MICROBIAL GENOMICS
Bases to Biology

Research Paper

The electrically conductive pili of *Geobacter* species are a recently evolved feature for extracellular electron transfer

Dawn E. Holmes,[1,2] Yan Dang,[1] David J. F. Walker[1] and Derek R. Lovley[1]

[1]Department of Microbiology, University of Massachusetts Amherst, Amherst, MA, USA

[2]Department of Physical and Biological Sciences, Western New England University, Springfield, MA, USA

Correspondence: Dawn E. Holmes (dholmes@wne.edu *or* dholmes1028@gmail.com)

DOI: 10.1099/mgen.0.000072

The electrically conductive pili (e-pili) of *Geobactersulfurreducens* have environmental and practical significance because they can facilitate electron transfer to insoluble Fe(III) oxides; to other microbial species; and through electrically conductive biofilms. E-pili conductivity has been attributed to the truncated PilA monomer, which permits tight packing of aromatic amino acids to form a conductive path along the length of e-pili. In order to better understand the evolution and distribution of e-pili in the microbial world, type IVa PilA proteins from various Gram-negative and Gram-positive bacteria were examined with a particular emphasis on Fe(III)-respiring bacteria. E-pilin genes are primarily restricted to a tight phylogenetic group in the order Desulfuromonadales. The downstream gene in all but one of the Desulfuromonadales that possess an e-pilin gene is a gene previously annotated as '*pilA–C*' that has characteristics suggesting that it may encode an outer-membrane protein. Other genes associated with pilin function are clustered with e-pilin and '*pilA–C*' genes in the Desulfuromonadales. In contrast, in the few bacteria outside the Desulfuromonadales that contain e-pilin genes, the other genes required for pilin function may have been acquired through horizontal gene transfer. Of the 95 known Fe(III)-reducing micro-organisms for which genomes are available, 80 % lack e-pilin genes, suggesting that e-pili are just one of several mechanisms involved in extracellular electron transport. These studies provide insight into where and when e-pili are likely to contribute to extracellular electron transport processes that are biogeochemically important and involved in bioenergy conversions.

Keywords: Geobacter; extracellular electron transfer; e-pilin; type IVa PilA; positive selection; evolution.

Abbreviation: e-pilin, electrically conductive pilin.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files.

Data Summary

1. Supplementary data (Tables S1–S2 and Figs S1–S4) and phylogenetic trees (Bayesian and maximum-likelihood) have been deposited in FigShare; https://figshare.com/s/87e875d0c5c97c2e5498.
2. Accession numbers from pilin gene sequences analyzed for this study are provided in Supplementary Table S1.

Received 31 March 2016; Accepted 31 May 2016

Introduction

The concept that electrically conductive pili (e-pili) can enable long-range electron transfer to insoluble minerals (Reguera *et al.*, 2005), to other cells (Rotaru *et al.*, 2014; Shrestha *et al.*, 2013; Summers *et al.*, 2010) and through electrically conductive biofilms (Malvankar *et al.*, 2011; Reguera *et al.*, 2006) is a paradigm shift in microbial electron transport. The distance over which e-pili can conduct electrons and the apparent competitive advantage of this capability in a number of anaerobic environments distinguish electron transfer via e-pili from more typical forms of short-range biological electron

transfer (Lovley & Malvankar, 2015; Malvankar & Lovley, 2014; Malvankar *et al.*, 2015; Vargas *et al.*, 2013).

To date, e-pili have only been documented in two species of the genus *Geobacter*, *Geobacter sulfurreducens* (Chun, 2014; Malvankar *et al.*, 2011; Reguera *et al.*, 2005) and *Gobacter metallireducens* (Y. Tan *et al.*, unpublish ed). The pili of the only other species of the genus *Geobacter* examined, *Geobacter uraniireducens,* are poorly conductive (Tan *et al.*, 2016). *G. uraniireducens* functions without e-pili because it utilizes a soluble electron shuttle to reduce Fe(III) oxides, does not form thick electrically conductive biofilms and does not participate in direct interspecies electron transfer (Rotaru *et al.* 2015; Tan *et al.,* 2016).

It has been suggested that electrically conductive filaments may facilitate extracellular electron transfer in other organisms (Castro *et al.*, 2014; Eaktasang *et al.*, 2016; Gorby *et al.*, 2006; Venkidusamy *et al.*, 2015; Wanger *et al.*, 2013), but in no instance has it been definitively demonstrated that the filaments are required for this process, and in many instances the composition of the filaments has yet to be determined. In contrast, many lines of evidence support the concept of long-range electron transport along the e-pili of *Geobacter sulfurreducens.* This includes the findings that: (1) deleting the gene for PilA, the pilus monomer, inhibited electron transport to Fe(III) oxide, interspecies electron exchange, and the development of thick electrically conductive biofilms (Malvankar *et al.*, 2011; Nevin *et al.*, 2009; Reguera *et al.*, 2005; Summers *et al.*, 2010); (2) genetically modifying *pilA* to yield pili with poor conductivity inhibited Fe(III) oxide reduction and reduced biofilm conductivity (Vargas *et al.*, 2013); (3) a strain of *Geobacter sulfurreducens* expressing the poorly conductive pili of *Pseudomonas aeruginosa* was ineffective in Fe(III) oxide reduction and current production (Liu *et al.*, 2014); (4) the individual pilin filaments are electrically conductive (Adhikari *et al.*, 2016; Malvankar *et al.*, 2011; Reguera *et al.*, 2005); and (5) the pili propagate charge similarly to carbon nanotubes (Malvankar *et al.*, 2014).

In the initial study on *Geobacter sulfurreducens* e-pili, it was noted that the PilA monomer of *Geobacter sulfurreducens* was much shorter than PilA from most micro-organisms for which a PilA sequence was available (Reguera *et al.*, 2005). It has been speculated that the shorter pilus monomer permits tighter packing, positioning aromatic amino acids (Lovley & Malvankar, 2015) and charged amino acids (Feliciano *et al.*, 2015), in a manner that promotes electron conduction along the length of the pilin. This hypothesis is supported by the finding that *G. metallireducens*, which also has a short PilA, produces e-pili (Y. Tan, unpublished), whereas the pili of *Geobacter uraniireducens*, which has a long PilA typical of that found in many micro-organisms, produces pili of low conductivity (Tan *et al.*, 2016).

Many more genome sequences have become available in the decade since the original phylogenetic analysis of the *Geobacter sulfurreducens* PilA protein (Reguera *et al.*, 2005). The purpose of this study was to further evaluate the phylogenetic distribution of the short *pilA* gene, (i.e. e-pilin) that appears to yield e-pili, to determine the distribution of e-pilin sequences in the microbial world and to obtain insight into the evolution of this unique electron transfer mechanism.

Impact Statement

It is becoming increasingly apparent that micro-organisms can make electrical connections with other cells as well as abiological materials, such as minerals and electrodes. This form of extracellular electron transfer plays an important role in natural biogeochemical cycles and is being adapted for practical applications in bioremediation, bioenergy and biomaterials. The electrically conductive pili (e-pili) of *Geobacter sulfurreducens* are an example of one type of electrical connection that has been studied intensively. However, the extent to which e-pili are distributed throughout the microbial world has been uncertain. The results presented here demonstrate that e-pili have arisen as a relatively recent evolutionary event and appear to be primarily restricted to a tight phylogenetic group within the deltaproteobacteria. This finding suggests that most bacteria other than species of the genus *Geobacter* have alternative strategies for making extracellular electrical connections and that electrically conductive filaments that have been observed in other organisms are likely to have evolved independently of e-pili and may have different mechanisms for conductivity.

Methods

Genome data analysis. Sequence data for all of the bacterial genomes was acquired from the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov) or from Genbank at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Initial analyses were done with analysis tools available on the Integrated Microbial Genomes (IMG) website (img.jgi.doe.gov). The Find Function search tool on IMG was used to identify proteins that carried the type IV pilin N-terminal methylation site GFxxxE (pfam13544), the N_methyl_3 domain (pfam13633), the PilA domain (COG4969), the pilin domain (Pfam001114)and/or the prepilin-type N-terminal cleavage/methylation domain (TIGR02532). These domains were also identified in various proteins with NCBI conserved domain search (Marchler-Bauer *et al.*, 2015) and Pfam search (Finn *et al.*, 2016) tools. These proteins were then individually screened to ensure that they were in fact type IV PilA proteins by comparison to previously characterized PilA proteins available in the NCBI Genbank database with the BLASTp and PSI-BLAST algorithms (Altschul *et al.*, 1997).

Open reading frames in genomes were also screened for the presence of type IV pilin-like motifs with PilFind (Imam *et al.*, 2011), FlaFind (Szabó *et al.*, 2007), Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and Motif Search (http://www.genome.jp/tools/motif/). A hidden Markov model was built from an alignment of 100 different Type IVa PilA protein sequences using the hmmbuild function in HMMER 3 (Eddy, 2008, 2011). Candidate genes in these genomes were identified by comparison to these alignments with the hmmsearch function and then further screened by comparison to sequences in the Genbank database with BLASTp and PSI-BLAST algorithms.

The same domain search tools used to identify type IVa pilA genes were also used to find genes coding for pilin accessory proteins. Open reading frames were scanned for the following motifs: *pilW* (pfam16074), *pilX* (pfam14341), *pilY1* (pfam05567), *pilD* (COG1989, pfam01478, and pfam06750), *pilS* (COG0642, pfam00512, pfam00672, and pfam02518), *pilR* (COG2204, pfam00072, pfam00158, and pfam02954), *pilQ* (COG4796, pfam00263, pfam07660, and pfam11741), *pilP* (COG3168 and pfam04351), *pilO* (COG3167 and pfam04350), *pilN* (COG3166 and pfam05137) and *pilM* (COG4972 and pfam11104). Genes from the *xap* operon were identified with the following motifs: *xapA* (TPR_8; pfam13181), *xapB* (ABC2_membrane_2 pfam;12679), *xapD* (ABC-type multidrug transport system, ATPase component; COG1131 and pfam00005), *xapE* (ubiA; COG0382 and pfam01040), *xapF* (Glycos_transf_2; pfam00535); *xapG* (ABC2_membrane; COG1682 and pfam01061), *xapH* (COG1134, pfam00005 and pfam14524), xapI (Methyltransf_21; pfam05050) and *xapJ* (Glyco_transf_9; COG0859 and pfam01075).

Alpha helices and beta strands were predicted with the Jnet algorithm (Cuff & Barton, 2000) on the JPred4 server (Drozdetskiy *et al.*, 2015) and transmembrane helices were predicted with TMpred (Hofmann & Stoffel, 1993), TMHMM (Krogh *et al.*, 2001), and HMMTOP (Tusnády & Simon, 2001). Signal peptides were identified with PSORTb v. 3.0.2 (Yu *et al.*, 2010) and Signal P v. 4.1 (Petersen *et al.*, 2011).

