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ORIGINAL ARTICLE

Electrically conductive pili from pilin genes of phylogenetically diverse microorganisms

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The possibility that bacteria other than *Geobacter* species might contain genes for electrically conductive pili (e-pili) was investigated by heterologously expressing pilin genes of interest in *Geobacter sulfurreducens*. Strains of *G. sulfurreducens* producing high current densities, which are only possible with e-pili, were obtained with pilin genes from *Flexistipes sinusarabici, Calditerrivibrio nitroreducens* and *Desulfurivibrio alkaliphilus*. The conductance of pili from these strains was comparable to native *G. sulfurreducens* e-pili. The e-pili derived from *C. nitroreducens*, and *D. alkaliphilus* pilin genes are the first examples of relatively long (>100 amino acids) pilin monomers assembling into e-pili. The pilin gene from *Candidatus* Desulfofervidus auxilii did not yield e-pili, suggesting that the hypothesis that this sulfate reducer wires itself with e-pili to methane-oxidizing archaea to enable anaerobic methane oxidation should be reevaluated. A high density of aromatic amino acids and a lack of substantial aromatic-free gaps along the length of long pilins may be important characteristics leading to e-pili. This study demonstrates a simple method to screen pilin genes from difficult-to-culture microorganisms for their potential to yield e-pili; reveals new sources for biologically based electronic materials; and suggests that a wide phylogenetic diversity of microorganisms may use e-pili for extracellular electron exchange.
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Introduction

Electrically conductive pili (e-pili) can have an important role in the biogeochemical cycling of carbon and metals and have potential applications as 'green' electronic materials (Lovley, 2011; Malvankar and Lovley, 2014; Lovley and Malvankar, 2015; Shi *et al.*, 2016; Lovley, 2017a, b, c). Therefore, a better understanding of the diversity of microorganisms capable of producing e-pili has many potential benefits. To date, e-pili have been most intensively studied in *Geobacter sulfurreducens* and the closely related *Geobacter metallireducens* (Reguera *et al.*, 2005; Malvankar *et al.*, 2011, 2014, 2015; Vargas *et al.*, 2013; Feliciano *et al.*, 2015; Adhikari *et al.*, 2016; Lampa-Pastirk *et al.*, 2016; Xiao *et al.*, 2016; Tan *et al.*, 2016b, 2017). These studies have demonstrated that the e-pili of both *G. sulfurreducens* and *G. metallireducens* are sufficiently conductive along their length, under physiologically relevant conditions, to account for maximum potential rates of extracellular electron transfer (Adhikari *et al.*, 2016; Lampa-Pastirk *et al.*, 2016; Tan *et al.*, 2016b, 2017). *Geobacter* strains that lack e-pili, or express pili with diminished conductivities, are incapable of direct interspecies electron transfer (DIET) and Fe(III) oxide reduction (Reguera *et al.*, 2005; Summers *et al.*, 2010; Tremblay *et al.*, 2012; Shrestha *et al.*, 2013; Vargas *et al.*, 2013; Liu *et al.*, 2014; Rotaru *et al.*, 2014a, b; Tan *et al.*, 2016b). e-Pili are also required for *Geobacter* species to generate high current densities when electrodes serve as the sole electron acceptor (Reguera *et al.*, 2006; Vargas *et al.*, 2013; Liu *et al.*, 2014; Tan *et al.*, 2016b). Increasing e-pili expression increases the conductivity of electrode biofilms and their current output (Yi *et al.*, 2009; Malvankar *et al.*, 2011, 2012; Leang *et al.*, 2013).

Aromatic amino acids are key components in electron transfer along the length of *G. sulfurreducens* e-pili (Vargas *et al.*, 2013; Adhikari *et al.*, 2016; Lampa-Pastirk *et al.*, 2016; Tan *et al.*, 2016a, 2017). There is debate whether the aromatic amino acids contribute to a traditional electron-hopping electron

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transport mechanism or whether overlapping π–π orbitals of the aromatics confer a metallic-like conductivity similar to that observed in synthetic conducting polymers (Malvankar *et al.*, 2011, 2014, 2015; Vargas *et al.*, 2013; Malvankar and Lovley, 2014; Lovley and Malvankar, 2015; Lampa-Pastirk *et al.*, 2016). However, the uncertainty over these mechanistic details should not obscure the fact that long-range electron transport via e-pili is a remarkable strategy for long-range biological electron transfer.

It has been speculated that the relatively small size (61 amino acids) of the *G. sulfurreducens* pilin is a key feature contributing to its assembly into e-pili (Reguera *et al.*, 2005; Malvankar *et al.*, 2015; Holmes *et al.*, 2016). Pili comprised of larger pilins, such as those found in *Shewanella oneidensis* (Reguera *et al.*, 2005), *Pseudomonas aeruginosa* (Reguera *et al.*, 2005; Liu *et al.*, 2014) and *G. uraniireducens* (Tan *et al.*, 2016b) have poor conductivity. On the basis of these results, it was proposed that smaller pilins might permit the tight packing of aromatic amino acids required for conductivity (Malvankar *et al.*, 2015; Xiao *et al.*, 2016). However, previously investigated poorly conductive pili comprised of longer pilins have either (1) a lower overall density of aromatic amino acids than those found in e-pili (that is, *P. aeruginosa* and *S. oneidensis*) or (2) a large gap within the pilin sequence devoid of aromatic amino acids, such as observed in the pilin of *G. uraniireducens* (Figure 1). These considerations suggest that the conductivity of a broader diversity of pili should be investigated.

Evaluating pili conductivity is technically challenging, especially with difficult-to-culture microorganisms. For example, it was suggested that e-pili were involved in DIET in an enriched consortium that contained a methane-oxidizing archaeon and its sulfate-reducing partner (Wegener *et al.*, 2015). The sulfate-reducing partner highly expressed a pilin gene when the two microorganisms were growing syntrophically and pili-like filaments were abundant in the co-culture (Wegener *et al.*, 2015). It was proposed that the pili of the sulfate reducer were conductive (Wegener *et al.*, 2015), but this was not verified, possibly due to difficulties in obtaining sufficient biomass.

