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Characterization of the genome from *Geobacter anodireducens*, a strain with enhanced current production in bioelectrochemical systems†

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Geobacter anodireducens is unique in that it can generate high current densities in bioelectrochemical systems (BES) operating under high salt conditions. This ability is important for the development of BES treating high salt wastewater and microbial desalination cells. Therefore, the genome of *G. anodireducens* was characterized to identify proteins that might allow this strain to survive in high salt BES. Comparison to other *Geobacter* species revealed that 81 of its 87 *c*-type cytochromes had homologs in *G. soli* and *G. sulfurreducens*. Genes coding for many extracellular electron transfer proteins were also detected, including the outer membrane *c*-type cytochromes OmcS and OmcZ and the soluble *c*-type cytochrome PgcA. *G. anodireducens* also appears to have numerous membrane complexes involved in the translocation of protons and sodium ions and channels that provide protection against osmotic shock. In addition, it has more DNA repair genes than most *Geobacter* species, suggesting that it might be able to more rapidly repair DNA damage caused in high salt and low pH anode environments. Although this genomic analysis provides invaluable insight into mechanisms used by *G. anodireducens* to survive in high salt BES, genetic, transcriptomic, and proteomic studies will need to be done to validate their roles.

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1 Introduction

Extracellular electron transfer (EET) is an important bioprocess that plays a role in global biogeochemical cycles, bioremediation applications, as well as anaerobic digestion.[1] EET is primarily carried out by dissimilatory metal-reducing bacteria (DMRB), which have the ability to transfer electrons from the cytoplasm to the outer cell surface in order to reduce extracellular electron acceptors including Fe(III), neighboring microorganisms *via* direct interspecies electron transfer (DIET), or electrodes in microbial fuel cells (MFCs).[2,3]

MFCs rely on microorganisms that colonize the anode to transfer electrons from the oxidation of organic matter. MFC technology has been developed for a variety of bioelectrochemical system (BES) applications including hydrogen production,[4] desalination,[5] organic product synthesis,[6] biosensors,[7–9] and wastewater treatment.[10] The study of EET by exoelectrogens has resulted in the development of the novel sub-discipline, electromicrobiology.[11–13] However, mechanisms used to transfer electrons from a microorganism's cytoplasm out to extracellular electron acceptors like anodes still remains an area of controversy. Therefore, identification and characterization of microbes that are superior in their EET capabilities is essential for understanding the EET mechanisms favored in the environment and for practical applications.

Mechanisms for EET have been most thoroughly studied in the genera *Geobacter* and *Shewanella*.[14] The model exoelectrogenic *Shewanella* species, *S. oneidensis*, uses a single known pathway to transfer electrons from an inner membrane cytochrome (CymA) to the MtrCAB porin-multiheme *c*-type cytochrome complex.[15] The Mtr proteins directly reduce flavins, which can act as soluble electron shuttles.[16] Approximately 75% of EET by *S. oneidensis* results from electron shuttles, which allows it to produce current in MFCs even though it

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cannot form thick biofilms.[17–19] *Geobacter* species, on the other hand, form very thick biofilms, and a recent study showed that *G. sulfurreducens* produced 1047% times more maximum current than *S. oneidensis*.[20]

Geobacter spp. are the most abundant microorganisms in anaerobic soils and sediments where microbial reduction of insoluble Fe(III) oxides is important.[1] To date, a total of 21 *Geobacter* species have been characterized (NCBI Taxonomy browser). Nearly all of the research regarding mechanisms of EET by *Geobacter* has been conducted in *G. sulfurreducens* strain PCA because it was the first to have a genome sequence, it can be genetically manipulated, and its metabolic traits such as rapid growth with fumarate as the electron acceptor make it easy to cultivate in the lab.[21,22] However, *G. sulfurreducens* is not the most environmentally representative strain as it does not reduce Fe(III) oxides or produce current as effectively as some other *Geobacter* species[23–27] and it rarely shows up in environmental samples.[3]

Although it is apparent that *Geobacter* species do not use soluble flavin shuttles like *Shewanella*, there does not seem to be one single pathway for EET amongst the genus. All *Geobacter* genomes sequenced to date appear to have genes that code for abundant multiheme *c*-type cytochromes and electrically conductive type IV pili (e-pili).[28–30] However, specific mechanisms used for EET seem to vary between species and even among strains of the same species.[31–33] For example, the soluble extracellular *c*-type cytochrome PgcA is required for Fe(III) oxide reduction in some strains of *G. sulfurreducens*[34] and in the absence of e-pili,[32] but it is not significant in other strains.[35] In addition, OmcS facilitates electron transfer to Fe(III) oxide[33] and anodes with thin biofilms formed by *G. sulfurreducens*,[36] while a different *c*-type cytochrome, OmcZ, is involved in EET in thick anode biofilms.[37,38] *G. metallireducens* does not have OmcS, although several other *c*-type cytochromes as well as e-pili are essential for EET.[32,39,40] Furthermore, some *Geobacter* strains that are capable of Fe(III) oxide reduction, are completely incapable of current generation. For example, *G. bemidjiensis* contains both e-pili and a *c*-type cytochrome in the OmcZ family, however it is not able to produce current.[23,41]