Phylogenetic analyses. Phylogenetic trees were generated with the maximum likelihood method using MEGA v. 6.0 software (Tamura *et al.*, 2013). Before trees were constructed, the Find Best DNA/Protein Models program was run on sequences previously aligned in GUIDANCE2. The PilA–C phylogeny was generated with the Dayhoff model (Dayhoff *et al.*, 1978) with Gamma Distributed rates among sites. The PilA phylogeny was generated with the LG model (Le & Gascuel, 2008) with Gamma Distributed with invariant sites set as the rate among sites. All tests of phylogeny were determined with the Bootstrap Method using 500 replicates. Relative tree node ages were determined with MEGA v. 6.0 and FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Amino acid and nucleotide sequence alignments were generated with MAFFT (Katoh & Standley, 2013) and PRANK (Löytynoja & Goldman, 2005) algorithms. GUIDANCE2 (Sela *et al.*, 2015) was used to identify and eliminate unreliable residues, columns and sequences in all alignments.

Tajima's Neutrality Test (Tajima, 1989) was conducted on amino acid and nucleotide sequence alignments in MEGA6 (Tamura *et al.*, 2013). Codon positions included were 1st +2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

Results and Discussion

Distribution of e-pilin genes in microbial enomes

More than 250 different genomes from a diversity of Gram-negative and Gram-positive bacteria (95 of which were Fe (III)-respiring bacteria) were scanned for the presence of type IVa pilin genes using previously characterized type IVa PilA sequences from such species as *Neisseria gonorrhoeae* (Craig *et al.*, 2006), *Geobacter sulfurreducens* (Reguera *et al.*, 2005), *Pseudomonas aeruginosa* (Craig *et al.*, 2003), *Myxococcus fulvus* (Sun *et al.*, 2000), *Escherichia coli* (Bieber *et al.*, 1998), and *Shewanella oneidensis* (Gorgel *et al.*, 2015) as queries. This analysis revealed that truncated PilA proteins, like those in *Geobacter sulfurreducens* and *Geobacter metallireducens* are primarily found in micro-organisms in the order Desulfuromondales (Table 1, Fig. 1). These truncated *pilA* genes are predicted to encode mature pilin structural proteins with only 60–90 amino acids, compared with the >120 amino acid residues typically found in PilA subunits of most other bacteria (Table 1). In the few instances in which pili conductivity has been directly measured, truncated *pilA* genes code for proteins that give rise to conductive pili (*Geobacter sulfurreducens* and *Geobacter metallireducens*), whereas longer *pilA* genes yield pili with poor conductivity (*Geobacter uraniireducens* and *Pseudomonas aeruginosa*) (Liu *et al.*, 2014; Tan *et al.*, 2016). Therefore, the truncated *pilA* genes were designated e-pilin to distinguish them from longer type IVa *pilA* genes that are more commonly found in bacteria.

All of the e-pilin sequences lack the C-terminal portion of the sequence found in longer, more typical, PilA proteins, which code for beta sheet structures that form a large globular head (Craig *et al.*, 2004; Giltner *et al.*, 2012). Analysis of the structure of the *Geobacter sulfurreducens* e-pilus suggested that the lack of this C-terminus sequence permits the pilin monomers to pack more tightly than the larger pilin monomers found in most bacteria, yielding a thinner pilus diameter and positioning amino acids in patterns that confer conductivity (Malvankar *et al.*, 2015).

As previously noted in the analysis of the *Geobacter sulfurreducens* PilA sequence (Malvankar *et al.*, 2015; Reardon & Mueller, 2013; Reguera *et al.*, 2005), the N-termini of the e-pilin proteins share many similarities with previously described type IVa PilA found in members of genera such as *Neisseria*, *Pseudomonas*, *Escherichia* and *Myxococcus* (Giltner *et al.*, 2012; Mattick, 2002). It is highly conserved and consists of one or two alpha helices and a transmembrane

Table 1. Predicted size and phylogenetic classification of type IVa pili detected in various Fe(III)-reducing bacteria

Fe(III)-reducing bacterium	Type IVa pilA type	Immature pilin size (aa)	Mature pilin size (aa)	Leader sequence length	Accession number	Number of beta strands at C terminus*
Geobacter bemidjiensis	e-pilin	76	66	10	Gbem_2590	1
Geobacter bremensis	e-pilin	74	64	10	K419DRAFT_00801	0
Pelobacter seleniigenes	e-pilin	70	59	11	N909DRAFT_0006	0
Geobacter sp. OR-1	e-pilin	74	64	10	WP_041974243	0
Geobacter sp. M18	e-pilin	74	64	10	GM18_2492	0
Geobacter sp. M21	e-pilin	74	64	10	GM21_1636	0
Desulfuromonas sp. TF	e-pilin	75	64	11	DTFDRAFT_03630	0
Geoalkalibacter ferrihydriticus	e-pilin	72	61	11	Ga0056053_00657	0
Geoalkalibacter subterraneus	e-pilin	79	66	13	WP_040199521	0
Desulfuromonas thiophila	e-pilin	70	59	11	Ga0056074_12312	0
Geobacter metallireducens	e-pilin	69	59	10	Gmet_1399	0
Geobacter lovleyi	e-pilin	70	60	10	Glov_2096	0
Geobacter sulfurreducens	e-pilin	90	61	29	GSU1496	0
Geobacter pickeringii	e-pilin	70	60	10	Ga0069007_111762	0
Desulfuromusa kysingii	e-pilin	72	60	12	Ga0056096_02700	0
Geobacter argillaceus	e-pilin	75, 75	65, 65	10, 10	Ga0052872_01800 Ga0052872_01802	0, 0
Geobacter soli	e-pilin	75	65	10	WP_039645155	0
Geopsychrobacter electrodiphilus	e-pilin	75	64	11	D888DRAFT_2042	0
Pelobacter propionicus	e-pilin	74	64	10	Ppro_1656	0
'Desulfuromonas *subbituminosa*'	Long type IVa *pilA*	132	125	7	Ga0064601_106193	3
Geobacter uraniireducens	Long type IVa *pilA*	203	193	10	Gura_2677	8
Geobacter daltonii	Long type IVa *pilA*	218	208	10	Geob_3369	10
'Desulfuromonas soudanensis'WTL	Long type IVa *pilA*	204	193	11	Ga0069009_112157	8
Pelobacter carbinolicus	Long type IVa *pilA*	138, 196	131, 185	7, 11	Pcar_2154, Pcar_2144	3, 7
Desulfobacter postgatei	Long type IVa *pilA*	216	205	11	DespoDRAFT_1114	8
Desulfuromonas acetoxidans				No type IVa *pilA* detected		
Desulfobulbus propionicus				No type IVa *pilA* detected		
Desulfobacterium autotrophicum	e-pilin	73	59	14	HRM2_27700	0
Thermincola ferriacetica	Long type IVa *pilA*	113	100	13	TferDRAFT_00608	2
'*Candidatus* Acidianus copahuensis'				No type IVa *pilA* detected		

OPEN MICROBIOLOGY

Table 1. cont.

Fe(III)-reducing bacterium	Type IVa pilA type	Immature pilin size (aa)	Mature pilin size (aa)	Leader sequence length	Accession number	Number of beta strands at C terminus*
Acidicaldus organivorans				No type IVa *pilA* detected		
Carboxydothermus ferrireducens	Long type IVa *pilA*	150	137	13	CarfeDRAFT_00001450	8
Deferrisoma camini	Long type IVa *pilA*	144, 131	130, 125	14, 5	DefcaDRAFT_3089, DefcaDRAFT_3087	2, 3
Desulfovibrio frigidus				No type IVa *pilA* detected		
Fervidicella metallireducens	Long type IVa *pilA*	126	114	12	Q428_01340	4
Desulfovibrio ferrireducens				No type IVa *pilA* detected		
Desulfovibrio vulgaris	Long type IVa *pilA*	141	128	13	Ga0076800_111227	6
'*Thermincola potens*'	Long type IVa *pilA*	146	138	8	WP_049771692	8
Carboxydothermus hydrogenoformans	Long type IVa *pilA*	150	137	13	CHY_0635	8
Shewanella oneidensis	Long type IVa *pilA*	138	126	12	SO0417	3
Anaeromyxobacter dehalogenans	Long type IVa *pilA*	195	188	7	A2cp1_0669	7
Ardenticatena maritima				No type IVa *pilA* detected		
Thermoanaerobacter ethanolicus	Long type IVa *pilA*	121	107	14	TeCCSD1DRAFT_1919	3
'*Thermoanaerobacter cellulolyticus*'	Long type IVa *pilA*	138	133	5	N907DRAFT_0879	6
Bacillus infernus				No type IVa *pilA* detected		
Bacillus subterraneus				No type IVa *pilA* detected		
Caloramator australicus	Long type IVa *pilA*	134	126	8	WP_008908368	5
'*Desulfotomaculum reducens*'	Long type IVa *pilA*	120	107	13	Dred_1042	2
Anaeromyxobacter sp. strain FW109-5	Long type IVa *pilA*	198	191	7	Anae109_0680	4
Desulfosporosinus orientis	Long type IVa *pilA*	141	129	12	Desor_0988	6
Acidithiobacillus ferrooxidans	Long type IVa *pilA*	178	163	15	AFE_0416	6
Acidithiobacillus ferrivorans	Long type IVa *pilA*	169	158	11	Ga0058672_17875	6

Table 1. cont.

Fe(III)-reducing bacterium	Type IVa pilA type	Immature pilin size (aa)	Mature pilin size (aa)	Leader sequence length	Accession number	Number of beta strands at C terminus*
Desulfosporosinus meridei	Long type IVa *pilA*	130, 133	114, 117	*15, 16*	Desmer_0976, Desmer_0977	4, 6
Aeromonas hydrophila	Long type IVa *pilA*	128	121	7	AHA_0692	3
Shewanella algae	Long type IVa *pilA*	138	126	12	BryDRAFT_00594	4
Rhodoferax ferrireducens	Long type IVa *pilA*	160	152	8	Rfer_1265	4
Acidicaldus organivorans				No type IVa *pilA* detected		
Acidimicrobium ferroxidans				No type IVa *pilA* detected		
Acidiphilum cryptum				No type IVa *pilA* detected		
'*Acidithrix ferrooxidans*'	Long type IVa *pilA*	177	144	33	AFO_01365	5
Acidobacterium capsulatum				No type IVa pilA detected		
Acidocella facilis				No type IVa *pilA* detected		
Alicyclobacillus contaminans				No type IVa *pilA* detected		
'*Alkaliphilus metalliredigens*'	Long type IVa *pilA*	112	98	14	Amet_3479	3
Clostridium beijerinckii	Long type IVa *pilA*	189	182	7	Cbei_4216	5
Desulfitobacterium hafniese	Long type IVa *pilA*	171	156	15	Dhaf_3553	8
Desulfitobacterium metallireducens	Long type IVa *pilA*	172	163	9	Desme_2113	9
Ferrimicrobium acidiphilum				No type IVa *pilA* detected		
Ferrimonas baelerica	Long type IVa *pilA*	170	165	5	Fbal_0401	7
Geothrix fermentans				No type IVa *pilA* detected		
Geovibrio thiophilus	Long type IVa *pilA*	147, 142	140, 134	7, 8	K300DRAFT_1049, K300DRAFT_1050	4, 3
Leptospirillum ferriphilum				No type IVa *pilA* detected		
Pantoea agglomerans	Long type IVa *pilA*	151	145	6	Ga0004745_2907	5
Rhodobacter capsulatus				No type IVa *pilA* detected		
Rhodopseudomonas palustris				No type IVa *pilA* detected		
Rhodopseudomonas sphaeroides				No type IVa *pilA* detected		
Sulfobacillus acidophilus				No type IVa *pilA* detected		
Sulfobacillus thermosulfidooxidans				No type IVa *pilA* detected		
Sulfurospirillum barnesii				No type IVa *pilA* detected		

OPEN MICROBIOLOGY

Table 1. cont.