The properties of proteins encoded in genomes of difficult-to-culture or as-yet-uncultured microorganisms can often be determined by expressing the genes of interest in more readily cultivated microorganisms (Handelsman, 2004). It is possible to screen pilin genes to determine whether they have the potential to yield pilin proteins that can assemble into e-pili by heterologously expressing the pilin gene of interest in *G. sulfurreducens* in place of the native *G. sulfurreducens* pilin gene (Vargas *et al.*, 2013; Liu *et al.*, 2014; Tan *et al.*, 2016a, b, 2017). Only if the pilin gene yields e-pili is *G. sulfurreducens* then capable of producing high current densities on graphite electrodes. For example, pili of *P. aeruginosa* are poorly conductive (Reguera *et al.*, 2005) and a strain of *G. sulfurreducens* expressing the pilin gene of *P. aeruginosa* produced low current densities (Liu *et al.*, 2014). Expression of the pilin gene from *G. metallireducens* in *G. sulfurreducens* yielded e-pili that were even more conductive than the e-pili originating from the native *G. sulfurreducens* pilin gene (Tan *et al.*, 2017). In contrast, the pilin gene of *G. uraniireducens*, which is phylogenetically distinct from the *G. sulfurreducens* and *G. metallireducens* pilin genes (Holmes *et al.*, 2016), yielded poorly conductive pili in *G. sulfurreducens* (Tan *et al.*, 2016b). As expected, the *G. sulfurreducens* strain expressing the *G. uraniireducens* pilin genes produced low current densities (Tan *et al.*, 2016b) as did a *G. sulfurreducens* strain expressing a synthetic pilin gene designed to yield poorly conductive pili (Vargas *et al.*, 2013). The poor conductivity of the *G. uraniireducens* pili was consistent with the finding that *G. uraniireducens* uses an electron shuttle for Fe(III) reduction and is

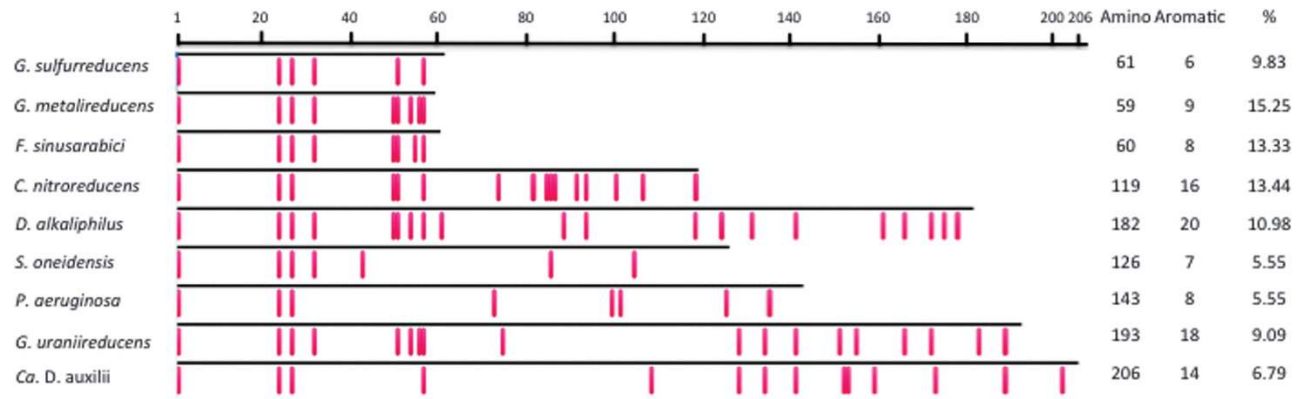

Figure 1 Comparison of aromatic amino-acid placement (red rectangles) within mature pilin protein sequences of *G. sulfurreducens*, *G. metallireducens*, *F. sinusarabici*, *C. nitroreducens*, *D. alkaliphilus*, *S. oneidensis*, *P. aeruginosa*, *G. uraniireducens* and *Candidatus* Desulfofervidus auxilii. Also shown are the total number of amino acids, the number of aromatic amino acids and the percentage of total amino acids that are aromatic.

incapable of producing high current densities on graphite electrodes (Rotaru *et al*., 2015; Tan *et al*., 2016b).

Therefore, the possibility that diverse microorganisms potentially involved in extracellular electron transfer might have the potential to produce e-pili was evaluated by expressing their pilin genes in *G. sulfurreducens*. The results suggest that pilin sequences phylogenetically distinct from the *G. sulfurreducens* pilin and much larger than the *G. sulfurreducens* pilin can, in some instances, yield e-pili.

Materials and methods

Bacterial strains, plasmids and culture conditions

The bacterial strains used in this study are listed in Supplementary Table S1. As previously described (Coppi *et al*., 2001), *G. sulfurreducens* was routinely cultured in liquid medium or on agar plates under strict anaerobic conditions in a medium with acetate as the electron donor and fumarate as the electron acceptor at 30 °C. *Escherichia coli* 5-alpha (New England Biolabs, Ipswich, MA, USA) chemically competent cells were used for Gibson Assembly cloning and cultured at 37 °C in Luria–Bertani medium. Gentamicin 20 μg ml^{-1} was added to cultures when required for plasmid or chromosomal selection.

Pilin gene sequences and phylogenetic analyses

Sequence data were acquired from the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov) or from the Genbank at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Amino-acid alignments were generated with MAFFT (Katoh and Standleyu, 2013) and PRANK (Löytynoja and Goldman, 2005) algorithms, and unreliable residues, columns and sequences in all alignments were identified and eliminated with GUIDANCE2 (Sela *et al*., 2015).