Geobacter anodireducens was the first *Geobacter* species isolated from an anode biofilm based on its ability to generate current.[26] In cyclic voltammetry, electrochemical impedance spectroscopy, and biomass tests, *G. anodireducens* SD-1 performed better than *G. sulfurreducens* PCA, *G. metallireducens* GS-15, and a mixed culture (wastewater). Current production was even greater in BESs operating with high salt concentrations.[42] *Geobacter anodireducens* SD-1 will be useful for providing additional insights into superior EET mechanisms and current generation in BESs. As more is learned, development of various new BES technologies can emerge. In this study, the genome of *G. anodireducens* SD-1 was analyzed and compared to genomes from closely related *Geobacter* species that are also able to generate current in MFCs in order to identify genes that might lead to the enhanced EET capabilities of SD-1.

2 Experimental

2.1 Physiological characterization

Geobacter anodireducens SD-1, *Geobacter sulfurreducens* PCA (ATCC 51573), and *Geobacter metallireducens* GS-15 (ATCC 53774) were acquired from Dr Bruce Logan's laboratory culture collection at Pennsylvania State University and grown anaerobically on bicarbonate-buffered medium (BCM-30)[43] with acetate (1 g L^{-1}) provided as the electron donor and Fe(III) citrate (20 mM) as the electron acceptor. All incubations were conducted under an 80 : 20 $N_2 : CO_2$ atmosphere at 30 °C in the dark.[26]

Electrical activities of SD-1, PCA, and GS-15 were tested in mini-BESs (5 ml) with graphite plate anodes and stainless steel mesh cathodes as previously described.[42,44] Microbial electrolysis cell (MEC) chambers were filled with either 50 mM phosphate buffer (PBM-50), 200 mM phosphate buffer (PBM-200), or saline water (SW, 50 mM PBS with 3.8% NaCl) and 1 g L^{-1} sodium acetate was provided as the electron donor. Reference electrodes (Ag/AgCl; +200 mV *vs.* standard hydrogen electrode (SHE); BASi) were used to record anode and cathode potentials. An applied potential of 0.7 V was supplied to each MEC. MECs were operated with a programmable power supply (model 3645A; Circuit Specialists Inc), and each circuit contained a 10 Ω resistor.

Voltage was recorded at 20 minute intervals with a multimeter (model 2700; Keithley Instruments Inc.), and current was calculated using Ohm's law ($I = V/R$). Volumetric current density (I_V; A m^{-3}) was determined by dividing current by liquid volume, and current density per area (I_A; A m^{-2}) was calculated by dividing current by anode surface area.

Fe(III) reduction was monitored by measuring Fe(II) that could be extracted in 0.5 M HCl after a 1 hour incubation with a ferrozine assay at an absorbance of 562 nm as previously described.[45]

2.2 *G. anodireducens* SD-1 genome sequencing, assembly and annotation

Total genomic DNA was extracted with a DNA extraction kit (Lifefeng), according to the manufacturer's protocol. The concentration and purity of DNA was measured with a NanoDrop spectrophotometer (ND 1000, Thermo Fisher Scientific, DE). The genome of strain *G. anodireducens* SD-1 was sequenced at the Life Science College of Zhejiang University using the PGM system (ABI, USA). The Ion Torrent data included a 350 bp paired-end library and a 3k bp mate-pair library, and a total of 0.8 G and 0.6 G of raw data were produced after filtering, respectively. These sequences were assembled into 2 contigs using the CLC Genomics Workbench 6.0 (CLCbio, Waltham, MA).

Preliminary annotation was performed using Rapid Annotation Subsystem Technology (RAST)[46] and NCBI. SignalIP v4.1 was used to identify genes with signal peptides, and THMMER 2.0 was used to define genes with transmembrane helices.[47,48] Translated amino acids were assigned to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using KASS (KEGG Automatic Annotation Server).[49]

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2.3 Comparative genomics

Genomes from *Geobacter anodireducens* SD-1 (NZ_CP014963 and NZ_CP014964), *G. soli* GSS01 (NZ_JXBL01000000), *G. sulfurreducens* PCA (NC_002939), and *G. metallireducens* GS-15 (NC_007517 and NC_007515) were downloaded from NCBI and screened by comparison to sequences in the NR database with the BLASTp algorithm[50] using NCBI BLAST-2.2.31+ standalone software.[51] *C*-type cytochrome proteins were identified by screening each protein for the heme binding motif (CXXCH). Tools on the IMG/M (Integrated Microbial Genomes & Microbiomes) website (https://img.jgi.doe.gov) were also used for comparison of the four genomes.

