repeated-batch cycles (50 mM PBS), while much lower current densities (34 to 44 A m⁻³) were produced using lactate, propionate and pyruvate that were not significantly different (P = 0.434) (Supporting Information Fig. S2). There was minimal current produced with formate, butyrate, or succinate (4 to 14 A m⁻³) in the first fed batch cycle, and lower current densities were obtained in subsequent cycles (Supporting Information Fig. S3). Strain SD-1 did not oxidize ethanol or glucose in MECs based on an absence of current or chemical oxygen demand removal after 11 days.

The electrochemical activity of strain SD-1 was further compared with two type strains, *G. sulfurreducens* PCA (Bond and Lovley, 2003) and *G. metallireducens* GS-15 (Bond et al., 2002) and a mixed culture (Fig. 1). In 50 mM PBS, current generation by strain SD-1 and the mixed culture were the same (219 ± 14 A m⁻³, P = 0.940), and 48% higher than that of strain GS-15 and 92% higher than that of strain PCA. In 30 mM bicarbonate buffer, the current produced by SD-1 was lower even though the solution conductivity (7.0 mS cm⁻¹) was similar to that of 50 mM PBS (7.5 mS cm⁻¹). Under these latter conditions, the concentration of the likely proton carrier in bicarbonate buffered medium (BCM; i.e. HCO₃⁻) was lower than that
in PBS (sum of HPO$_4^{2-}$ and H$_2$PO$_4^-$). In the BCM, the highest current was produced by the mixed culture (175 ± 9 A m$^{-3}$), followed by SD-1, GS-15 and PCA.

Strain SD-1 produced a stable and much greater current density in PBS-H (200 mM) of 290 ± 29 A m$^{-3}$. This was surprising given previous reports that G. sulfurreducens PCA and G. metallireducens GS-15 are adversely affected by high PBS concentrations, since a high phosphate salt concentration can be toxic to these species (Call and Logan, 2011). While the mixed culture produced 189 ± 44 A m$^{-3}$, strains PCA and GS-15 generated < 70 A m$^{-3}$ in the first cycle with PBS-H, and no current in subsequent cycles (Supporting Information Fig. S4). Taking into account the electrochemical benefits of a high-conductivity solution like PBS-H, the activities of the mixed culture, strain PCA, and strain GS-15, were greatly inhibited by PBS-H, as shown by more positive anode potentials in this solution [−0.109 ± 0.007 V to −0.015 ± 0.007 V for PCA, and −0.125 ± 0.017 V to −0.016 ± 0.030 V for GS-15] (Supporting Information Table S1). These more positive anode potentials of strain SD-1 become only slightly more positive in PBS-H (from −0.188 ± 0.003 V to −0.150 ± 0.003 V).

To further verify the salt tolerance ability of strain SD-1 in PBS-H, all cultures were tested in a higher salinity solution (SW). Strains PCA and GS-15 did not generate current in SW, and therefore further electrochemical tests with these strains were discontinued with these solutions. Some key proteins of strain PCA and GS-15 in electron transfer or metabolic processes might be lost or reduced in activities in such high-salt solutions. SD-1 produced 158 ± 4 A m$^{-3}$ in SW, which was comparable to that of the mixed culture (147 ± 19 A m$^{-3}$; $P = 0.625$) but lower than that obtained using only 50 mM PBS. The anode potentials of strain SD-1 and mixed culture both became more positive in the SW compared with 50 mM PBS (Supporting Information Table S1). These more positive anode potentials indicated that the activities of SD-1 and the mixed culture were impaired by the use of 650 mM NaCl.

**Cyclic voltammetry (CV) analysis**

All CVs had typical sigmoidal shapes, consistent with previous CV studies of G. sulfurreducens and biofilms dominated by Geobacter (Fig. 2A and B) (Marsili et al., 2010; Sun et al., 2012). Strain SD-1 and the mixed culture produced similar peak currents of c. 165 ± 2 A m$^{-3}$, followed by strain GS-15 and PCA in 50 mM PBS. In the high-salt concentration solutions, the maximum current densities of SD-1 (451 A m$^{-3}$ in PBS-H and 122 A m$^{-3}$ in SW) were both higher than those of the mixed culture (401 A m$^{-3}$ in PBS-H and 95 A m$^{-3}$ in SW). Thus, the relative performance of these different strains showed agreement with MEC results.

In 50 mM PBS, at anode potentials more positive than −0.088 V, current produced by the mixed culture was higher than that of strain SD-1 (Fig. 2A). The improved current by the mixed culture was due to trace amounts of the alternate electron donor, hydrogen (produced as a result of current production in MEC), even though we attempted to remove hydrogen from solution by gas sparging prior to CV tests. Another CV test was conducted after hydrogen had built up in head space for 2 h. The peak currents of mixed culture significantly increased from 167 to 237 A m$^{-3}$ and PCA increased from 30 to 58 A m$^{-3}$ respectively, while that of strain SD-1 and GS-15 was unchanged relative to the original scans (Supporting Information Fig. S5).