Phylogenetic trees were generated with the maximum likelihood method using MEGA v. 7.0 software (Kumar *et al*., 2016). Before trees were constructed, the Find Best DNA/Protein Models program was run on sequence alignments. The evolutionary history of the PilA protein was inferred with the maximum likelihood method based on the Whelan and Goldman model (Whelan and Goldman, 2001) and the bootstrap consensus tree was inferred from 100 replicates (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with a superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+*G*, parameter = 0.9542)). The analysis involved 79 amino-acid sequences. All positions with <95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position. There were a total of 55 positions in the final data set.

Construction of G. sulfurreducens strains expressing heterologous pilins

The strains of *G. sulfurreducens* heterologously expressing the pilin genes of other microorganisms were constructed with a slight modification of the previously described approach (Tan *et al*., 2017). Pilin genes (gene accession numbers in parentheses) were expressed from the following: *Flexistipes sinusarabici* DSM 4947 (Flexsi_2288); *Calditerrivibrio nitroreducens* DSM 19672 (Calni_0149); *Desulfurivibrio alkaliphilus* AHT2 (DaAHT2_2283) and *Candidatus* Desulfofervidus auxilii strain HS1 (HS1_000117). To ensure that the pre-pilin would be correctly recognized and transported to the cellular membrane for processing, the signal peptide from *G. sulfurreducens* (MLQKLRNRKG) was included in all strains. This signal peptide is cleaved during post-translational processing and is not part of the mature pilin sequence. The mature pilin-coding sequence from the organisms of interest was inserted downstream from the *G. sulfurreducens* signal peptide sequence in the pYT-1 plasmid (Supplementary Figure S1). The region between the *Sac*II and *Not*I restriction sites, which included the *G. sulfurreducens* promoter region (P*pilA*), the *G. sulfurreducens* signal peptide sequence and the heterologous *pilA* sequence, was synthesized as a gene fragment (Thermo Fisher Scientific, Waltham, MA, USA). Two 15-bp regions homologous to sequences located 5′ and 3′ of the restriction sites on the pYT-1 plasmid were also included in the synthesized gene fragment to facilitate Gibson Assembly. The synthesized DNA was inserted into plasmid pYT-1 between the *Sac*II and *Not*I restriction sites with the Gibson Assembly Kit ((Gibson *et al*., 2009); New England Biolabs). Correct sequence and assembly was verified by sequencing DNA fragments amplified with *pilA*-Screen-F and *pilA*-Screen-R primers (Supplementary Table S2). The recombinant plasmid was then linearized with restriction enzyme *Sac*I and transformed into wild-type *G. sulfurreducens* competent cells by electroporation as previously described (Liu *et al*., 2014).

To ensure that the heterologous gene had correctly integrated into the *G. sulfurreducens* chromosome, sequences were verified with *pilA*-InScreen-F and *pilA*-InScreen-R primers (Supplementary Table S2). Strain purity was also confirmed by sequencing with this primer set throughout the experiment; that is, before placing the strain in the bioelectrochemical device, during the current production experiment and before pili were collected from cells.

Current production
The capacity of the strains to produce current was determined as previously described (Nevin *et al.*, 2009). Cells were grown in two-chambered H-cell systems with a continuous flow of medium with acetate (10 mM) as the electron donor and graphite stick anodes (65 cm^2) poised at 300 mV versus Ag/AgCl as the electron acceptor.

Microscopy
As previously described (Nevin *et al.*, 2011), anode biofilms were imaged with confocal laser scanning microscopy using the LIVE/DEAD BacLight viability stain kit (Molecular Probes, Eugene, OR, USA). Cells from the anode biofilms were examined by transmission electron microscopy as previously described (Tan *et al.*, 2017). Cells placed on carbon-coated copper grids were stained with 2% uranyl acetate and visualized with a JEOL (Welwyn garden city, UK) 2000fx transmission electron microscope operated at 200 kV accelerating voltage.

Pili preparation
Pili were obtained from anode biofilms as previously described (Tan *et al.*, 2017). Briefly, pili were sheared from the cells in a blender in 150 mM ethanolamine buffer (pH 10.5), the cells were removed with centrifugation and the pili were precipitated from the supernatant with the addition of ammonium sulfate, followed by centrifugation. The precipitate was resuspended in ethanolamine buffer, centrifuged to remove additional debris and the ammonium sulfate precipitation step was repeated. The pili were then resuspended in ethanolamine buffer and stored at 4 °C. Before conductance measurements the pili were dialyzed in deionized water at 4 °C with a Slide-A-Lyzer MINI Dialysis Device (Thermo Fisher Scientific) with 7 kDa molecular weight cutoff columns. The purity of the pili preparations was evaluated with transmission electron microscopy, as previously described (Tan *et al.*, 2016a). To determine the protein concentration, 20 µl of the pili preparation was air dried, dissociated in 17.2 µl of 1% SDS at pH 1.5 at 95 °C and then neutralized with 2.8 µl of 1 N NaOH. The protein concentration was analyzed with the Pierce nano bicinchoninic acid assay (Thermo Fisher) All pili preparations were normalized to 500 µg protein per µl.

Pili conductance
The method for measuring pili conductance was a modification of the previously described (Malvankar *et al.*, 2011; Vargas *et al.*, 2013) method for estimating the conductance of pili networks. This method is preferable for screening studies because measuring the conductivity of multiple individual pili is extremely laborious, expensive and technically difficult. Conductance was measured with the four-point probe method to eliminate the possibility of contact resistance.