3 Results and discussion

3.1 Similarities to *Geobacter soli* GSS01

Geobacter anodireducens SD-1 was isolated from a MFC biofilm inoculated with effluent collected from a primary clarifier at the Pennsylvania State University Wastewater Treatment Plant.[52] Intriguingly, its genome is most similar to another *Geobacter* species, *Geobacter soli* GSS01, isolated from a completely different environment. *Geobacter soli* GSS01 was isolated from a soil sample taken from the humic layer of an underground ancient forest in China.[27] Comparisons of the two genomes with the Pairwise ANI (ANI; a measure of nucleotide-level genomic similarity between the coding regions of two genomes) tool available on the IMG/MER website (www.img.jgi.org) revealed that 99.64% of the genes are similar.

The physiologies of *G. anodireducens* and *G. soli* also appear to be quite similar (Table 1). Both strains can couple the oxidation of acetate with a variety of extracellular electron acceptors including Fe(III), S^0, Mn(IV), and AQDS, and neither strain can respire fumarate. However, *G. soli* can grow with nitrate provided as an electron acceptor,[27] while *G. anodireducens* cannot couple the oxidation of organic compounds with nitrate respiration.[26]

G. anodireducens and *G. soli* are both much more similar to *G. sulfurreducens* than to *G. metallireducens*. Pairwise ANI values from comparisons of *G. anodireducens* to *G. sulfurreducens* and *G. metallireducens* were 93.10 and 78.65, respectively. Similar to most members of the genus *Geobacter*, all four strains can utilize both soluble and insoluble Fe(III) as the sole electron acceptor coupled with the oxidation of acetate,[25–27,53] and are capable of producing current on the anode of MFCs (Table 1).[11,26,54]

Table 1 Major physiological characteristics of *G. anodireducens* SD-1 (ref. 26), *G. sulfurreducens* PCA,[25] *G. metallireducens* GS-15 (ref. 55), and *G. soli* GSS01 (ref. 27)[a]

Characteristic	SD-1	PCA	GS-15	GSS01
Acetate (electron donor)	Yes	Yes	Yes	Yes
Hydrogen (electron donor)	Yes	Yes	No	ND
Ferric citrate (electron acceptor)	Yes	Yes	Yes	Yes
Fumarate (electron acceptor)	No	Yes	No	No
Nitrate (electron acceptor)	No	No	Yes	Yes
Sulfur (electron acceptor)	Yes	Yes	No	Yes
Mn(IV) (electron acceptor)	Yes	Yes	Yes	Yes
AQDS (electron acceptor)	Yes	Yes	Yes	Yes
MFC anode (electron acceptor)	Yes	Yes	Yes	Yes
NaCl tolerance	3.0%	1.7%	0.5%	ND
Presence of plasmid	Yes	No	Yes	No

[a] ND: not determined.

3.2 Physiological comparison of *Geobacter anodireducens* to *G. sulfurreducens* and *G. metallireducens*

Due to the in depth characterization and completed genome sequences of *G. sulfurreducens* PCA and *G. metallireducens* GS-15, further physiological comparisons with these species were done in order to uncover potential mechanisms used for enhanced EET in high salinity environments. As previous research has shown, *G. anodireducens* SD-1 grew fastest of the three strains with soluble Fe(III) citrate provided as the electron acceptor. It had a doubling time of only 5.4 hours, while generation times of *G. metallireducens* GS-15 and *G. sulfurreducens* PCA were only 6.11 and 9.11 hours, respectively (ESI Fig. S1A†).[26] In addition, strain SD-1 consistently produced the highest currents in BES with high-salt solutions (50 or 200 mM phosphate buffer solution, and high salinity water) (ESI Fig. S1B†).[42]

Geobacter anodireducens shares some physiological traits with both *G. sulfurreducens* and *G. metallireducens*. All three species can grow with a number of insoluble extracellular electron acceptors including current-harvesting anodes, Fe(III), Mn(IV), and AQDS (Table 1).[25,26,35,53] However, among the three species only *G. anodireducens* and *G. sulfurreducens* can grow with elemental sulfur provided as an electron acceptor.[25,26] Fumarate is a soluble electron acceptor that can only be used by *G. sulfurreducens*,[25] while nitrate is a soluble electron acceptor that can only be utilized by *G. metallireducens*.[53] Another important difference between these strains is that *G. anodireducens* is much more salt tolerant compared to the other two strains.[42] Similar to *G. metallireducens*, a plasmid was identified in the *G. anodireducens* genome.

In terms of environmental niche, *G. anodireducens* SD-1 survived at pH and temperature ranges similar to those of *G. metallireducens* and *G. sulfurreducens*.[25,26,53] However, SD-1 was able to grow with up to 3% NaCl,[26] which is significantly higher than *G. sulfurreducens* and *G. metallireducens* which could only tolerate 1.7% NaCl[25] and 0.5% NaCl,[53] respectively. Furthermore, SD-1 generated significant current in BES with 200 mM phosphate buffer and 650 mM NaCl, while neither *G. sulfurreducens* nor *G. metallireducens* could grow in the high salt solution and both were significantly impaired in 200 mM phosphate solutions (ESI Fig. S1B†).[42] Salt concentrations have been shown to influence microbial metabolism in MFCs and a recent study demonstrated that NaCl concentrations should remain below 0.1 M for optimal electricity generation.[55] SD-1's ability to withstand such high salt concentrations will make the strain useful in future application of microbial desalination cells and microbial reverse electrodialysis cells[56] as well as BES used for bioremediation of high salt solutions.[57]

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3.3 General genomic features

The complete genome of SD-1 consists of a circular chromosome of 3 555 507 bp with a GC content of 61.8% and a circular plasmid of 110 507 bp with a GC content of 52.2%. In total, 3434 genes have been identified on the chromosome and 130 genes have been detected on the plasmid. These genome statistics are similar to those from the *G. soli*, *G. sulfurreducens*, and *G. metallireducens* genomes, except that *G. metallireducens* is the only other species with a plasmid (Table 2).