Fe(III)-reducing bacterium	Type IVa pilA type	Immature pilin size (aa)	Mature pilin size (aa)	Leader sequence length	Accession number	Number of beta strands at C terminus*
Thermoanaerobacter siderophilus				No type IVa *pilA* detected		
Thermus scotoductus	Long type IVa *pilA*	117	111	6	WP_038068616	4
Serratia fonticola				No type IVa *pilA* detected		
Tepidimicrobium xylanilyticum	Long type IVa *pilA*	142	127	15	Ga0056071_02505	2
Clostridium celerecrescens				No type IVa *pilA* detected		
Enterococcus gallinarum				No type IVa *pilA* detected		
Pelosinus fermentans				No type IVa *pilA* detected		
Shewanella amazonensis	Long type IVa *pilA*	157	145	12	Sama_0370	4
Shewanella putrefaciens	Long type IVa *pilA*	152	136	16	Sput200_3560	4
Shewanella baltica	Long type IVa *pilA*	187	171	16	Sbal175DRAFT_2072	7
Shewanella peizotolerans	Long type IVa *pilA*	110	95	15	swp_4760	3
Shewanella decolorationis	Long type IVa *pilA*	136	124	12	SdecDRAFT01_04314	4
Shewanella frigidimarina	Long type IVa *pilA*	148	136	12	Sfri_3782	4
Shewanella loihica	Long type IVa *pilA*	145	134	11	Ga0069557_1249	4
Shewanella pealeana	Long type IVa *pilA*	185	172	13	Spea_3315	

*Secondary structure predictions were made with the Jnet algorithm (Cuff & Barton, 2000) on the Jpred4 server ((Drozdetskiy *et al.*, 2015)

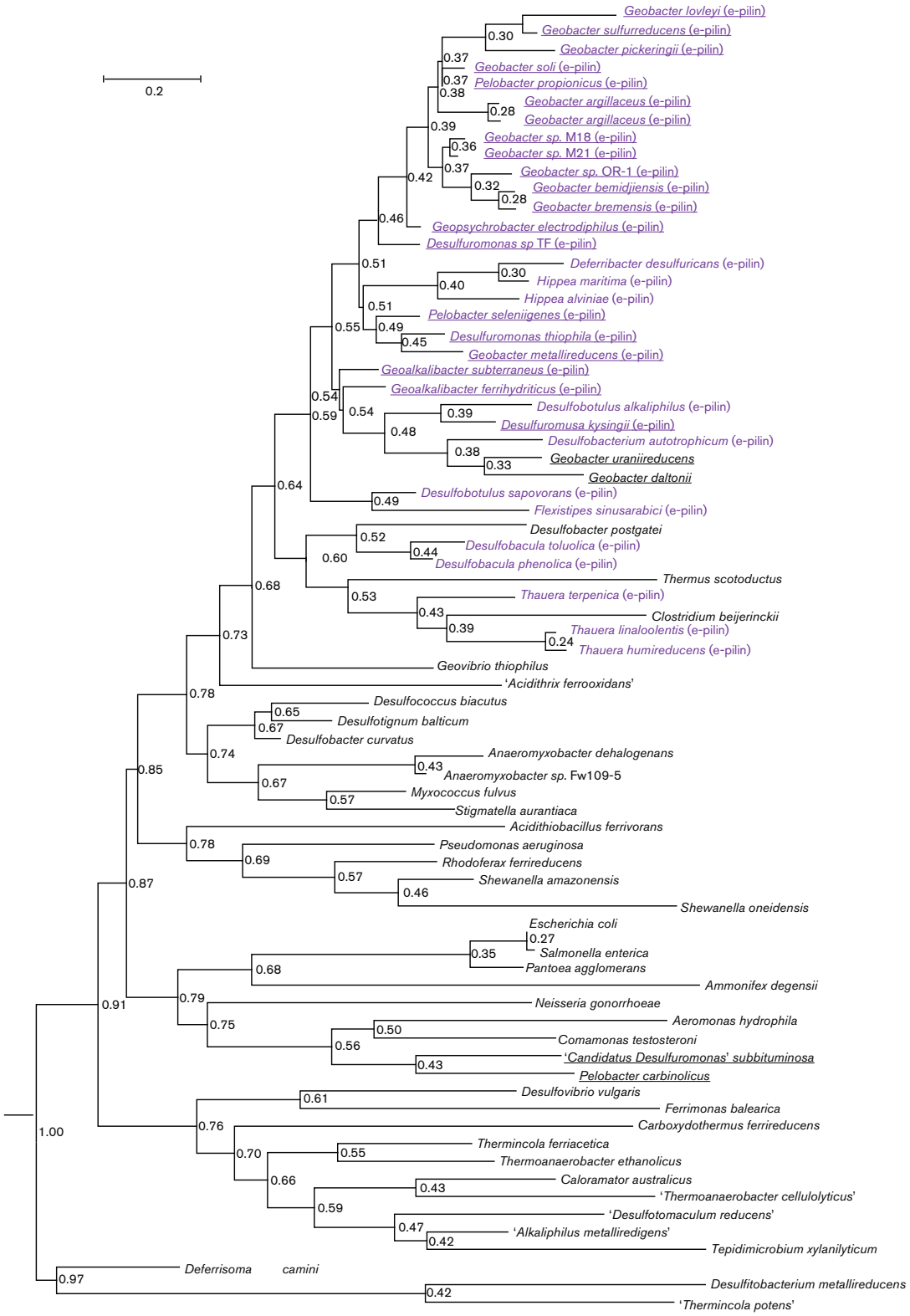


Fig. 1. Phylogenetic tree generated with the maximum-likelihood algorithm comparing type IVa PilA proteins from various Fe(III)-reducing species and species with well-characterized type IVa PilA proteins. *Desulfitobacterium metallireducens* and '*Thermincola potens*' were used as outgroups. Organisms with e-pilin proteins are identified with purple font, and all species from the order Desulfuromondales are underlined. Relative divergence times are shown for each node and the root age was set at 1.0.

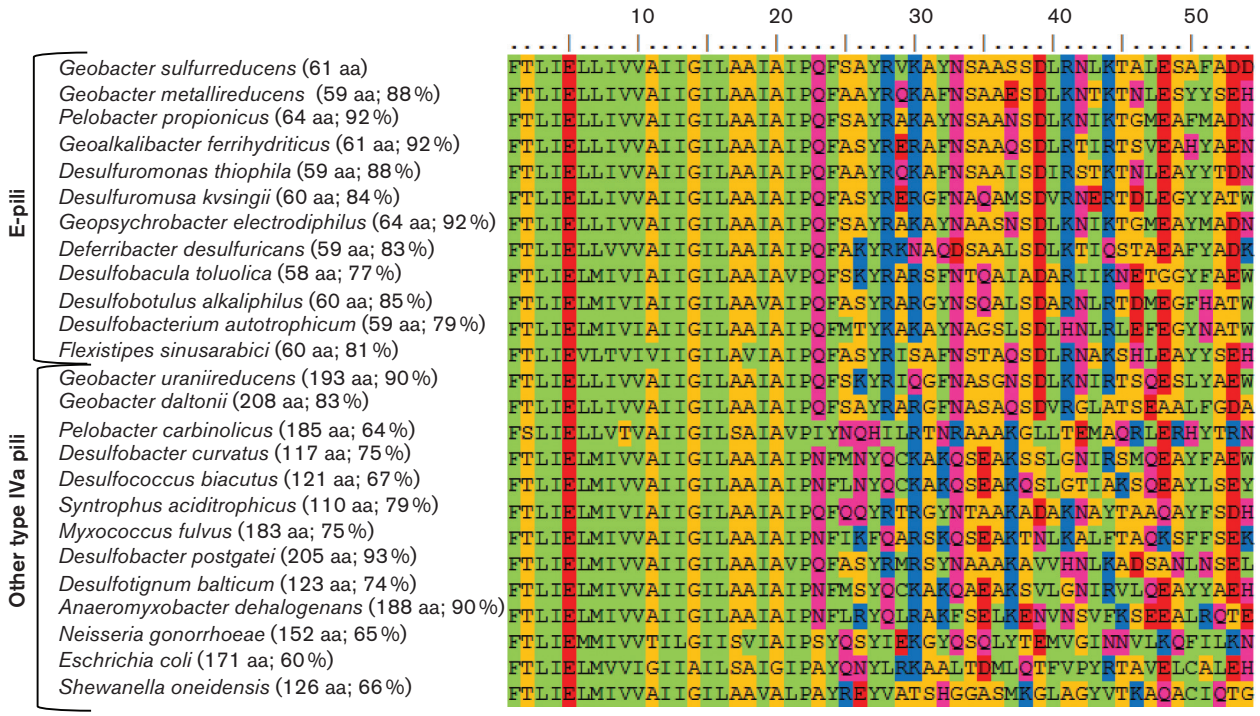


Fig. 2. Alignment of the N-terminus (first 54 amino acids) of mature type IVa PilA proteins from various taxonomic groups. The numbers in parentheses represent the size of the mature pilin protein and the percentage similarity to PilA from *Geobacter sulfurreducens*. The Lesk color scheme was used to shade the amino acids. All small nonpolar residues (G, A, S, T) are orange; all hydrophobic residues (C, V, I, L, P, F, Y, M, W) are green; all polar residues (N, Q, H) are magenta; all negatively charged (D, E) are red; and all positively charged residues (K, R) are blue.

motif and aligns closely with other previously described type IVa pilin proteins (Figs 2 and S1, available in the online Supplementary Material).

Consistent with other type IVa pilins, all of the e-pilin proteins have a phenylalanine at the N terminus (Fig. 2), and the majority have leader peptides with less than 12 amino acids (Table 1). The exception is the leader peptide of *Geobacter sulfurreducens*, which is 29 amino acids long and more characteristic of a type IVb PilA.

The size of these type IVa e-pilins is similar to that of the major subunits of Tad pili (Flp) found in *Aggregatibacter actinomycetemcomitans* (Kachlany *et al.*, 2001; Tomich *et al.*, 2007) and *Peudomonas aeruginosa* (Bernard *et al.*, 2009; Burrows, 2012), which range in size from 50 to 80 amino acid residues. However, Flp pili are type IVb pili with longer leader peptides and a different nonpolar hydrophobic residue (alanine or valine) at the N-terminus following removal of the signal sequence (Fig. 3).