The electrodes were fabricated with photolithography and were 50 nm thick with 10 nm titanium used as an adhesion layer and 40 nm gold (Supplementary Figure S2). The substrate was 300 nm-thick silicon dioxide grown on antimony-doped silicon wafers with a wet chemical process. Pads for the four-point probe electrodes were 1 mm by 1 mm in length and the electrodes were non-equidistant with 15 µm between the two inner electrodes and 3 µm between each of the inner and outer electrodes. Electrical contacts on the pads were made with 1 µm diameter tungsten probes (Signatone, Gilroy, CA, USA) connected to a Keithley 4200 Semiconductor Characterization System Parametric Analyzer (Tektronix Inc., Beaverton, OR, USA) with triaxial cables.

Pili preparations (2 µl, 500 µg protein per µl) were drop cast onto the gold electrode arrays and dried for 1 h at 24 °C. Another 2 µl of the pili preparation was then drop cast on the electrode array and dried in a desiccator for ca. 12 h.

Each pili preparation was drop cast onto three different devices and current–voltage (*I*–*V*) curves for each device were obtained in triplicate with a Keithley 4200 Semiconductor Characterization System setup with four probes using a $\pm 30 \times 10^{-8}$ V sweep with a 5 s delay and a 250 s hold time. The conductance value of the pili was extracted from the slope of the linear fit of the current–voltage response of the sample as

$$G = \frac{I}{V}$$

where G is the conductance, I is the current and V is the voltage.

Results and discussion

e-Pili from the Geobacter-like pilin of F. sinusarabici
Many *Geobacter* species and closely related microorganisms have pilin genes that are closely related to the *G. sulfurreducens* pilin gene that yields e-pili (Holmes *et al.*, 2016). These include other members of the Desulfuromonadales family in the Deltaproteobacteria, such as some *Geoalkalibacter*, *Desulfuromusa* and *Desulfuromonas* species that are already known to participate in extracellular electron transfer (Holmes *et al.*, 2016). However, a few species outside the Desulfuromonadales also have pilin genes closely related to those found in *G. sulfurreducens* (Holmes *et al.*, 2016). For example, *F. sinusarabici,* which is in the phylum Deferribacteres, is phylogenetically distant from *Geobacter* species based on 16S rRNA gene sequences, but contains a pilin gene closely related to the *G. sulfurreducens* pilin gene (Holmes *et al.*, 2016) that is predicted to yield a pilin monomer with a density

of aromatic amino-acid intermediate between the *G. sulfurreducens* and *G. metallireducens* pilins (Figure 1).

Even under optimal culture conditions *F. sinusarabici* grows poorly, not even producing visible turbidity (Fiala *et al.*, 1990). Thus, it was not possible to obtain sufficient biomass to evaluate the conductivity of *F. sinusarabici* pili directly. Therefore, the pilin gene of *F. sinusarabici* was heterologously expressed in *G. sulfurreducens* in place of the native *G. sulfurreducens* pilin gene. The *G. sulfurreducens* strain expressing the *F. sinusarabici* pilin gene grew on anodes, and produced current densities comparable to wild-type *G. sulfurreducens* (Figure 2). Such high current densities have only been seen in previous studies when *G. sulfurreducens* expresses e-pili (Reguera *et al.*, 2006; Vargas *et al.*, 2013; Liu *et al.*, 2014; Tan *et al.*, 2016b, 2017), which permit cells at distance from the anode to contribute to current production (Lovley, 2012). The thick biofilms of the *G. sulfurreducens* strain expressing the *F. sinusarabici* pilin gene (Figure 3a) were comparable to those previously reported (Vargas *et al.*, 2013) for the control strain expressing the *G. sulfurreducens* wild-type pilin gene.

The *G. sulfurreducens* strain expressing the *F. sinusarabici* pilin gene expressed abundant pili (Figure 4a). The purified pili (Supplementary Figure S3) had a conductance comparable to that of the pili preparations from the control strain of *G. sulfurreducens* expressing its native pilin gene (Figure 5). These results demonstrate that the *F. sinusarabici* pilin gene encodes a pilin monomer that can assemble into e-pili. Close relatives of *F. sinusarabici* include *Geovibrio* and *Deferribacter* species, which are known to be capable of extracellular electron transfer (Alauzet and Jumas-Bilak, 2014). The one available *Deferribacter* genome contains a pilin gene homologous to the *G. sulfurreducens* pilin gene (Holmes *et al.*, 2016). Further investigation into the possibility that *F. sinusarabici* uses e-pili for extracellular electron transfer is warranted, but such studies will be challenging because of its poor growth and the lack of tools for genetic manipulation.

e-Pili from larger, more phylogenetic distant pilins

The possibility that pilins that are larger than the truncated e-pilin of *G. sulfurreducens* might also yield e-pili has only been previously explored with a few pilin sequences (Liu *et al.*, 2014; Tan *et al.*, 2016b). Therefore, larger pilin sequences were further investigated with an emphasis on microorganisms that (1) are thought to be capable of extracellular electron exchange and (2) have aromatic-rich pilin proteins.

For example, *C. nitroreducens*, which like *F. sinusarabici* is in the phylum Deferribacteres, is capable of extracellular electron transfer to electrodes (Fu *et al.*, 2013), and the density of aromatic

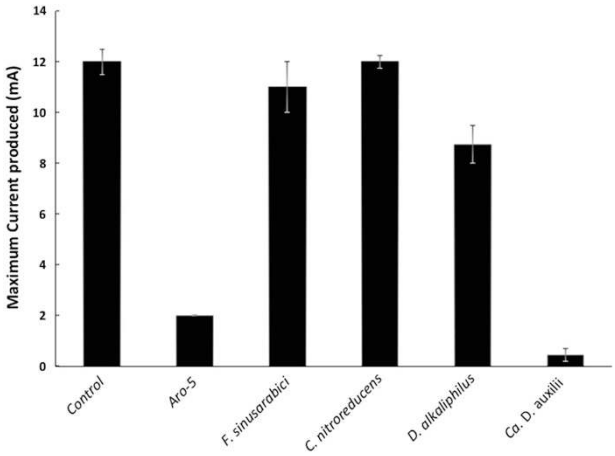

Figure 2 Current production by *G. sulfurreducens* strains expressing either the native wild-type gene (control), a synthetic pilin gene (*Aro-5*) designed to be poorly conductive (Vargas *et al.*, 2013) or the pilin gene of *F. sinusarabici*, *C. nitroreducens*, *D. alkaliphilus* or *Ca.* D. auxilii. Results shown are the mean of duplicate determinations for each strain with the bars designating the two current values. Aro-5 results from Vargas *et al.* (2013).