The Function Category Comparison tool on the IMG/MER website was used to classify genes from various COG pathways in the four different genomes (ESI Table S1†). The comparison revealed that both the *G. anodireducens* and *G. soli* genomes have genes coding for two ATP synthase complexes, the F- and V-type ATPases. The only other *Geobacter* species that have both ATPase complexes are *G. uraniireducens* and *Geobacter* sp. M18.[58] The F-type ATPase complex is found in most bacteria, eukaryotic mitochondria, and chloroplasts[58–60] while the V-type complex is found in archaea, a few bacteria, and eukaryotic vacuoles.[58,61–63] Studies have found that both of these ATPases can translocate protons or sodium ions across the membrane to drive the synthesis or hydrolysis of ATP.[58,59,63] Analysis of subunit *c* from the F- and V-type ATPase complexes revealed that neither appears to have Na^+-binding sites,[58] suggesting that both of these ATPase complexes are proton-dependent.

Another difference among these genomes is the presence of genes for a multisubunit NA^+/H^+ antiporter in *G. anodireducens*, *G. soli*, and *G. sulfurreducens*, but not *G. metallireducens*. There are 7 Mrp (multiple resistance and pH) subunits (MrpA-G) required for enzymatic activity[64] and *G. anodireducens* has genes for all 7 of these subunits (Ga0133348_111263–Ga0133348_111270). The Mrp complex is a Na^+/H^+ antiporter that utilizes the proton motive force to efflux intracellular sodium ions, and its functions include sodium tolerance and pH homeostasis.[65–68] This complex is likely to be important for the high salt tolerance of *G. anodireducens*.[42]

An additional notable difference is that the number of fatty acid metabolism genes is significantly higher (∼3 times) in the *G. metallireducens* genome than the other three genomes. This can be explained by the fact that *G. metallireducens* is able to utilize a number of aromatic hydrocarbons (*i.e.* benzoate, toluene, phenol, *p*-cresol, benzene) as electron donors[53,69] and these pathways share many genes with the fatty acid degradation pathway.[70–72]

Table 2 Basic genome statistics from *G. anodireducens* SD-1, *G. soli* GSS01, *G. sulfurreducens* PCA, and *G. metallireducens* GS-15

	Total number of bases	Number of genes	% G + C
SD-1 chromosome	3 555 507	3434	61.8%
SD-1 plasmid	110 507	130	52.2%
GSS01 chromosome	3 657 100	3388	61.8%
PCA chromosome	3 814 128	3711	60.9%
GS-15 chromosome	3 997 420	3617	59.5%
GS-15 plasmid	13 762	18	52.5%

3.4 Metabolic pathways

All four *Geobacter* species have genes coding for all of the enzymes in the tricarboxylic acid (TCA) cycle (Fig. 1). The *G. metallireducens* genome, however, has significantly more TCA cycle genes (ESI Table S1†), as it has multiple copies of many of the enzymes. For example, *G. metallireducens* has two citrate synthase (*gltA*) genes and two sets of genes coding for the succinate dehydrogenase/fumarate reductase complex.[24]

Similar to *G. metallireducens*, neither *G. anodireducens* nor *G. soli* are capable of growth with fumarate provided as the electron acceptor.[26,27] Both of these organisms have genes that code for a succinate dehydrogenase complex which also functions as a respiratory fumarate reductase in *G. sulfurreducens*; Ga0133348_112411–112413 in *G. anodireducens* and Ga0077628_111869–111871 in *G. soli* (Fig. 2). However, their genomes lack the gene that codes for the anaerobic C4-dicarboxylate transporter (*dcuB*), which is required for the transport of aspartate, malate, fumarate, and succinate by many bacterial species.[73–75] When the *dcuB* gene from *G. sulfurreducens* is expressed on a vector introduced into *G. metallireducens*, fumarate respiration is possible.[76] It is likely that both of these strains would also be able to respire fumarate if the *G. sulfurreducens dcuB* gene was introduced into their chromosomes.

The respiratory nitrate reduction pathway has been characterized in *G. metallireducens* and genes for the nitrate reductase complex (*narGYJI*) and the nitrate/nitrite antiporter (*narK*) have been identified.[24] Analysis of the other three genomes did not reveal the presence of any genes that code for proteins involved in nitrate respiration. This is consistent with the fact that neither *G. sulfurreducens* nor *G. anodireducens* can grow with nitrate provided as an electron acceptor. However, it is interesting that *G. soli* was reported to grow with nitrate provided as the sole electron acceptor.[27] Further investigation into the mechanism for nitrate reduction used by *G. soli* is required.