Positive selection of the e-pilin gene in the Desulfuromondales

With a few exceptions, most of the e-pilin genes cluster in the same phylogenetic clade, suggesting that they evolved from a common ancestral *pilA* gene (Fig. 1). This clade branches off from other type IVa PilA proteins at a relative time of 0.55 when the root node divergence age is set at 1.0. The majority of nodes for longer PilA proteins found in various Fe(III)-reducing bacteria and other well-studied bacteria such as members of the genera *Neisseria*, *Shewanella*, *Pseudomonas* and *Myxococcus*, on the other hand, have relative times that are > 0.7.

Tajima's test of neutrality (Tajima, 1989) indicated that the Desulfuromondales e-pilin amino acid and nucleotide sequences are undergoing positive selection; Tajima's D=6.03 for the protein alignment and Tajima's D=3.5 for the nucleotide alignment. These results suggest that there is a strong selective pressure on the conservation of e-pilin genes within the order Desulfuromondales and that these organisms have been exposed to environmental conditions under which e-pili provide a competitive advantage.

Most (77 %) of the bacteria in the order Desulfuromondales possess e-pilin genes. The exceptions are *Gobacter uraniireducens, Geobacter daltoni*, '*Desulfuromonas soudanensis*', '*Desulfuromonas subbituminosa*', *Desulfuromonas acetoxidans* and *Pelobacter carbinolicus*. With the exception of a gene coding for an IS4 family transposase located directly upstream from the *Geobacter uraniireducens pilA* gene (Gura_2677), there is little evidence of horizontal gene transfer within pilin gene clusters in these six species (Fig. 4, Table S1, available in the online Supplementary Material).

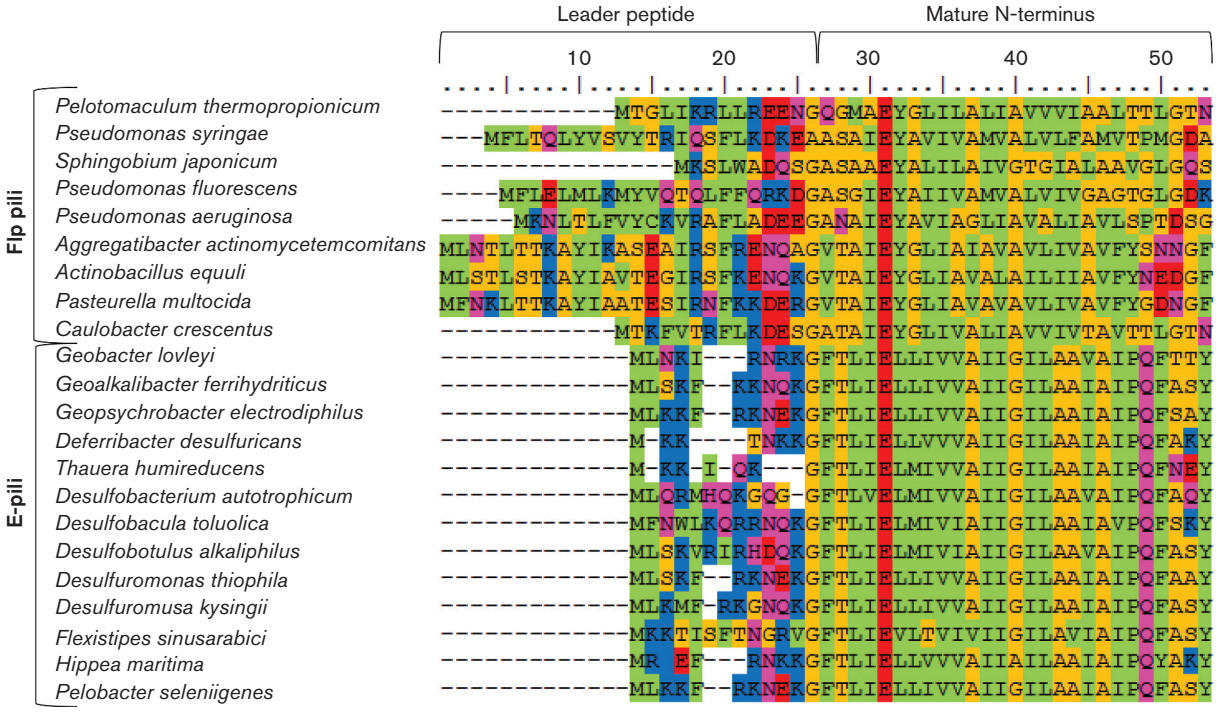


Fig. 3. Alignment of the N-terminus of immature Flp pili and E-pili from various taxonomic groups. The Lesk color scheme was used to shade the amino acids. All small nonpolar residues (G, A, S, T) are orange; all hydrophobic residues (C, V, I, L, P, F, Y, M, W) are green; all polar residues (N, Q, H) are magenta; all negatively charged (D, E) are red; and all positively charged residues (K, R) are blue.

Desulfuromonas. acetoxidans does not possess a type IVa *pilA* gene, and the *pilA* gene cluster found in the '*Desulfuromonas subbituminosa*' genome is more similar to those of other deltaproteobacteria (Fig. S2). Pilus gene clusters associated with '*Desulfuromonas soudanensis*', *Pelobacter carbinolicus*, *Geobacter daltonii* and *Geobacter uraniireducens* are characteristic of species of the order Desulfuromondales with e-pilin genes (Fig. S2).

Hypothetical protein gene associated with e-pilin genes

In the genomes of all members of the order Desulfuromondales with e-pilin genes, except the genome of *Geobacter soli*, the adjacent gene downstream from e-pilin, is a gene that codes for a small (86–136 amino acids) hypothetical protein with multiple beta strands and a signal peptide (Table 2). This gene was annotated as '*pilA–C*' in the *Geobacter sulfurreducens* genome, with the expectation that its protein was a component of the *Geobacter sulfurreducens* pilin protein. However, this annotation is incorrect because denatured pili do not contain a protein of the predicted size (Reguera *et al.*, 2005; Tan *et al.*, 2016; Veazey *et al.*, 2011).

Although both the C-terminus of long type IVa PilA and 'PilA–C' proteins have similar topology (beta strands and alpha helices), it is unlikely that they evolved from the same protein. If the '*pilA–C*' gene was derived from the C-terminus of a typical type IV PilA gene it would only be composed of beta strands and the proteins would be more homologous (Fig. 5). However, all of the 'PilA–C' proteins have one to three alpha helices (Fig. S3) and the proteins form two distinct phylogenetic clades. Because of the great divergence between '*pilA–C*' and the C-terminus of typical long *pilA* genes, it was difficult to reconstruct an accurate phylogenetic tree comparing all of the taxonomic groups discussed thus far in this paper. Therefore, a relative time tree comparing 'PilA–C' and the C-terminus of long PilA proteins was constructed with only sequences from members of the genus *Geobacter*. It is apparent that even within the same genus these two proteins are highly divergent and that they evolved separately around the same time forming two distinct phylogenetic clades (Fig. 5).

There is little homology between 'PilA–C' and other characterized proteins, however, all of the Desulfuromondales 'PilA–C' proteins except *P. propionicus* have signal peptides, suggesting that they are either extracellular or localized to the outer membrane, and their N-termini are similar to the porin-like protein OmpX from *Klebsiella pneumonia* and *Escherichia coli* (Fig. 6). The 'PilA –C' protein shares many other features with porin proteins (Galdiero *et al.*, 2012); multiple beta strands, a signal sequence of approximately 21 amino acids at its N-terminus, low hydrophobicity and few to no cysteine residues. The only porin-related feature that is lacking in 'PilA–C' is a C-terminal phenylalanine, which is important for membrane insertion. A porin-like protein (usher protein; PapC) is associated with type I pili and P-

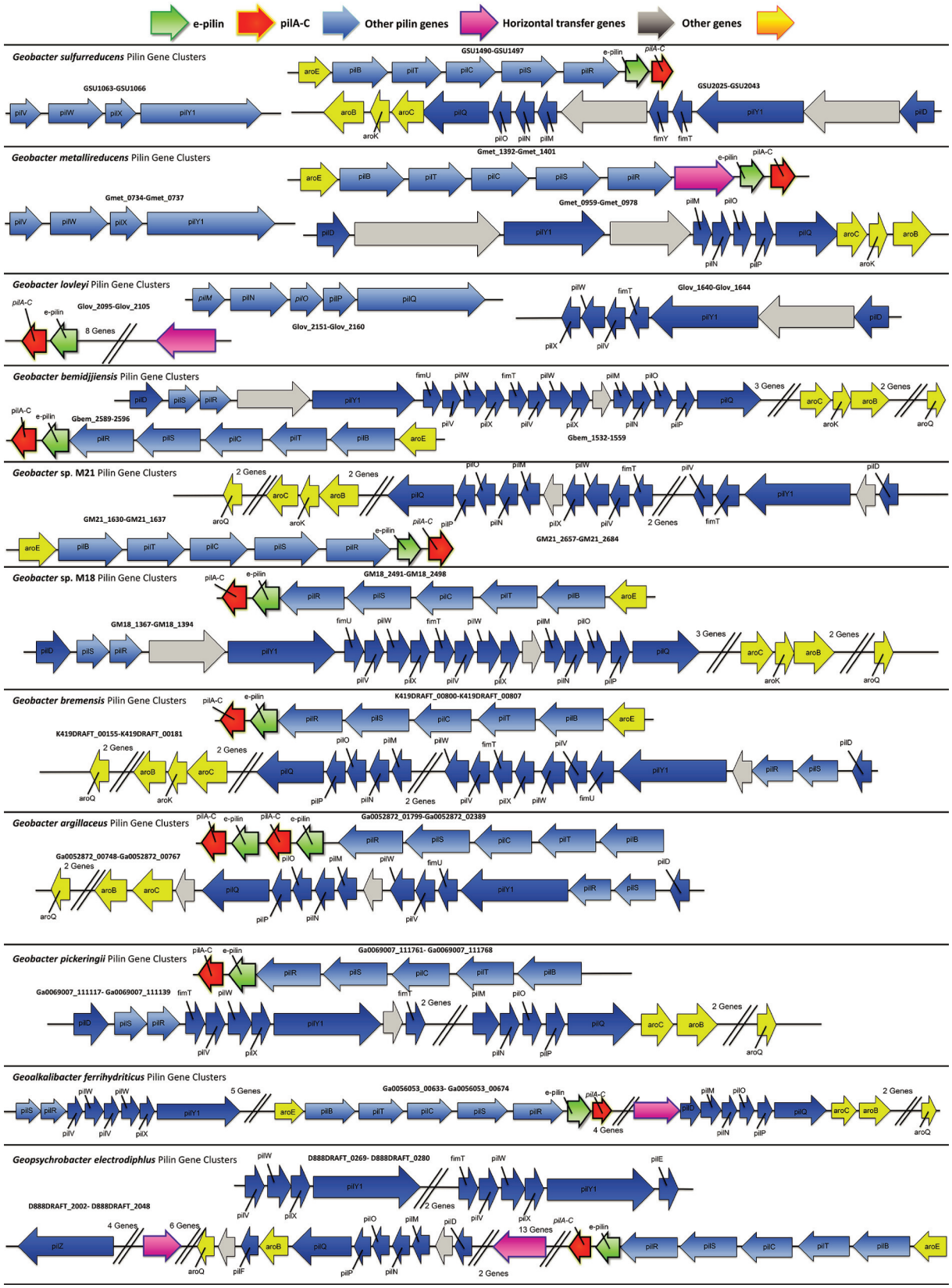


Fig. 4. Distribution of pilin genes within the genomes of bacteria that are predicted to possess e-pili.