First derivative CV (DCV) analysis in 50 mM PBS showed that strain SD-1 had the same peak height and mid-point potential of −0.18 V (the corresponding anode potential of the peak value of I) as the mixed culture (Fig. 2C). The DCV peaks obtained for the type Geobacter cultures were much lower than that of strain SD-1, with more positive mid-point potentials of −0.14 V for strain PCA and −0.16 V for strain GS-15. A high peak in the DCV indicates an improved electrochemical activity of the anode biofilm at that potential. Strain SD-1 also had the same peak height and mid-point potential as the mixed culture in each high-salt solution (c. −0.16 V in PBS-H and c. −0.13 V in SW) (Fig. 2D). With the increase in solution conductivity (from PBS-H to SW), the mid-point potential increased, the peak height decreased and the peak width narrowed. The narrower peak with a higher salt concentration indicated that SD-1, or the specific bacteria in the mixed culture, transferred electrons over a more limited range of potentials, or that less (or different) exoelectrogenic bacteria were now present in the mixed culture. The increased mid-point potential from −0.16 to −0.14 V indicated that the activity of cultures, especially the c-type cytochrome, was inhibited by high-salt concentration, since the redox potential of a c-type cytochrome was reported as −0.167 V (Cord-Ruwisch et al., 1998; Seeliger et al., 1998).

**Electrochemical impedance spectroscopy (EIS) analysis**

The changes in the components of the internal resistance were further examined using EIS. In the 50 mM PBS, solution resistances (where the impedance crossed the real axis on Nyquist plots, Supporting Information Fig. S6) were identical for all cultures (22 ± 1 Ω), which was expected based on the use of the same reactors and solution conditions (Fig. 3A). The total anode resistance of strain SD-1 was the smallest (115 Ω), followed by the mixed culture (146 Ω), strain GS-15 (259 Ω), and strain
PCA (1351 Ω). The charge transfer resistance was the primary component of the total anode resistance, with the lowest charge transfer resistance of 67 Ω for strain SD-1 followed by mixed culture, GS-15, and PCA.

In the high-salt solutions, the solution resistances decreased with an increase in solution conductivity, as expected (10 ± 1 Ω, PBS-H; and 4 ± 0 Ω, SW). Compared with that in 50 mM PBS, only SD-1 in PBS-H had a lower anode resistance of 51 Ω, with the lowest charge transfer resistance of 23 Ω and the lowest diffusion resistance of 17 Ω (Fig. 3A and B), consistent with the anode performance based on MEC and CV tests as discussed above. Thus, the better electrochemical activity of strain SD-1 in PBS-H was due to its low impedance. The anode resistances of strain SD-1 and mixed culture substantially increased as the solution salinity increased in SW, which indicated that the anode activities were inhibited when using such high-salt concentrations.

**Effects of pre-culture conditions**

The maximum current densities of strain PCA in 50 mM PBS was significantly lower than previously described (Call and Logan, 2011). One difference here was that the PCA inoculum was pre-cultured on ferric citrate rather than fumarate, because strains SD-1 and GS-15 cannot use fumarate as an electron acceptor. In order to test whether cultivation of ferric citrate might have affected subsequent performance of strain PCA, new reactors were inoculated with PCA pre-cultured in a fumarate-acetate medium. The electrochemical activity of PCA pre-cultured on fumarate was increased, with a current density of 212 ± 15 A m⁻². This current density was similar to that obtained with strain SD-1 and the mixed culture. However, pre-culturing strain PCA on fumarate did not change its inability to generate current in 200 mM PBS (Call and Logan, 2011).
Concluding remarks

An increase in solution conductivity can produce an increase in current generation when the solution resistance is a significant portion of the total resistance (Logan, 2008). This ability of strain SD-1 to tolerate high-salt concentrations may make it useful in other BES systems that can have highly saline anolytes, such as microbial desalination cells, microbial reverse electrodialysis cells and microbial reverse-electrodialysis electrolysis cell (Logan and Rabaey, 2012) and MECs or MFCs used for bioremediation of salty solutions (Lovley, 2011).