3.5 Electron transport genes involved in extracellular electron transfer

Similar to other characterized *Geobacter* species, *G. anodireducens* appears to excel at its ability to exchange electrons with the extracellular environment.[26] Numerous studies have identified *c*-type cytochromes that are involved in extracellular electron transfer to or from such substrates as Fe(III) oxide, Fe(III) citrate, Mn(IV) oxide, electrodes, Fe(0), Fe(III) containing sediments, and other microorganisms in such organisms as *G. sulfurreducens*,[33,37,77–79] *G. metallireducens*,[32,40] *G. soli*,[54] and *G. uraniireducens*.[35]

The *G. anodireducens* genome has 87 genes that could potentially code for *c*-type cytochrome proteins (ESI Table S2†). Four of these cytochromes are predicted to be localized to the inner membrane, 24 are extracellular or outer membrane associated, and 24 are periplasmic. More than half (53.5%) of these *c*-type cytochromes have 5 or more heme groups and 30.2% have 2 to 4 hemes. Many of these *c*-type cytochromes have homologs in both *G. soli* and *G. sulfurreducens* (ESI Table S2A†). In addition, many of the *c*-type cytochromes that genetic, transcriptomic, proteomic, and genomic studies have identified as

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Fig. 1 Identification of genes that code for proteins from the tricarboxylic acid (TCA) cycle in the *G. anodireducens* SD-1 genome.

being critical for extracellular electron exchange by *Geobacter* have homologs in *G. anodireducens* (Table 3, ESI Table S2†).

In order for *Geobacter* to use extracellular substrates as terminal electron acceptors, they need to be able to transfer electrons from the quinone/quinol pool in the inner membrane, across the periplasm and then across the outer membrane to the extracellular environment.[3,80] Two inner membrane *c*-type cytochromes in *G. sulfurreducens*, the inner membrane cytochrome c (ImcH) and a cytochrome protein with *b*- and *c*-type domains (CbcL), appear to be involved in the early steps of

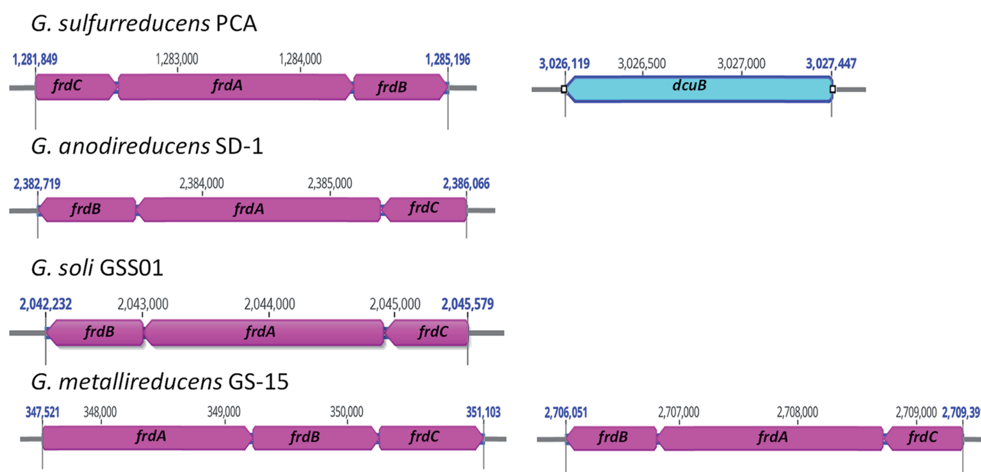

Fig. 2 Location of genes that code for fumarate reductase subunits (*frdABC*) in *G. sulfurreducens* PCA, *G. anodireducens* SD-1, *G. soli* GSS01, and *G. metallireducens* GS-15 genomes. *G. sulfurreducens* PCA is the only one of the four genomes that contains the gene that codes for the anaerobic C4-dicarboxylate transporter (*dcuB*) and *G. metallireducens* is the only one of the four with two copies of the *frdABC* operon.

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Table 3 Homologs in *G. anodireducens* for genes from *G. sulfurreducens* and *G. metallireducens* that code for proteins that show impaired growth on extracellular electron acceptors when they are deleted from the chromosome