Fig. 4. (cont.)

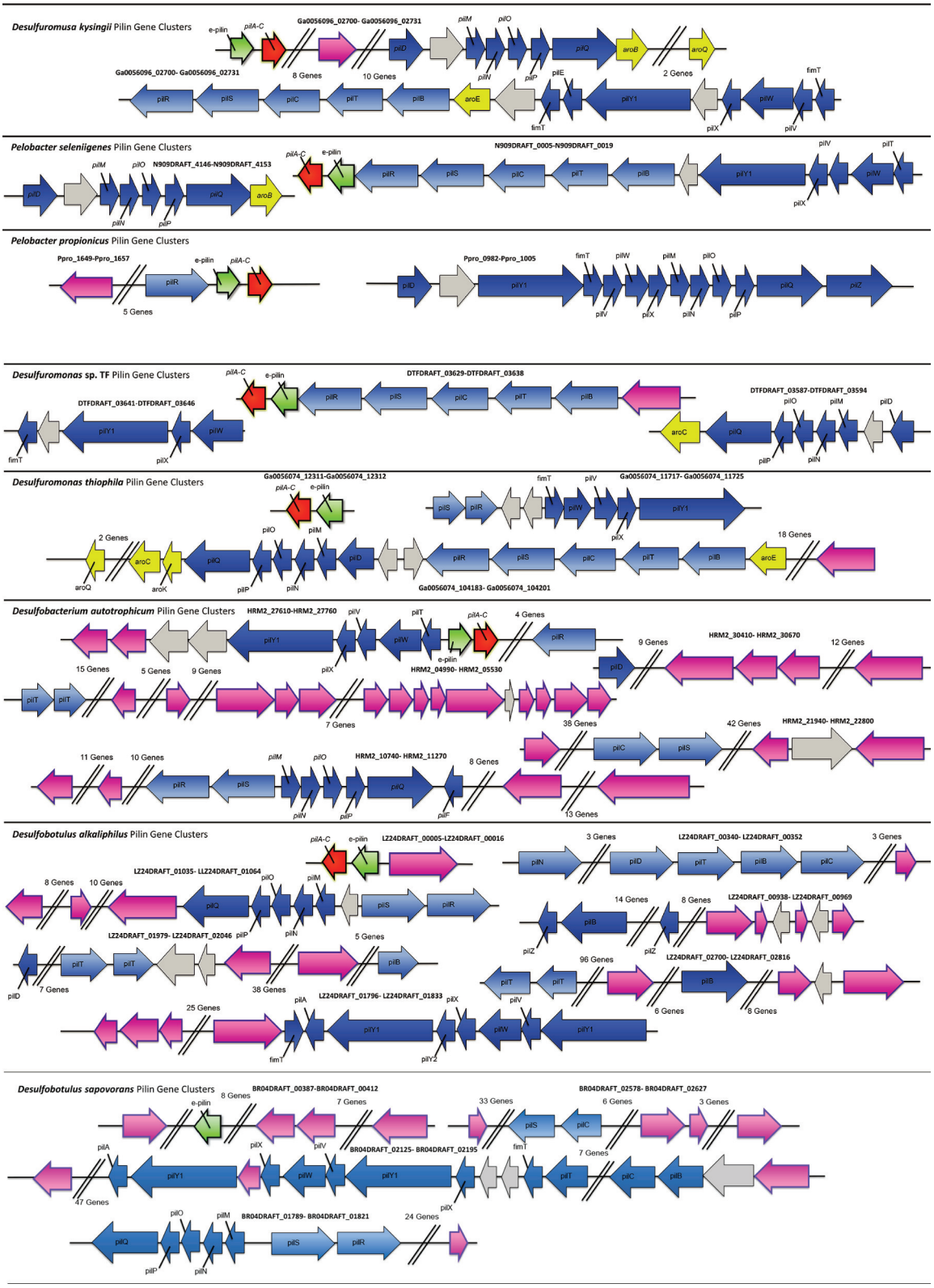
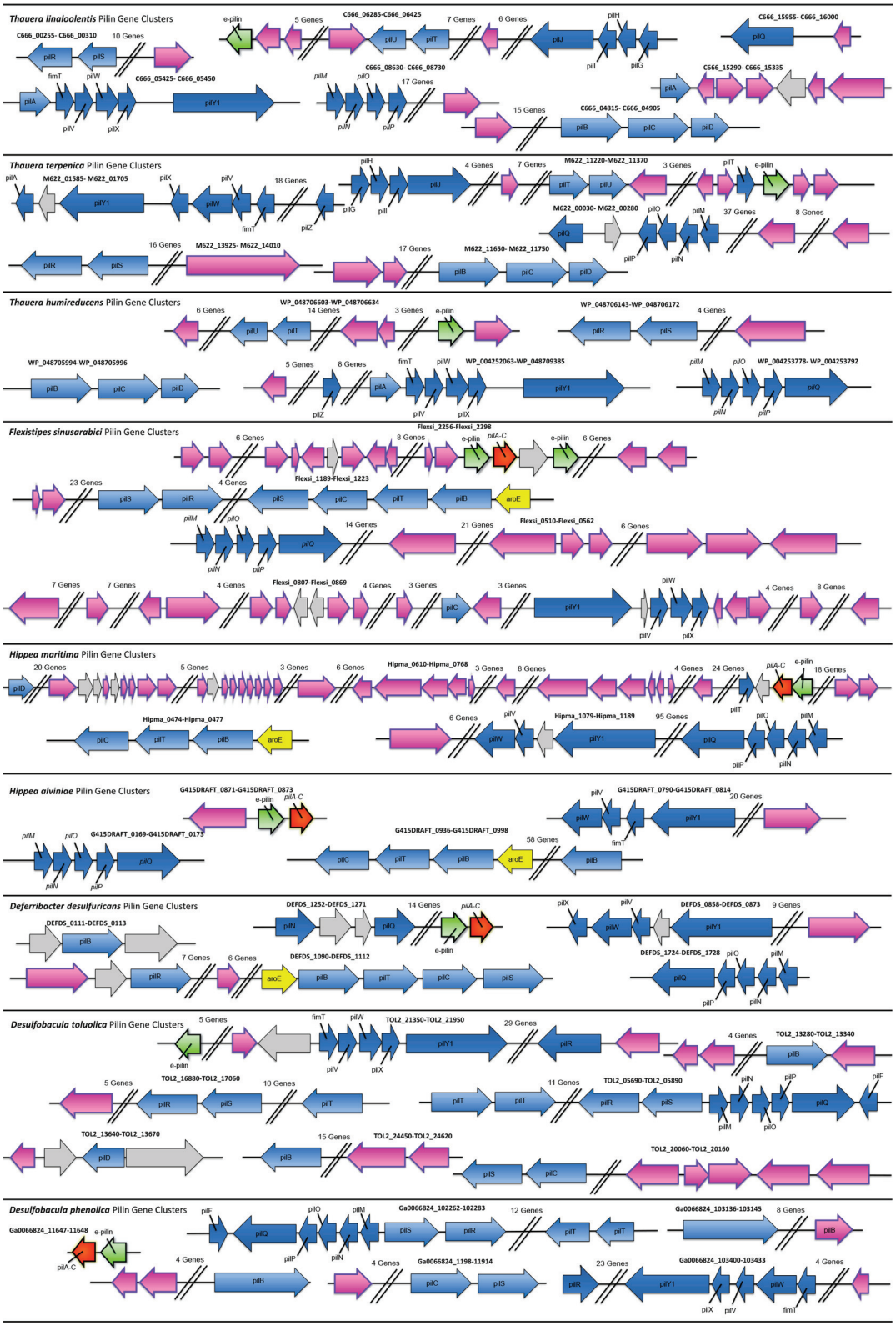

Desulfuromusa kysingii Pilin Gene Clusters
Pelobacter seleniigenes Pilin Gene Clusters
Pelobacter propionicus Pilin Gene Clusters
Desulfuromonas sp. TF Pilin Gene Clusters
Desulfuromonas thiophila Pilin Gene Clusters
Desulfobacterium autotrophicum Pilin Gene Clusters
Desulfobotulus alkaliphilus Pilin Gene Clusters
Desulfobotulus sapovorans Pilin Gene Clusters

Fig. 4. (cont.)


Thauera linaloolentis Pilin Gene Clusters
Thauera terpenica Pilin Gene Clusters
Thauera humireducens Pilin Gene Clusters
Flexistipes sinusarabici Pilin Gene Clusters
Hippea maritima Pilin Gene Clusters
Hippea alviniae Pilin Gene Clusters
Deferribacter desulfuricans Pilin Gene Clusters
Desulfobacula toluolica Pilin Gene Clusters
Desulfobacula phenolica Pilin Gene Clusters

pili in *E. coli* (Ford *et al.*, 2012; Waksman & Hultgren, 2009). PapC facilitates translocation of the major pilin subunit across the outer membrane and acts as an assembly platform for the pilus. Although no sequence homology between PilA–C and PapC is apparent, it is possible that they play a similar role in pilus assembly.

Another difference between type I pilin and type IV e-pilin systems is the absence of a chaperone protein in the e-pilin gene cluster. The type I chaperone (PapD) has two immunoglobulin-like domains and facilitates pilin subunit folding and delivery to PapC. Only two e-pilin-harboring taxa, *Geobacter bemidjiensis* (Gbem_1552) and strain M18 (GM18_1387), have PapD-like domain proteins. These PapD-like proteins are not located in the e-pilin gene cluster, rather they are found in the vicinity of *pilMNOPQ* and only have a single immunoglobulin-like domain. The *pilVWXY1* gene cluster in many other e-pilin-harboring members of the order Desulfuromondales, on the other hand, contains a gene coding for another chaperone, thiol:disulfide interchange protein (DsbC), which helps fold secreted proteins, such as pili, by enabling the formation of disulfide bonds (Zapun *et al.*, 1995). While most type IV pilin proteins have disulfide bonds (Giltner *et al.*, 2012), including all of the long type IVa pili discussed here, e-pili lack the cysteine residues that would be required for the formation of disulfide bonds. Therefore, it seems that this protein is not likely to be involved in posttranslational modification of e-pili.

As with the e-pilin gene, Tajima's test of neutrality (Tajima, 1989) suggests that the '*pilA–C*' gene is undergoing positive selection within the order Desulfuromondales; Tajima's D=5.71 for the protein alignment and Tajima's D=3.88 for the nucleotide alignment. The frequent occurrence of '*pilA–C*' genes in species of the order Desulfuromondales with genomes that encode e-pili suggests that '*pilA–C*' is likely to play an important role in proper e-pilin expression or function. Further study of the location and function of the protein encoded by the '*pilA–C*' gene is warranted.