There are many additional factors that suggest SD-1 could be more than just a *G. sulfurreducens* variant strain. In all tests, the electrochemical activity of strain SD-1 was comparable to or even higher than that of a mixed culture and two type strains of *Geobacter*. Strain SD-1 cannot grow in fumarate-acetate medium, which is the standard medium for *G. sulfurreducens*. The performance of *G. sulfurreducens* strain PCA in MECs was also greatly inhibited by pre-culturing conditions (ferric citrate compared with fumarate), while strain SD-1 was unaffected by the presence of ferric citrate. In addition, further analyses based on DNA–DNA hybridization analysis and physiological and biochemical characterizations indicated that it had many differences from the type strain of *G. sulfurreducens* PCA (D. Sun et al., submitted). This new strain of *Geobacter* may be useful as an additional microorganism for electromicrobiology tests, as well as for providing additional insights into the extracellular electron transfer mechanisms and current generation in BESs.

Experimental procedures

**Mini-MEC construction**

Mini-MECs (5 ml) were constructed as previously described (Call and Logan, 2011), with graphite plate anodes and stainless steel mesh cathodes. A typical added voltage in MEC anode studies of 0.7 V was applied here using a power supply. All MECs were connected in parallel to the power supply, with each circuit containing a 10 Ω resistor to monitor voltage. Current was calculated using Ohm’s law (I = U/R), and current density was normalized by the liquid volume (5 ml) or the cathode area (1.5 cm²). Ag/AgCl reference electrodes (+ 200 mV versus SHE; BASi) were used to record electrode potentials in some tests. Before being inserted into the MECs, the Ag/AgCl electrodes were immersed in 70% ethanol for 6 h to sterilize them.

**Isolation**

The initial inoculum was domestic wastewater that was then enriched over a 6-month period in a MFC as previously described with sodium formate (1.5 g l⁻¹) in a 50 mM PBS nutrient medium (Sun et al., 2012). The 50 mM PBS nutrient medium contained (per litre): 2.45 g NaH₂PO₄·H₂O, 4.58 g Na₂HPO₄, 0.31 g NH₄Cl, 0.13 g KCl, 12.5 ml metal salts and 5 ml vitamins (pH = 7; conductivity = 7.5 mS cm⁻¹) (Sun et al., 2012). The same medium was used for the isolation in mini-MEC, except that either 1 g l⁻¹ sodium acetate or 3 g l⁻¹ sodium formate was used as an electron donor. Graphite fibres from the anode in the formate-fed MFC were cut and transferred to a serum bottle containing anaerobic 50 mM PBS. The bottle was vortexed to produce a cell suspension, and then serially diluted to an end-point dilution of 10⁻⁸ with anaerobic 50 mM PBS. In the first isolation cycle, the same samples at each dilution were separately inoculated into both formate-fed and acetate-fed MECs at same time, and then the reactors were operated at 30°C. Exoelectrogenic growth was monitored based on current generation. The MEC with the highest dilution that produced current was again vortexed and serially diluted by the above procedure until a pure...
culture was obtained as indicated by using microscopy and by 16S rRNA clone sequencing.

All MECs were filled with 100% nitrogen in the headspace, and sterilized by autoclaving before being inoculated. Standard anaerobic techniques were used throughout isolation procedures. All transfer ratios were 10% v/v.

16S rRNA gene sequencing

Cell suspensions of anode biofilms that represented the highest dilution with current generation during each cycle were obtained by vortexing mini-MECs. Deoxyribonucleic acid was extracted, amplified and purified as previously described (Sun et al., 2012), except that a pair of universal bacterial primers 27F (5'-AGAGTTTGATCCTGAGCTCAG-3') and 1541R (5'-AAGAGGTTGATCCTGAGCC-3') (Zuo et al., 2008) were used for the PCR amplification. When a clear sequence was obtained, the PCR products were used to generate a clone library (48 clones) to verify purity. The clone analysis, plasmid extraction and sequencing were conducted as previously described (Sun et al., 2012). The sequences were analysed by alignments with those deposited in GenBank (nucleotide collection) using the MEGABLAST algorithm of the National Center for Biotechnology Information.

Current generation

Isolates, G. sulfurreducens PCA and G. metallireducens GS-15 were pre-cultured in a ferric-acetate medium (except as noted), and then inoculated into MECs at their stationary phase. Geobacter sulfurreducens PCA was also grown in an additional test on a fumarate-acetate medium, and then used in MECs. Current production in MECs was examined by a wastewater inoculum (mixed culture) obtained from the effluent from the primary clarifier at the Pennsylvania State University Wastewater Treatment Plant. In addition to acetate and formate, the other electron donors tested were sodium forms of propionate, butyrate, lactate, pyruvate and succinate, and ethanol and glucose, all at concentrations of 1 g l⁻¹ in 50 mM PBS except that for sodium formate (1.5 g l⁻¹). The ferric-acetate medium contained (per litre): 1 g NaH₂PO₄, 0.1 g KCl, 10 ml metal salts and 10 ml vitamin. The fumarate-acetate medium was the same with the ferric-acetate medium except that 8 g l⁻¹ sodium fumarate was used instead of ferric citrate.