Locus ID	SD-1 Locus ID	Abbreviation	Annotation	Impaired growth substrate(s)	References
GSU1761	Ga0133348_111806	*pgcA*	Soluble extracellular cytochrome c, class I	Fe(III) oxide	31 and 34
GSU2724–GSU2726	No homologs	*extEFG*	Outer membrane electron conduit complex	Fe(III) citrate,	88
GSU2642–GSU2645	Ga0133348_11946–11949	*extABCD*	Porin–cytochrome (Pcc) complex	Anode	88
GSU2936–GSU2940	Ga0133348_113069–113073	*extHIJKL*	Porin–cytochrome (Pcc) complex	Fe(III) citrate, anode	88
GSU2731–GSU2739	Ga0133348_11855–11861	*omcBC complex*	Two tandem porin-cytochrome (Pcc) complexes	Fe(III) oxide, Fe(III) citrate, anode	85 and 87–91
GSU3259	Ga0133348_113383	*imcH*	Inner membrane c-type cytochrome protein	Fe(III) citrate, Mn(IV) oxide	82
GSU0274	Ga0133348_11226	*cbcL*	*b*/*c*-type cytochrome domain protein	Fe(III) oxide	83
GSU2504	Ga0133348_111108	*omcS*	Outer membrane *c*-type cytochrome		33
GSU2076	Ga0133348_111435	*omcZ*	Outer membrane *c*-type cytochrome	Anode	37 and 38
GSU0618	Ga0133348_11695	*omcE*	Outer membrane *c*-type cytochrome	Fe(III) oxide, Mn(IV) oxide, anode, AQDS	32 and 33
GSU1496	Ga0133348_112105	*pilA-N*	Pilin domain protein	Fe(III) oxide, anode	37, 39 and 101
GSU0612	Ga0133348_11688	*ppcA*	Periplasmic cytochrome c, class III	Fe(III) citrate	35 and 84
GSU1394	Ga0133348_112215	*ompB*	Multicopper oxidase protein	Fe(III) oxide, Mn(IV) oxide	100
GSU2657	No homolog	*ompC*	Multicopper oxidase protein	Fe(III) oxide	99
GSU1501	Ga0133348_112101	*xapD*	ATP dependent transporter involved in exopolysaccharide biosynthesis	Fe(III) oxide, anode	105 and 106
GSU1704	Ga0133348_111888	*esnA*	Mcp protein	Anode	87
GSU2220	Ga0133348_111374	*esnB*	cheW scaffolding protein	Anode	87
GSU2222	Ga0133348_111372	*esnC*	cheA histidine kinase	Anode	87
GSU3376	Ga0133348_1182	*esnD*	Diguanylate cyclase	Anode	87
Gmet_0557	Ga0133348_113044	*omcP*	Outer membrane c-type cytochrome	Fe(III) oxide	32 and 35
Gmet_0558	No homolog	*omcO*	Outer membrane c-type cytochrome	Fe(III) oxide	32 and 35
Gmet_1867	Ga0133348_111778		*c*-type cytochrome	Fe(III) oxide	32
Gmet_1868	Ga0133348_111777	—	*c*-type cytochrome	Fe(III) oxide	32
GSU2505	Ga0133348_111106	—	NHL repeat domain protein	Anode	105
GSU3361	Ga0133348_1198	—	Transglutaminase domain protein	Anode	105
Gmet_2029	Ga0133348_111539	—	Exopolysaccharide biosynthesis protein	Fe(III) oxide	32

electron transfer to extracellular substrates.[81–83] ImcH is required for reduction of extracellular electron acceptors with reduction potentials greater than 0.1 V *versus* the standard hydrogen electrode (SHE) such as Fe(III) citrate and insoluble Mn(IV) oxides,[82] while CbcL is important for electron transfer to low potential acceptors such as Fe(III) oxides.[83] Homologs for both *imcH* and *cbcL* are present in the *G. anodireducens*, *G. soli*, and *G. metallireducens* genomes (Table 3, ESI Table S2B†). All four genomes also have homologs for PpcA, another periplasmic *c*-type cytochrome that is required for efficient reduction of Fe(III) citrate,[84] but not insoluble Fe(III)-oxide or an electrode.[35]

Once periplasmic and inner membrane cytochromes shuttle electrons across the periplasm, they need to then be transferred across the outer membrane. Studies have suggested that porin cytochrome (Pcc) protein complexes, found in all *Geobacter* species that have been sequenced to date, act as electron conduits for transfer across the outer membrane.[85,86] These conduits are composed of a periplasmic multiheme *c*-type cytochrome, a porin like outer membrane protein, and an outer membrane *c*-type cytochrome.[85,87–91] The *G. sulfurreducens* genome has 5 gene clusters that code for putative Pcc complexes; *ombB–omaB–omcB* (GSU2737–GSU2739), *ombC–omaC–omcC* (GSU2731–GSU2733), *extEFG* (GSU2724–GSU2726), *extABCD* (GSU2642–GSU2645), and *extHIJKL* (GSU2936–GSU2940), each of which appears to shuttle electrons to different acceptors.[87,88] The *G. anodireducens*, *G. soli*, and *G. metallireducens* genomes have homologs for all of the *pcc* complexes found in *G. sulfurreducens* except *extEFG* (Table 3, ESI Table S2B†).

OmcB, which is part of the *ombB–omaB–omcB* complex in *G. sulfurreducens*, is localized to the exterior surface of the outer membrane[92] and is thought to transfer electrons to extracellular multiheme *c*-type cytochromes that act as terminal reductases.[33,85] There is no homolog for *omcB* in *G. metallireducens*, although another *c*-type cytochrome (Gmet_0910) is found in a syntenous location.[24] However, deletion of this gene did not impair Fe(III) oxide reduction.[32] Both *G. anodireducens* (Ga0133348_11857) and *G. soli* (Ga0077628_11353) have *omcB* homologs with 84.09% and 83.82% amino acid sequence identity to that of *G. sulfurreducens*.