Additional conserved gene organization associated with e-pilin genes

The genome region around the e-pilin genes in species of the order Desulfuromondales species is highly conserved (Fig. 4, Table S1). The e-pilin gene is found within a cluster of genes including *pilB* and *pilT* (both ATPase proteins involved in pilin assembly and retraction), *pilC* (platform protein), *pilS* and *pilR* (two-component regulator proteins involved in pilin expression) and genes involved in synthesis and export of surface polysaccharides and biofilm formation (*xap* genes) (Rollefson *et al.*, 2011). A gene coding for shikimate dehydrogenase (*aroE*) is also frequently found in this gene cluster. This gene codes for a protein involved in the synthesis of aromatic amino acids, which are required for e-pili conductivity (Adhikari *et al.*, 2016; Vargas *et al.*, 2013).

Additional genes coding for proteins required for proper assembly and function of e-pili are the same as those from other type IVa pili such as *Myxococcus*, *E. coli*, *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* (Ayers *et al.*, 2010; Nivaskumar & Francetic, 2014). PilY1 is a pilin tip adhesion protein that helps pili adhere to surfaces. PilV, PilW and PilX are all minor pilin proteins. PilQ is a secretin protein that allows translocation of the pilin subunits across the membrane. PilD is a prepilin peptidase. PilM, PilN, PilO and PilP form a secretin-associated sub complex. Desulfuromondales pilin accessory and minor pilin proteins are homologous to well characterized pilin genes (Table S2, Fig. S4).

In the majority of species of the order Desulfuromondales, genes coding for these pilin assembly proteins are found in one or two gene clusters (Fig. 4). These clusters also possess genes coding for other aromatic amino acid synthesis proteins (*aroC, aroK* and *aroB*) and numerous genes coding for glycosyltransferase proteins that may be involved in glycosylation of pilin proteins.

Many of the Desulfuromondales pilin accessory proteins were approximatley 45–80 % similar to proteins from *E. coli*, *Pseudomonas aeruginosa*, *Nisseria gonorrhoeae* and *Myxococcus fulvus* with the exception of the minor pilin proteins PilX, PilY1, PilW and PilV (Table S2). For the most part, Desulfuromondales pilin accessory proteins form unique phylogenetic clades (Fig. S4). Although e-pilin proteins from species from other orders cluster with species from the order Desulfuromondales, their pilin accessory proteins fall into separate clades. In addition, long type IVa PilA proteins from species of the order Desulfuromondales do not cluster with e-pilin proteins, but most of their pilin accessory proteins cluster with those of e-pilin-harboring Desulfuromondales.

Horizontal transfer of e-pilin outside the *Desulfuromondales*

Few bacteria outside the Desulfuromondales have e-pilin genes. Those that do include *Desulfobacterium autotrophicum*, *Desulfobotulus alkaliphilus*, *Desulfobotulus sapovorans*, *Thauera linaloolentis*, *Thauera terpenica*, *Thauera humireducens*, *Flexistipes sinusarabici*, *Hippea maritima*, *Hippea alvinae*, *Deferribacter desulfuricans*, *Desulfobacula toluolica*, *Desulfobacula phenolica* and *Desulfurobacula* sp. TS (Fig. 1, Table 3). With the exception of *Desulfobacterium autotrophicum* (Lovley *et al.*, 1993), none of these organisms are known to grow via Fe(III) respiration. However, one of the primary functions of e-pili may be interspecies electron exchange (Rotaru *et al.*, 2015), a recently discovered microbial capability (Summers *et al.*, 2010) for which few microorganisms have been evaluated. Many of the organisms outside the Desulfuromondales that have e-pilin genes were isolated from habitats where species of the order Desulfuromondales are typically found and there is evidence of horizontal gene transfer in regions of their genomes where the e-pilin genes are located.

For example, the genome of *Flexistipes sinusarabici* contains two e-pilin genes, one of which is located next to a putative '*pilA-C*', that cluster with e-pili from members of the genus

Table 2. Predicted properties of 'PilA–C' proteins from members of the genus *Geobacter* and other bacteria with e-pilin genes

Organism	GeneID	beta-strands*	alpha helices*	Size (aa)	Signal peptide?†
Desulfuromusa kysingii	Ga0056096_02701	7	2	119	+
Geopsychrobacter electrodiphilus	D888DRAFT_2041	4	3	125	+
Pelobacter seleniigenes	N909DRAFT_0005	5	2	128	+
Geobacter bemidjiensis	Gbem_2589	8	3	136	+
Geobacter bremensis	K419DRAFT_00800	5	3	129	+
Geobacter sp. OR-1	WP_041974245	5	1	122	+
Geobacter sp. M18	GM18_2491	5	3	120	+
Geobacter sp. M21	GM21_1637	4	2	117	+
Geobacter soli	Not present				
Pelobacter propionicus	Ppro_1657	3	2	86	–
Geobacter argillaceus	Ga0052872_01799	6	2	121	+
Geobacter argillaceus	Ga0052872_01801	5	2	121	+
Desulfuromonas sp. TF	DTFDRAFT_03629	4	2	124	+
Geoalkalibacter ferrihydriticus	Ga0056053_00658	5	1	106	+
Geoalkalibacter subterraneus	WP_040199522	6	2	121	+
Desulfuromonas thiophila	Ga0056074_12311	6	1	109	+
Geobacter metallireducens	Gmet_1400	6	1	113	+
Geobacter lovleyi	Glov_2095	4	3	119	+
Geobacter sulfurreducens	GSU1497	6	2	124	+
Geobacter pickeringii	Ga0069007_111761	5	1	109	+
Desulfobotulus alkaliphilus	LZ24DRAFT_00005	7	1	138	+
Flexistipes sinusarabici	Flexsi_2289	5	4	128	–
Desulfobotulus sapovorans	Not present				
Desulfobacterium autotrophicum	HRM2_27710	6	2	120	+
Desulfobacula phenolica	Not present				
Desulfobacula toluolica	Not present				
Thauera linaloolentis	Not present				
Hippea maritima	Hipma_0736	6	2	107	+
Hippea alviniae	G415DRAFT_0873	4	2	109	+
Deferribacter desulfuricans	DEFDS_1271	4	1	107	+
Thauera humireducens	Not present				
Thauera terpenica	Not present				

*Alpha helices and beta strands were identified with the Jnet algorithm (Cuff & Barton, 2000) on the Jpred 4 server (Drozdetskiy, 2015).
†Signal peptides were predicted with PSORTb v. 3.0 (Wagner, 2010) and SignalP 4.1 (Nielsen,1997).

Geobacter and closely related organisms. However, there are nine prophage/transposon-related genes in the vicinity of these genes, compared with few, if any, within genomes of members of the genus Desulfuromondales (Fig. 4, Table S1). Furthermore, genes coding for other pilin components are scattered throughout the *Flexistipes sinusarabici* genome and are flanked by numerous horizontal transfer genes.

The *Deferribacter desulfuricans* genome contains both the e-pilin and '*pilA*-C' genes together in a cluster. Other genes required for pilin assembly are scattered throughout the genome in four other clusters and prophage- and transposon-related genes are found in two of these clusters (Fig. 4, Table S1).

In a similar manner, the genomes from *Hippea maritima* and *Hippea alviniae* contain both e-pilin and *pilA–C* genes, but other pilin-related genes are scattered throughout their genomes and flanked by horizontal transfer genes (Fig. 4, Table S1). Genes for E-pilin and 'PilA–C' are not present in the two other available genomes of members of the genus *Hippea*, *Hippea jasoniae* and "'*Hippea medeae*'. 'Hippea *medeae*' (D891DRAFT_0589), which has not been cultured, has a pilin protein that clusters with non-delta-related long type IVa pili from such genera as *Pseudomonas* (Fig. 1). No type IVa pili genes were detected in the genome of *Hippea jasoniae*.

Desulfobacterium autotrophicum is one of the few microbes that appears to have obtained the e-pilin gene via horizontal gene transfer with an adjacent '*pilA–C*' gene (Fig. 4, Table S1) that has been verified to reduce Fe(III) (Lovley *et al.*, 1993) and Mn(IV) (Lovley & Phillips, 1994). Other

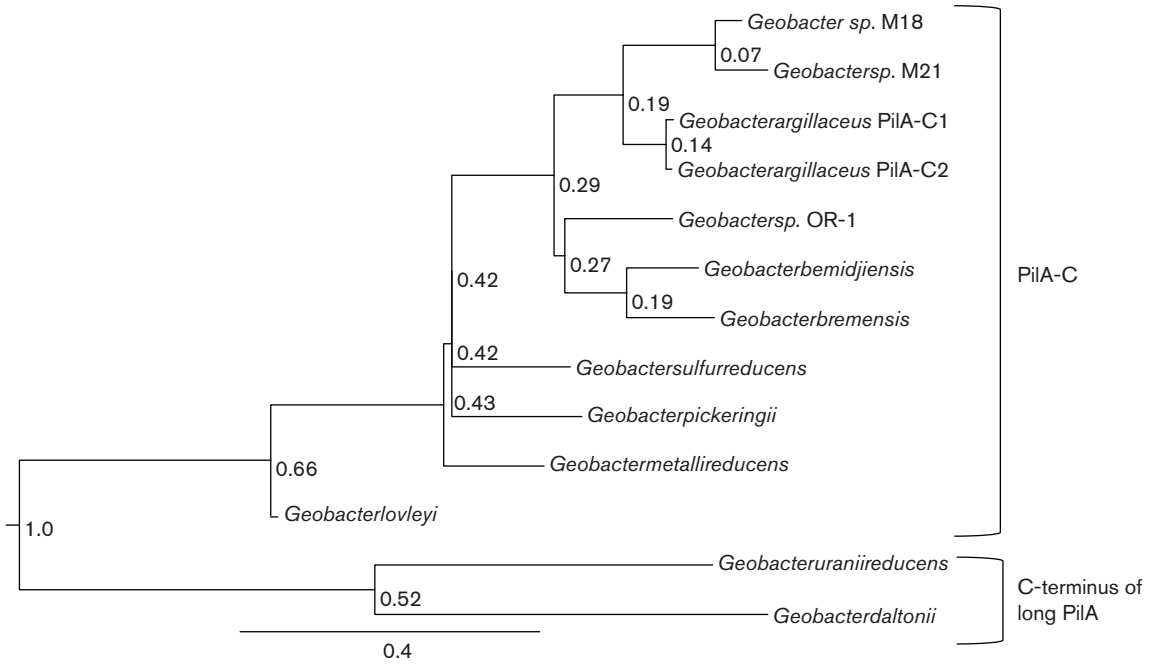


Fig. 5. Phylogenetic tree generated by the maximum-likelihood algorithim comparing amino acid sequences from the C-terminus of *Geobacter* PilA to *Geobacter* 'PilA–C'. Values represent relative times when each branch node evolved compared with a root node age set at 1.0. *Geobacter uraniireducens* and *Geobacter daltonii* were used as outgroups.

pilin genes are scattered throughout the genome in five different gene clusters flanked with numerous horizontal transfer genes.