amino acids in the *C. nitroreducens* pilin protein is higher than in the *G. sulfurreducens* pilin (Figure 1). However, the *C. nitroreducens* pilin contains nearly twice as many amino acids as the *G. sulfurreducens* pilin. A strain of *G. sulfurreducens* expressing the *C. nitroreducens* pilin gene, rather than the native *G. sulfurreducens* pilin gene, produced current densities similar to the control strain expressing the native pilin gene (Figure 2) with thick biofilms (Figure 3b). The cells produced abundant pili (Figure 4b). The conductance of the purified pili preparations (Supplementary Figure S3) from the anode biofilm was almost twice as that of the control strain expressing the native *G. sulfurreducens* pilin gene (Figure 5). These results demonstrate for the first time that pilin monomers much larger than the pilin of *G. sulfurreducens* can yield e-pili.

Further investigation of the role of e-pili in *C. nitroreducens* will be challenging because of the lack of tools for genetic manipulation of this organism and because this microorganism is difficult to grow to high densities. However, the possibility of *C. nitroreducens* expressing e-pili should be considered when evaluating its ecological niche or possibilities for increasing its current output in microbial fuel cells.

It has been proposed that cable bacteria accept electrons from sulfide-oxidizing bacteria via DIET (Vasquez-Cardenas *et al.*, 2015). Genome sequences of cable bacteria have yet to be published but a genome is available for *D. alkaliphilus* (Melton *et al.*, 2016), which is closely related to some species of cable bacteria (Müller *et al.*, 2016). Similar to *Geobacter* species, *D. alkaliphilus* is in the Deltaproteobacteria. However, the pilin of *D. alkaliphilus* is more closely related to the pilin of *G. uraniireducens,* which yields poorly conductive pili (Tan *et al.*, 2016b), than to the *G. sulfurreducens* pilin (Figure 6).

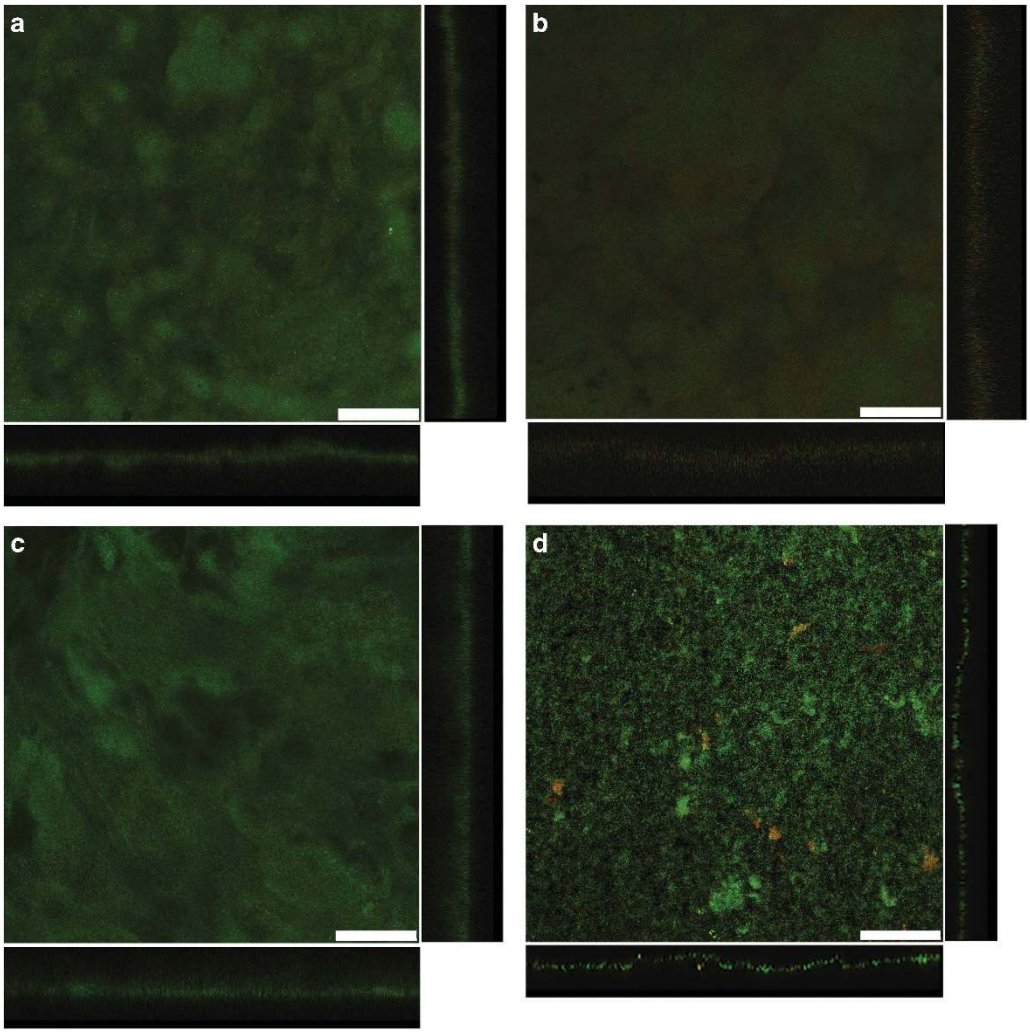

Figure 3 Scanning laser confocal microscopy images of anode biofilms of *G. sulfurreducens* strains heterologously expressing pilin genes from (**a**) *F. sinusarabici*, (**b**) *C. nitroreducens*, (**d**) *D. alkaliphilus* and (**d**) *Ca.* D. auxilii. Top-down three-dimensional, lateral side views (right image) and horizontal side views (bottom image) show cells stained with LIVE/DEAD BacLight viability stain. Bar, 50 μm. Mean and s.d. for biofilm thickness at 100 points in each biofilm: *F. sinusarabici,* 26.1 ± 5.2 μm; *C. nitroreducens*, 41.0 ± 6.6 μm; *D. alkaliphilus*, 41.7 ± 6.6 μm; *Ca.* D. auxilii, 10.7 ± 2.1 μm.