There also appears to be a considerable amount of variation in terminal *c*-type cytochromes between species and even within the same species. In *G. sulfurreducens*, the multiheme outer membrane *c*-type cytochrome, OmcS, is required for reduction of Fe(III)- and Mn(IV) oxides,[33] the uptake of electrons during

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DIET[79] and Fe(0),[77] and anodes operated in fuel cell mode with thin biofilms.[36] Immunogold labeling has shown that OmcS can localize along electrically conductive pili found on the *G. sulfurreducens* surface,[93] and recent studies have suggested that it can form cytochrome based conductive filaments.[94,95] OmcS also appears to be involved in electron transfer to insoluble Fe(III) oxide and anodes by *G. soli*.[54] *G. anodireducens* also has a homolog for OmcS (Ga0133348_111108) with amino acid sequence identity of 95.16%. However, *omcS* homologs are not present in genomes from *G. metallireducens* or most other *Geobacter* species (Table 3, ESI Table S2†).[3]

Although most species within the genus *Geobacter* do not have OmcS, they all produce extracellular multiheme *c*-type cytochromes that can serve as terminal reductases. For example, the multiheme *c*-type cytochrome GscA (Gbem_3371) found in *G. bemidjiensis* was able to restore Fe(III) oxide reduction in the OmcS-deficient strain of *G. sulfurreducens*.[96] In addition, while OmcS is required for efficient electron transfer from *G. sulfurreducens* to Fe(III) oxide,[33] and in the reverse direction from an electron-donating partner during DIET,[79] another extracellular multiheme *c*-type cytochrome (Gmet_2896) is needed for DIET and Fe(III) reduction by *G. metallireducens*.[32,40] The outer membrane multiheme *c*-type cytochromes OmcP (GSU2913) and OmcO (GSU2912) are also not required for reduction of Fe(III) oxide by *G. sulfurreducens*,[35] but their homologs (Gmet_0557 and Gmet_0558) are required for Fe(III) oxide respiration by *G. metallireducens*.[32] *G. anodireducens* has a homolog for *omcP* (Ga0133348_113044) and two homologs for *omcO* (Ga0133348_113042 and Ga0133348_113043).

PgcA (GSU1761) is another extracellular *c*-type cytochrome that plays a role in Fe(III) reduction in some strains of *Geobacter* but not others.[31,34] It is a soluble *c*-type cytochrome that is secreted into the extracellular environment to facilitate Fe(III) reduction.[31,34] *G. uraniireducens* also releases a soluble electron shuttle that promotes Fe(III) oxide reduction,[97] however, this shuttle was never characterized. The *G. uraniireducens* genome contains a gene that codes for a PgcA homolog (Gura_0706) which could have served as the shuttle and was significantly expressed in cells grown in Fe(III)-containing sediments.[35,98] *G. metallireducens* does not have a PgcA homolog, however, homologs are found in both *G. anodireducens* (Ga0133348_111806) and *G. soli* (Ga0077628_111213) genomes (Table 3, ESI Table S2†).

Another multiheme outer surface *c*-type cytochrome, OmcZ, is required for electron transfer to anodes in flow-through systems with thick biofilms (>50 μm),[37] but not for reduction of anodes with thin biofilms, Fe(III)- or Mn(IV) oxides by *G. sulfurreducens*.[35,36] The homolog for *omcZ* in *G. metallireducens* (Gmet_0930) however, was essential for Fe(III) oxide reduction.[32] Homologs for *omcZ* are found in all genomes analyzed in this study (Table 3, ESI Table S2†).

Other electron transport proteins that have been implicated in extracellular electron transfer from the *G. sulfurreducens* outer membrane include two outer membrane multicopper proteins (OmpB and OmpC),[99,100] OmcE,[33] and electrically conductive pili.[101–103] All of the genomes have genes coding for OmcE and OmpB, but only the *G. metallireducens* genome has an *ompC* homolog (ESI Table S1†). A unique extracellular electron transfer component characteristic of *Geobacter* species is the presence of pilin monomers that have a structure that enables them to be electrically conductive.[30,101–103] Conductive PilA subunits have >9% aromatic amino acid residues localized in specific regions along the protein and they do not have large gaps between the aromatic residues.[29,30,104] Both the *G. anodireducens* and *G. soli* mature PilA monomers have 10.8% aromatic amino acids and their largest gap is 22 amino acids, which is the same as both *G. metallireducens* and *G. sulfurreducens*. Therefore, the *G. anodireducens* and *G. soli* pili are likely to be conductive.