Two genomes of members of the genus *Desulfobotulus* are available. Both the e-pilin and '*pilA-C*' genes are found in *Desulfobotulus alkaliphilus* but only the e-pilin gene is present in the *Desulfobotulus sapovorans* genome. Similar to other species that are not members of the order Desulfuromondales, other pilin assembly genes are scattered throughout the genome and are flanked with genes indicative of horizontal transfer (Fig. 4, Table S1).

E-pilin genes were also detected in the genomes of three other sulfate reducers from the genus *Desulfobacula*, *Desulfobacula toluolica*, *Desulfobacula phenolica* and strain TS (Kim *et al.*, 2014; Kuever *et al.*, 2001; Rabus *et al.*, 1993). Both *Desulfobacula toluolica* and *Desulfobacula phenolica* have an e-pilin gene, but no gene for 'PilA–C,' and strain TS only has a partial e-pilin gene (missing three amino acids at the N-terminus) that is located near a gap in the genome's draft assembly (Tables 2 and 3). While a small hypothetical protein with five beta strands is found directly downstream of the e-pilin gene in *Desulfobacula phenolica*, it is unlikely to be '*pilA–C* ' because it lacks a signal peptide. The *Desulfobacula phenolica* e-pilin gene is also unusual and has a beta strand at its C-terminus. The genome of strain TS is not sufficiently assembled for analysis, however both *Desulfobacula toluolica* and *Desulfobacula phenolica* have the full suite of genes required for pilus assembly scattered throughout the genome in seven different gene clusters that are

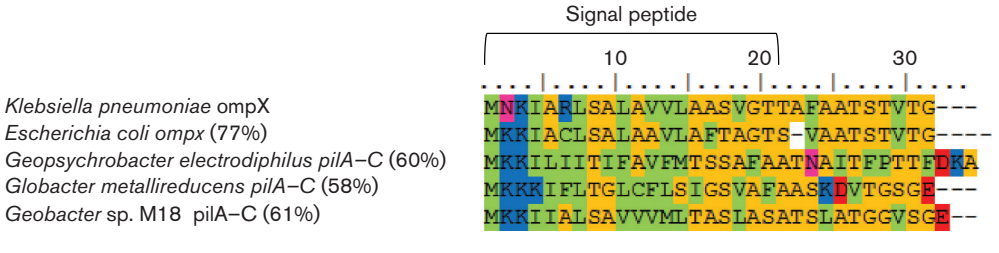


Fig. 6. Alignment of N-termini from OmpX and PilA-C proteins. The numbers in parentheses represent the percentage similarity to OmpX from *Klebsiella pneumoniae*. The Lesk color scheme was used to shade the amino acids. All small nonpolar residues (G, A,S, T) are orange; all hydrophobic residues (C, V, I, L, P, F, Y, M, W) are green; all polar residues (N, Q, H) are magenta; all negatively charged (D, E) are red; and all positively charged residues (K, R) are blue.

Table 3. Characteristics of e-pilin genes found in non-Fe(III)-reducing bacterial species

Organism	Type IVa pilin type	Immature pilin size (aa)	Mature pilin size (aa)	Leader sequence length	Accession number	#beta strands at C terminus*
Desulfobacterium autotrophicum	epilin	73	59	14	HRM2_27700	0
Hippea maritima	epilin	68	59	9	Hipma_0737	0
Hippea alviniae	epilin	70	61	9	G415DRAFT_0872	0
Deferribacter desulfuricans	epilin	67	59	8	DEFDS_1270	0
Flexistipes sinusarabici	epilin	74, 70	67, 60	7, 10	Flexsi_2291, Flexsi_2288	1, 0
Desulfobacula toluolica	epilin	71	58	13	TOL2_21350	0
Desulfobacula phenolica	epilin	72	59	13	Ga0066824_11648	1
Desulfobacula sp. TS	epilin		Partial sequence		Ga0097800_108051	0
Desulfobotulus alkaliphilus	epilin	73	60	13	LZ24DRAFT_00006	0
Desulfobotulus sapovorans	epilin	71	58	13	BR04DRAFT_00394	0
Thauera linaloolentis	epilin and long type 4a pilin	59, 150, 168	52, 144, 161	7, 6, 7	C666_06285, C666_05425, C666_15290	0, 4, 5
Thauera terpenica	epilin and long type 4a pilin	59, 137	52, 128	7, 9	M622_11345, M622_01585	0, 3
Thauera humireducens	epilin and long type 4a pilin	150, 60	144, 52	6, 8	WP_048709378, WP_048706629	4, 1

*Beta strands were identified with the Jnet algorithm (Cuff & Barton, 2000) on the Jpred 4 server(Drozdetskiy, 2015).

flanked by prophage- and transposon related genes (Fig. 4, Table S1).

Genes coding for e-pili were also found in the genomes of *Thauera linaloolentis*, *Thauera humireducens* and *Thauera terpenica*, but none of these genes are accompanied by '*pilA–C*.' The e-pilin gene and other pilin gene clusters in all three of these genomes is flanked by prophage- and transposon-related genes and the GC content of the e-pilin gene from *Thauera humireducens*, *Thauera terpenica* and *Thauera linaloolentis* is 15 %, 10 % and 19 % lower than the GC content of their respective genomes. All three of these genomes also have genes coding for long type IVa PilA proteins (*Thauera linaloolentis* has two long *pilA*). The e-pilin gene seems to be isolated within each of the genomes, whereas the long *pilA* gene clusters with genes required for proper pilin assembly (Fig. 4 and Table S1). Furthermore, the putative e-pilin protein in *Thauera humireducens* has a beta strand at its C-terminus similar to that found in long PilA proteins, suggesting that this putative e-pilin gene may instead be a truncation of a long *pilA* gene duplication.

Available genomes from two other denitrifying species of the genus *Thauera*, *Thauera phenylacetica* and *Thauera aminoaromatica*, do not contain genes for e-pilin, but do have genes coding for long type IVa pili.

Most Fe(III)-reducing bacteria lack e-pili

Genes for identifiable e-pilin are not present in the majority of known Fe(III)-reducing micro-organisms with sequenced genomes (Table 1). Only 21 % of the available 95 genomes contain e-pilin genes (Table 1). About half of the genomes without e-pilin genes contain genes for long type IVa PilA (Table 1).

There are a number of instances in which e-pili are not required for extracellular electron transfer. For example, some soluble natural organic compounds, such as humic substances, can act as electron shuttles (Lovley *et al.*, 1996). They are reduced at the outer cell surface (Voordeckers *et al.*, 2010) and then carry electrons to the surface of Fe (III) oxides (Lovley *et al.*, 1996) or other cells (Smith *et al.*, 2015), alleviating the need for long-range electron transport via e-pili. Other environmental components, such as sulfur compounds, may also act as electron shuttles (Nevin & Lovley, 2000). Alternatively, natural organic matter may chelate Fe(III) which can also be reduced at the cell surface (Nevin & Lovley, 2002). Direct contact of the outer cell surface with an insoluble electron acceptor, such as when *Geobacter sulfurreducens* cells are in direct contact with electrodes (Bond & Lovley, 2003), is another possibility, but the importance of this type of electron transfer in nature is not well understood. These alternative strategies for long-range electron transport may be most favored when rates of microbial metabolism are slow. For example, species of the

genus *Rhodoferax*, which lack e-pili, predominated in a subsurface environment where rates of Fe(III)-reduction were low, but when Fe(III) reduction was artificially stimulated in this same environment as a bioremediation strategy there was a strong selection for species of the genus *Geobacter* (Mouser *et al.*, 2009; Zhuang *et al.*, 2011).

There is also the possibility that some Fe(III)-reducing micro-organisms that do not have e-pili employ other, independently evolved, types of electrically conductive filaments. For example, *Rhodopsuedomonas palustris* strain RP2 specifically produced electrically conductive filaments under Fe (III)-reducing conditions (Venkidusamy *et al.*, 2015). It has yet to be determined whether these filaments are required for Fe(III) reduction, and the composition of the filaments is as yet unknown, but given that available *Rhodopseudomonas palustris* genomes lack e-pilin genes (Table 1), the filaments of *Rhodopseudomonas palustris* strain RP2 could offer an alternative strategy for extracellular electron exchange. Other examples of electrically conductive filaments putatively involved in extracellular electron exchange are being increasingly documented (Castro *et al.*, 2014; Eaktasang *et al.*, 2016; Li & Li, 2014). Further evaluation of the mechanisms for extracellular electron transfer in these organisms is also warranted.

Conclusions

The results suggest that e-pili of *Geobacter sulfurreducens* and *Geobacter metallireducens*, and presumably close relatives, are a relatively recent evolutionary development. The ability to transport electrons over multiple cell distances to minerals or other cells may be one of the key attributes that permits members of the genus *Geobacter* to outcompete other micro-organisms when there is strong selective pressure for rapid growth on Fe(III) minerals; electron exchange via direct interspecies electron transfer; or under artificial conditions when an electrode is provided as an electron acceptor and the growth of electrically conductive biofilms is favored (Lovley *et al.*, 2011). The acquisition of e-pilin genes in micro-organisms outside the order Desulfuromondales has only been experimentally linked to the capacity for extracellular electron transfer in a few strains. Further investigation of the physiological capabilities of other species with a focus not only on Fe(III) oxide reduction, but also on direct interspecies electron transfer is warranted.

In addition to their significance in anaerobic microbial ecology, e-pili are of interest as a sustainable bioelectronic material. The genomic analysis reported here has identified many new e-pilin genes that may have value for the development of conductive materials. Studies to screen the conductivity of these newly identified e-pili are underway.

Acknowledgements

This research was supported by grants N00014-13-1-0550 and N00014-13-1-0549 from the Office of Naval Research.

References

Adhikari, R. Y., Malvankar, N. S., Tuominen, M. T. & Lovley, D. R. (2016). Conductivity of individual *Geobacter* pili. *RSC Adv.* **6**, 8354–8357.

Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.

Ayers, M., Howell, P. L. & Burrows, L. L. (2010). Architecture of the type II secretion and type IV pilus machineries. *Future Microbiol* **5**, 1203–1218.

Bernard, C. S., Bordi, C., Termine, E., Filloux, A. & de Bentzmann, S. (2009). Organization and PprB-dependent control of the *Pseudomonas aeruginosa tad* locus, involved in Flp pilus biology. *J Bacteriol* **191**, 1961–1973.

Bieber, D., Ramer, S. W., Wu, C. Y., Murray, W. J., Tobe, T., Fernandez, R. & Schoolnik, G. K. (1998). Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. *Science* **280**, 2114–2118.

Bond, D. R. & Lovley, D. R. (2003). Electricity production by *Geobacter sulfurreducens* attached to electrodes. *Appl Environ Microbiol* **69**, 1548–1555.

Burrows, L. L. (2012). *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annu Rev Microbiol* **66**, 493–520.

Castro, L., Vera, M., Muñoz, JÁ., Blázquez, M. L., González, F., Sand, W. & Ballester, A. (2014). *Aeromonas hydrophila* produces conductive nanowires. *Res Microbiol* **165**, 794–802.