Similar to the *G. uraniireducens* pilin, the *D. alkaliphilus* pilin is much larger than the pilins that have been shown to yield e-pili in *Geobacter* species (Figure 1). However, unlike the *G. uraniireducens* pilin, the density of aromatic amino acids in the *D. alkaliphilus* pilin is comparable to that of *G. sulfurreducens* (Figure 1). Expression of the *D. alkaliphilus* pilin gene in *G. sulfurreducens* yielded a strain that produced thick anode biofilms (Figure 3c) that generated high current densities (Figure 2). Pili were abundant (Figure 4c). The purified pili preparations (Supplementary Figure S3) had a conductance nearly equal to native *G. sulfurreducens* pili (Figure 5). These results provide another example of a large pilin monomer yielding e-pili.

It does not appear that the ability of *D. alkaliphilus* to reduce Fe(III) oxides or produce current has been examined, but *D. alkaliphilus* does contain porin–cytochrome genes closely related to *Geobacter* genes that are known to be involved in extracellular electron transfer (Shi *et al.*, 2014). Furthermore, *D. alkaliphilus* grows with elemental sulfur/polysulfide as the electron acceptor (Sorokin *et al.*, 2008) and microorganisms that can reduce elemental sulfur/polysulfide are often also capable of electron transfer to Fe(III) or other extracellular electron acceptors (Lovley *et al.*, 2004). If cable bacteria closely related to *D. alkaliphilus* have similar pilin genes, this might provide a mechanism for the proposed DIET between cable bacteria and sulfur oxidizers (Vasquez-Cardenas *et al.*, 2015). The fact that cable bacteria or their putative sulfur-oxidizing partners are not available in pure culture currently limits further investigation into this possibility.

Pilin of Ca. D. auxilii does not yield e-pili

It has been proposed that the sulfate-reducer *Ca.* D. auxilii has e-pili that facilitate DIET in a

consortium with a methane-oxidizing archaeon (Wegener *et al*., 2015; Krukenberg *et al*., 2016). The phylogeny of *Ca*. D. auxilli is still under evaluation, but it is either a member of the Deltaproteobacteria or the closely related Thermodesulfobacteria (Krukenberg *et al*., 2016). It was not possible for us to obtain a sample of the consortium to directly evaluate the conductivity of the pili, but *G. sulfurreducens* readily