3.6 Other proteins involved in extracellular electron transfer

In addition to the involvement of electron transport proteins in reduction of extracellular acceptors, proteins involved in biofilm formation also appear to be important. For example, XapD (GSU1501), which is an ATP-dependent transporter that is encoded by a gene located within an operon with extracellular polysaccharide (*xapABCDEFGH*) biosynthesis genes, is required for reduction of anodes and Fe(III) oxide.[105,106] A number of genes involved in extracellular polysaccharide biosynthesis were also more significantly transcribed by *G. metallireducens* cells grown with Fe(III) oxide provided as the electron acceptor than cells grown with Fe(III) citrate.[32] In fact, deletion of Gmet_2029 which codes for a lipopolysaccharide biosynthesis chain length determinant protein completely inhibited Fe(III) oxide reduction by *G. metallireducens*.[32] All four *Geobacter* genomes in this study had genes coding for both the XapD protein and Gmet_2029 (Table 3, ESI Table S2B†).

Another series of proteins involved in electron transfer to poised anodes are part of an electrode sensing network (Esn) and include an MCP (EsnA, GSU1704), a CheW-like scaffolding protein (EsnB, GSU2220), a CheA-like histidine kinase (EsnC, GSU2222), and a diguanylate cyclase (EsnD, GSU3376).[87] It has been proposed that these Esn proteins regulate the accumulation of cyclic di-GMP, which is involved in biofilm formation.[87,107,108] All of the genomes analyzed in this study have genes coding for all of the Esn proteins (Table 3, ESI Table S2B†).

Other proteins implicated in electrode and/or Fe(III) reduction by *G. sulfurreducens* that are not considered electron transport proteins include an NHL repeat domain protein (GSU2505) located just downstream from the *omcS* gene that might be required for proper assembly and/or expression of OmcS, and a transglutaminase domain protein (GSU3361) likely involved in posttranslational modification of proteins involved in extracellular electron transport.[105] All four genomes from this study have both of these genes (Table 3, ESI Table S2B†).

3.7 Proteins involved in stress responses and DNA repair

One significant difference that was apparent when TIGR (The Institute for Genomic Research) categories were compared with the Function Category Comparison tool was that SD-1 has more genes coding for proteins involved in DNA replication, recombination and repair (143 genes compared to 86 in PCA, 94 in GS-15, and 98 in SS01). Many DNA recombination and repair genes (*uvrA*, *uvrD*, *recJ*, *recN*, *polA*, *priA*, *mutS*, and *pmbA*) are duplicated

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in the genome (ESI Table S3†), which is a genome trait that is not observed in the other 3 *Geobacter* species. Additionally, out of the 130 putative open reading frames detected in the plasmid genome, 17 genes coded for proteins involved in DNA replication, recombination or repair (ESI Table S4†). These proteins included the DNA repair proteins RadC and MutS, DNA primase, an ATP-dependent helicase and DNA polymerase III and IV subunits. This redundancy in DNA repair genes might give *G. anodireducens* an upper edge in stressful conditions like high salt environments or on anode surfaces where protons accumulate and lower the pH,[109] as increased levels of these proteins might allow cells to rapidly repair DNA damage.

The mechanosensitive ion channel (Msc) proteins are involved in protection from osmotic shock and are induced in response to high salt concentrations in some bacteria.[110] Six different genes coding for mechanosensitive ion channel proteins were found in the *G. anodireducens* (Ga0133348_112933, Ga0133348_111255, Ga0133348_111279, Ga0133348_111853, Ga0133348_111966, Ga0133348_112030), *G. soli* (Ga0077628_112329, Ga0077628_11701, Ga0077628_11719, Ga0077628_111256, Ga0077628_111444, Ga0077628_111502) and *G. sulfurreducens* (GSU2794, GSU1557, GSU1633, GSU1723, GSU2316, GSU2357) genomes compared to only three in the *G. metallireducens* genome (Gmet_2522, Gmet_1942, Gmet_2581), suggesting that these proteins might also help *Geobacter* survive osmotically stressful environments like those with elevated salt concentrations.

Studies have also shown that many other stress response genes including oxidative stress, heat shock, and universal stress genes are more significantly expressed on anode surfaces and when Fe(III) serves as an electron acceptor.[98,111,112] Analysis of other stress response genes (*i.e.* oxidative stress, heat shock proteins, and universal stress proteins) did not reveal any apparent differences among the four *Geobacter* species (ESI Table S3†).

4 Conclusions

G. anodireducens is a recently isolated *Geobacter* species that could be used for practical applications due to its generation of high current densities and its ability to withstand high salt concentrations in BESs. The genome of *G. anodireducens* was compared to *G. sulfurreducens* PCA, *G. metallireducens* GS-15, and *G. soli* GSS01 in order to uncover potential traits that give SD-1 its superior exoelectrogenic capabilities. The analysis identified many genes likely involved with EET including 87 genes encoding for *c*-type cytochromes, many of which are homologous to those previously found to be essential for EET in other *Geobacter* species. In addition, *G. anodireducens* has substantially more genes for DNA repair and osmotic shock protection than other *Geobacter* species, likely explaining its ability to survive in the stressful environment found in high salt BES. This study provides an instrumental foundation for future molecular and biochemical analyses of this strain.

Conflicts of interest

There are no conflicts of interest to declare.

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