Microbial electrolysis cells inoculated with each culture were initially acclimated using acetate (1 g l⁻¹) in 50 mM PBS. Except for the isolation and electron donor tests, MECs operated with 1 g l⁻¹ acetate were tested with four different kinds of solutions (pH = 7): 50 mM PBS, 200 mM PBS (PBS-H), 30 mM BCM and saline water (SW: 50 mM PBS and 650 mM NaCl). PBS-H contained 9.8 g l⁻¹ NaH₂PO₄·H₂O and 18.32 g l⁻¹ NaH₂PO₄ (conductivity = 20 mS cm⁻¹). BCM (30 mM) was the same as the ferric-acetate medium except for the exclusion of the ferric citrate (conductivity = 7.0 mS cm⁻¹). Saline water contained 2.45 g l⁻¹ NaH₂PO₄·H₂O, 4.58 g l⁻¹ Na₂HPO₄ and 38.03 g l⁻¹ (650 mM) NaCl (conductivity = 55 mS cm⁻¹). PBS and SW were sparged and maintained under 100% nitrogen while BCM was sparged and maintained under a CO₂/N₂ (20/80). In order to keep the chambers anaerobic and to remove any gas produced in the former cycle, reactors were successively vacuumed and filled three times with anaerobic gas. Duplicate reactors were operated for two separate cycles at 30°C. One-way analysis of variance was conducted using Microsoft Office Excel 2011 to determine if the effects of electron donor and anode strains on current generation were significant (defined as a P-value < 0.05).

Electrochemical analysis

Cyclic voltammetry, DCV and EIS were examined using a potentiostat (VMP3; BioLogic, Clax, France; EC-Lab V10.02 software). Before tests, the reactors were emptied, sparged with nitrogen and refilled with fresh medium. The anode was the working electrode, the cathode was the counter electrode, and a Ag/AgCl electrode was used as the reference electrode. For CV and DCV analyses, the reactors were set at open circuit voltage conditions for 1 h, and then scanned from −0.50 to +0.30 V at a rate of 1 mV s⁻¹. Electrochemical impedance spectroscopy was conducted at a set potential equal to the anode operating potential corresponding to the maximum current density obtained in MECs, over a frequency range of 200 kHz to 10 mHz, with a sinusoidal perturbation of 10 mV amplitude. The EIS spectra were fitted into an equivalent circuit containing a solution resistance (Rₛ), two charge transfer resistances (R_dl, and R_sw), a diffusion resistance (R_d) and double layer capacitance (Q) (Supporting Information Fig. S7) (Xia et al., 2013). After all electrochemical tests were finished, electrode potentials were adjusted for the accuracy of the Ag/AgCl electrode.

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References


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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Current generation as a function of time in mini-MECs with different dilutions of an anode biofilm following the first isolation cycle (F: formate, AC: acetate). (A) Mini-MECs were fed with formate and inoculated with 10−2–10−4 and 10−6–10−8 dilution samples, or (B) were fed with acetate and inoculated with 10−6–10−8 dilution samples.

**Fig. S2.** Maximum volumetric current densities by strain SD-1 in mini-MECs (Eφ = 0.7 V, 50 mM PBS) using different electron donors. The data are means of duplicate or more reactors in two separate cycles, with error bars based on standard deviations for acetate, lactate, propionate and pyruvate, or the first cycle for succinate, formate and butyrate (see Supporting Information Fig. S3 for details) when successive cycles failed to produce repeatable cycles of current.

**Fig. S3.** Current generation in the first two cycles by strain SD-1 in mini-MECs using succinate, formate and butyrate.

**Fig. S4.** Current generation in the first two cycles by strain SD-1 in Mini-MECs using succinate, mixed culture, PCA and GS-15 in mini-MECs using PBS-H.

**Fig. S5.** CVs (1 mV s−1) conducted under accumulated hydrogen condition by operating at 0.7 V for 2 h in a new cycle after of strain SD-1, *G. sulfurreducens* PCA, *G. metallireducens* GS-15 and mixed culture (MC); (B) first derivative analysis (DCV) of CV curves from (A).

**Fig. S6.** Nyquist plots of EIS spectra for the anodes of strain SD-1, *G. sulfurreducens* PCA, *G. metallireducens* GS-15 and mixed culture (MC) in 50 mM PBS, PBS-H and saline water (SW).

**Fig. S7.** Equivalent circuit for anode EIS.

**Table S1.** Anode potentials of strains SD-1, PCA, GS-15 and mixed cultures corresponding to the maximum current densities obtained in Fig. 1. (NC indicates measurements were not conducted).