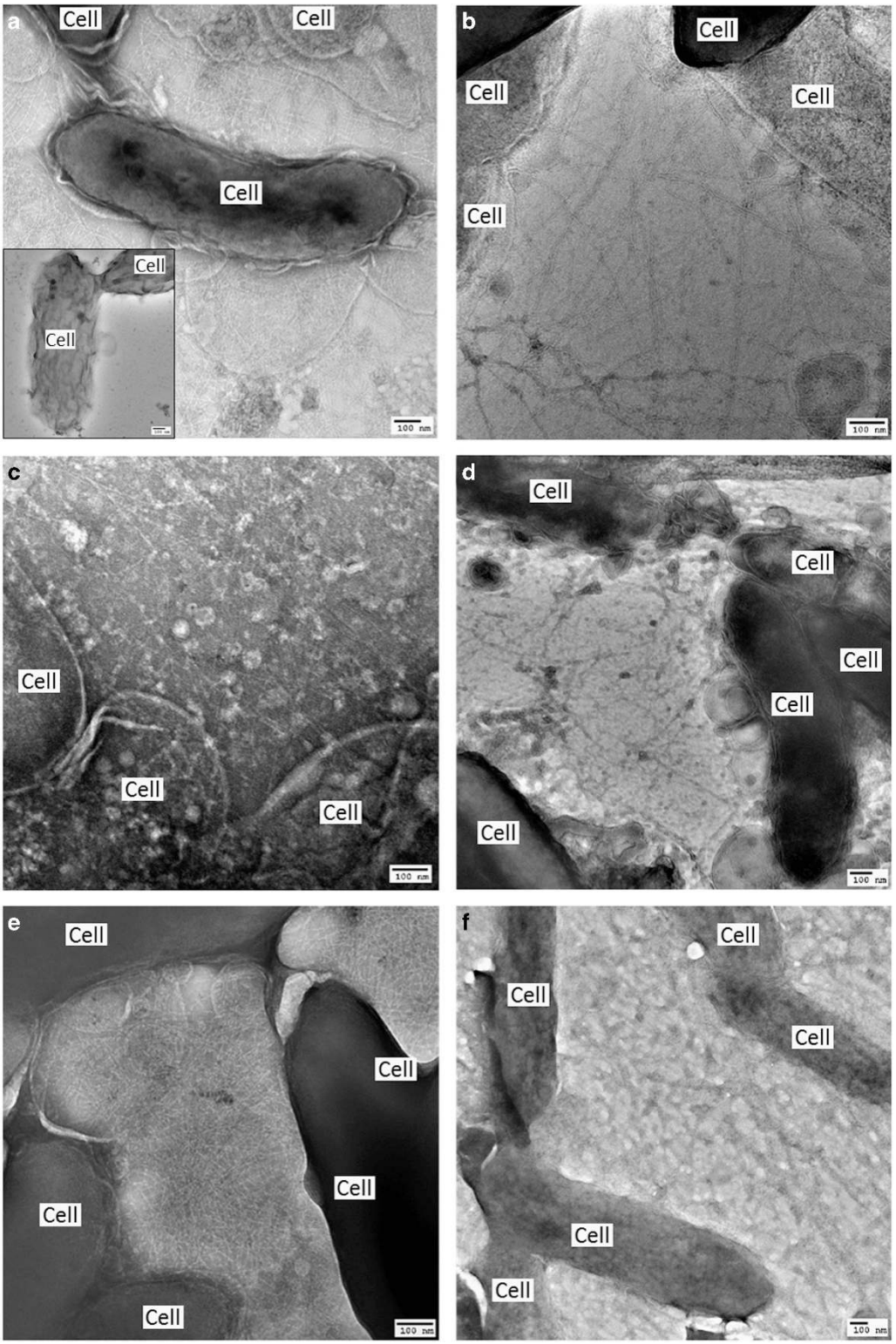

Figure 4 Transmission electron micrographs of *G. sulfurreducens* strains. (**a**) Control strain expressing wild-type pilin gene (insert: strain with pilin gene deleted). Heterologous expression of pilin genes from the following organisms: (**b**) *F. sinusarabici*, (**c**) *C. nitroreducens,* (**d**) *D. alkaliphilus*, (**e**) *Ca*. D. auxilii or (**f**) the synthetic Aro-5 sequence.

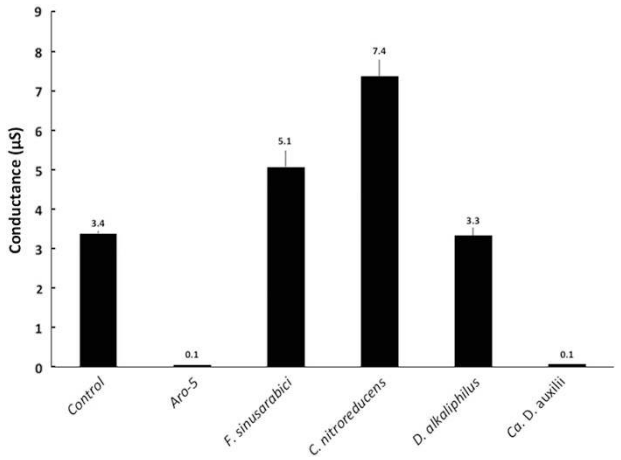

Figure 5 Conductance of pili preparations from *G. sulfurreducens* strains expressing either the native wild-type gene (control), a synthetic pilin gene (*Aro-5*) designed to be poorly conductive (Vargas *et al*., 2013) or the pilin gene of *F. sinusarabici*, *C. nitroreducens*, *D. alkaliphilus* or *Ca.* D. auxilii. Results shown are the mean and s.d. of triplicate measurements on triplicate electrode arrays.

expressed pili (Figure 4d; Supplementary Figure S3) from the *Ca.* D. auxilli pilin gene that was previously reported (Wegener *et al*., 2015) to be highly expressed during syntrophic growth. This strain of *G. sulfurreducens* produced low current densities (Figure 2) with relatively thin biofilms (Figure 3d), comparable to the previously reported (Liu *et al*., 2014) biofilms produced by a strain of *G. sulfurreducens* expressing the pilin from *P. aeruginosa*, which yield poorly conductive pili. The conductance of the pili was also low (Figure 5). There is the possibility that the *Ca.* D. auxilli pilin monomers assemble differently in *Ca.* D. auxilli than they do in *G. sulfurreducens* to yield conductive pili in the natural host. However, this seems unlikely due to the finding that *G. sulfurreducens* expressed e-pili from the pilin monomer of *D. alkaliphilus*, which is comparable in size. The density of aromatic amino acids in the *Ca.* D. auxilli pilin is much lower than that found in e-pili (Figure 1), suggesting that even when expressed in the native host the pilin of *Ca.* D. auxilli is unlikely to assemble into a conductive filament. These results suggest that the hypothesis that *Ca.* D. auxilli exchanges electrons with its archeal partner via *Ca.* D. auxilli e-pili (Wegener *et al*., 2015) needs to be revisited. One possible approach would be to measure conductance of the consortium as such measurements can be more technically feasible than studying pili conductance directly (Morita *et al*., 2011; Shrestha *et al*., 2014).

Conclusions

These results demonstrate that microorganisms outside the *Geobacteraceae* contain pilin genes that can encode e-pili and that pilin monomers much larger than those of *G. sulfurreducens* can yield e-pili. Thus, the diversity of microorganisms that might electrically communicate with other species, or exchange electrons with insoluble minerals, through e-pili may extend well beyond the small percentage of microbes with genes for short e-pilins similar to those found in *G. sulfurreducens*.

The poor understanding of the mechanisms for conductivity for even *G. sulfurreducens* e-pili precludes definitively specifying the features of pilin monomers that confer conductivity to e-pili. However, the limited data set available suggests that pilins with high densities of aromatic amino acids, without large gaps of aromatic-free regions in the pilin monomer, can assemble into e-pili. The phylogenetic distance between the longer pilin sequences found to yield e-pili and the shorter pilin sequences closely related to *G. sulfurreducens* (Figure 6) suggest that aromatic-rich e-pili may have independently evolved several times within bacteria.

The results offer a simple screening strategy to evaluate whether difficult-to-culture microorganisms contain pilin genes that might enable them to participate in extracellular electron exchange with e-pili. The genetic manipulation of *G. sulfurreducens* to heterologously express pilin genes of interest is straightforward and the requirement of *G. sulfurreducens* for e-pili to produce high current densities provides a clear phenotype. Thus, the approach described here provides a good first step for hypothesis testing and is preferable to the common practice of suggesting that filaments are 'nanowires' based solely on visual appearance. Ideally, detailed investigation of the function of the e-pili from any microorganism should also include estimates of the conductivity of individual e-pili under physiologically relevant conditions (Adhikari *et al*., 2016; Tan *et al*., 2016a, b, 2017). However, such measurements are technically challenging and the necessary equipment and expertise are not readily available to many microbiologists.

Diverse microorganisms such as *Aeromonas hydrophila* (Castro *et al*., 2013), *Acidithiobacillus ferroxidans* (Li and Li, 2014), *Desulfovibrio desulfuricans* (Eaktasang *et al*., 2016) and *Rhodopseudomonas palustris* (Venkidusamy *et al*., 2015) can produce electrically conductive protein filaments. However, uncertainties about the physiological roles of these filaments exist because of the following: (1) the protein composition of the filaments has not been determined; (2) a role for the filaments in extracellular electron transfer has not been verified; and (3) electron transport along the length of filaments under physiologically relevant conditions has yet to be shown. Expressing pilin genes from these microbes in *G. sulfurreducens* may help better define these filaments.

e-Pili are a potential source of 'green' electronic materials that can be sustainably mass-produced with renewable feedstocks without toxic components in the final product (Lovley, 2017b). The e-pili revealed here greatly expand the options for starting materials and the screening method described will

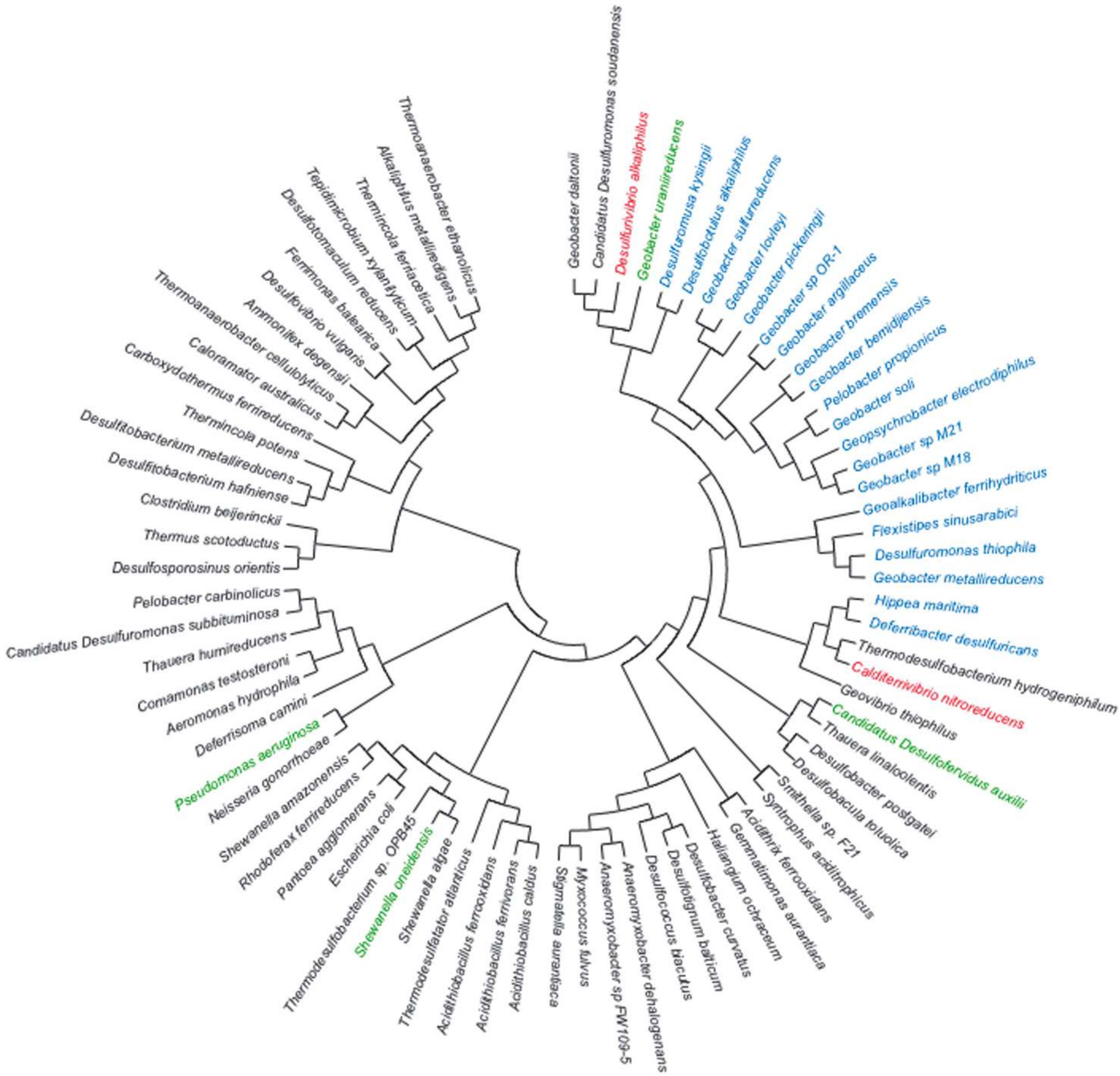

Figure 6 Phylogeny of pilin protein sequences from a diversity of bacteria.Short (ca. 60 amino acids) pilins closely related to *G. sulfurreducens* are shown in blue, including the pilin from *F. sinusarabici* (bold). The phylogenetic placement of the longer pilins from *C. nitroreducens* and *D. alkaliphilus* that assembled into conductive pili in *G. sulfurreducens* are in red. The pilins from *Ca.* D. auxilii, *S. oneidensis* and *P. aeruginosa* that did not yield conductive pili in this or previous studies are shown in green.

facilitate searching the microbial world, including the metagenomes of uncultured microorganisms, for additional conductive protein wires.

Conflict of Interest

The authors declare no conflict of interest.

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