Chun, A. L. (2014). Bacterial nanowires: an extended membrane. *Nat Nanotechnol* **9**, 750.

Craig, L., Pique, M. E. & Tainer, J. A. (2004). Type IV pilus structure and bacterial pathogenicity. *Nat Rev Microbiol* **2**, 363–378.

Craig, L., Taylor, R. K., Pique, M. E., Adair, B. D., Arvai, A. S., Singh, M., Lloyd, S. J., Shin, D. S., Getzoff, E. D. & other authors (2003). Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. *Mol Cell* **11**, 1139–1150.

Craig, L., Volkmann, N., Arvai, A. S., Pique, M. E., Yeager, M., Egelman, E. H. & Tainer, J. A. (2006). Type IV pilus structure by cryo-electron microscopy and crystallography: implications for pilus assembly and functions. *Mol Cell* **23**, 651–662.

Cuff, J. A. & Barton, G. J. (2000). Application of multiple sequence alignment profiles to improve protein secondary structure prediction. *Proteins* **40**, 502–511.

Dayhoff, M. O., Schwartz, R. M. & Orcutt, B. C. (1978). A Model of Evolutionary Change in Proteins. In *Atlas of Protein Sequence and Structure*, pp. 345–352. Edited by M. O. DayhoffWashington DC: National Biomedical Research Foundation.

Drozdetskiy, A., Cole, C., Procter, J. & Barton, G. J. (2015). JPred4: a protein secondary structure prediction server. *Nucleic Acids Res* **43**, W389–394.

Eaktasang, N., Kang, C. S., Lim, H., Kwean, O. S., Cho, S., Kim, Y. & Kim, H. S. (2016). Production of electrically-conductive nanoscale filaments by sulfate-reducing bacteria in the microbial fuel cell. *Bioresour Technol* **210**.

Eddy, S. R. (2008). A probabilistic model of local sequence alignment that simplifies statistical significance estimation. *PLoS Comput Biol* **4**, e1000069.

Eddy, S. R. (2011). Accelerated profile HMM searches. *PLoS Comput Biol* **7**, e1002195.

Feliciano, G. T., Steidl, R. J. & Reguera, G. (2015). Structural and functional insights into the conductive pili of *Geobacter sulfurreducens*

revealed in molecular dynamics simulations. *Phys Chem Chem Phys* **17**, 22217–22226.

Finn, R. D., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Mistry, J., Mitchell, A. L., Potter, S. C., Punta, M., Qureshi, M. & other authors (2016). The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Res* **44**, D279–285.

Ford, B., Verger, D., Dodson, K., Volkan, E., Kostakioti, M., Elam, J., Pinkner, J., Waksman, G. & Hultgren, S. (2012). The structure of the PapD–PapGII pilin complex reveals an open and flexible P5 pocket. *J Bacteriol* **194**, 6390–6397.

Galdiero, S., Falanga, A., Cantisani, M., Tarallo, R., Della Pepa, M. E., D'Oriano, V. & Galdiero, M. (2012). Microbe–host interactions: structure and role of Gram-negative bacterial porins. *Curr Protein Pept Sci* **13**, 843–854.

Giltner, C. L., Nguyen, Y. & Burrows, L. L. (2012). Type IV pilin proteins: versatile molecular modules. *Microbiol Mol Biol Rev* **76**, 740–772.

Gorby, Y. A., Yanina, S., McLean, J. S., Rosso, K. M., Moyles, D., Dohnalkova, A., Beveridge, T. J., Chang, I. S., Kim, B. H. & other authors (2006). Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *Proc Natl Acad Sci U S A* **103**, 11358–11363.

Gorgel, M., Ulstrup, J. J., Bøggild, A., Jones, N. C., Hoffmann, S. V., Nissen, P. & Boesen, T. (2015). High-resolution structure of a type IV pilin from the metal-reducing bacterium *Shewanella oneidensis*. *BMC Struct Biol* **15**, 4.

Hofmann, K. & Stoffel, W. (1993). TMbase - A database of membrane spanning proteins segments. *Biol Chem Hoppe Seyler* **374**, 166.

Imam, S., Chen, Z., Roos, D. S. & Pohlschröder, M. (2011). Identification of surprisingly diverse type IV pili, across a broad range of Gram-positive bacteria. *PLoS One* **6**, e28919.

Kachlany, S. C., Planet, P. J., Desalle, R., Fine, D. H., Figurski, D. H. & Kaplan, J. B. (2001). *flp-1*, the first representative of a new pilin gene subfamily, is required for non-specific adherence of *Actinobacillus actinomycetemcomitans*. *Mol Microbiol* **40**, 542–554.

Katoh, K. & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**, 772–780.

Kim, S. J., Park, S. J., Jung, M. Y., Kim, J. G., Min, U. G., Hong, H. J. & Rhee, S. K. (2014). Draft genome sequence of an aromatic compound-degrading bacterium, *Desulfobacula* sp. TS, belonging to the *Deltaproteobacteria*. *FEMS Microbiol Lett* **360**, 9–12.

Krogh, A., Larsson, B., von Heijne, G. & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305**, 567–580.

Kuever, J., Könneke, M., Galushko, A. & Drzyzga, O. (2001). Reclassification of *Desulfobacterium phenolicum* as *Desulfobacula phenolica* comb. nov. and description of strain Sax^T as *Desulfotignum balticum* gen. nov., sp. nov. *Int J Syst Evol Microbiol* **51**, 171–177.

Le, S. Q. & Gascuel, O. (2008). An improved general amino acid replacement matrix. *Mol Biol Evol* **25**, 1307–1320.

Li, Y. & Li, H. (2014). Type IV pili of *Acidithiobacillus ferrooxidans* can transfer electrons from extracellular electron donors. *J Basic Microbiol* **54**, 226–231.

Liu, X., Tremblay, P. L., Malvankar, N. S., Nevin, K. P., Lovley, D. R. & Vargas, M. (2014). A *Geobacter sulfurreducens* strain expressing *Pseudomonas aeruginosa* type IV pili localizes OmcS on pili but is deficient in Fe(III) oxide reduction and current production. *Appl Environ Microbiol* **80**, 1219–1224.

Lovley, D. R. & Malvankar, N. S. (2015). Seeing is believing: novel imaging techniques help clarify microbial nanowire structure and function. *Environ Microbiol* **17**, 2209–2215.

Lovley, D. R. & Phillips, E. J. (1994). Novel processes for anaerobic sulfate production from elemental sulfur by sulfate-reducing bacteria. *Appl Environ Microbiol* **60**, 2394–2399.

Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P. & Woodward, J. C. (1996). Humic substances as electron acceptors for microbial respiration. *Nature* **382**, 445–448.

Lovley, D. R., Roden, E. E., Phillips, E. J. P. & Woodward, J. C. (1993). Enzymatic iron and uranium reduction by sulfate-reducing bacteria. *Marine Geology* **113**, 41–53.

Lovley, D. R., Ueki, T., Zhang, T., Malvankar, N. S., Shrestha, P. M., Flanangan, K. A., Aklujkar, M. A., Butler, J. E., Giloteaux, L. & other authors. (2011). Geobacter: the microbe electric's physiology, ecology, and practical applications. *Adv Micro Physiol* **59**, 1–100.

Löytynoja, A. & Goldman, N. (2005). An algorithm for progressive multiple alignment of sequences with insertions. *Proc Natl Acad Sci U S A* **102**, 10557–10562.

Malvankar, N. S. & Lovley, D. R. (2014). Microbial nanowires for bioenergy applications. *Curr Opin Biotechnol* **27**, 88–95.

Malvankar, N. S., Vargas, M., Nevin, K. P., Franks, A. E., Leang, C., Kim, B. C., Inoue, K., Mester, T., Covalla, S. F. & other authors (2011). Tunable metallic-like conductivity in microbial nanowire networks. *Nat Nanotechnol* **6**, 573–579.

Malvankar, N. S., Vargas, M., Nevin, K., Tremblay, P. L., Evans-Lutterodt, K., Nykypanchuk, D., Martz, E., Tuominen, M. T. & Lovley, D. R. (2015). Structural basis for metallic-like conductivity in microbial nanowires. *MBio* **6**, e00084.

Malvankar, N. S., Yalcin, S. E., Tuominen, M. T. & Lovley, D. R. (2014). Visualization of charge propagation along individual pili proteins using ambient electrostatic force microscopy. *Nat Nanotechnol* **9**, 1012–1017.

Marchler-Bauer, A., Derbyshire, M. K., Gonzales, N. R., Lu, S., Chitsaz, F., Geer, L. Y., Geer, R. C., He, J., Gwadz, M. & other authors (2015). CDD: NCBI's conserved domain database. *Nucleic Acids Res* **43**, D222–226.

Mattick, J. S. (2002). Type IV pili and twitching motility. *Annu Rev Microbiol* **56**, 289–314.

Mouser, P. J., N'Guessan, A. L., Elifantz, H., Holmes, D. E., Williams, K. H., Wilkins, M. J., Long, P. E. & Lovley, D. R. (2009). Influence of heterogeneous ammonium availability on bacterial community structure and the expression of nitrogen fixation and ammonium transporter genes during in situ bioremediation of uranium-contaminated groundwater. *Environ Sci Technol* **43**, 4386–4392.

Nevin, K. P. & Lovley, D. R. (2000). Potential for nonenzymatic reduction of Fe(III) via electron shuttling in subsurface sediments. *Environ. Sci. Technol* **34**, 2472–2478.

Nevin, K. P. & Lovley, D. R. (2002). Mechanisms for Fe(III) oxide reduction in sedimentary environments. *Geomicrobiol J* **19**, 141–159.

Nevin, K. P., Kim, B. C., Glaven, R. H., Johnson, J. P., Woodard, T. L., Methé, B. A., Didonato, R. J., Covalla, S. F., Franks, A. E. & other authors (2009). Anode biofilm transcriptomics reveals outer surface components essential for high density current production in *Geobacter sulfurreducens* fuel cells. *PLoS One* **4**.

Nivaskumar, M. & Francetic, O. (2014). Type II secretion system: a magic beanstalk or a protein escalator. *Biochim Biophys Acta* **1843**, 1568–1577.

Petersen, T. N., Brunak, S., von Heijne, G. & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* **8**, 785–786.

Rabus, R., Nordhaus, R., Ludwig, W. & Widdel, F. (1993). Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. *Appl Environ Microbiol* **59**, 1444–1451.

Reardon, P. N. & Mueller, K. T. (2013). Structure of the type IVa major pilin from the electrically conductive bacterial nanowires of *Geobacter sulfurreducens*. *J Biol Chem* **288**, 29260–29266.

Reguera, G., McCarthy, K. D., Mehta, T., Nicoll, J. S., Tuominen, M. T. & Lovley, D. R. (2005). Extracellular electron transfer via microbial nanowires. *Nature* **435**, 1098–1101.

Reguera, G., Nevin, K. P., Nicoll, J. S., Covalla, S. F., Woodard, T. L. & Lovley, D. R. (2006). Biofilm and nanowire production leads to increased current in *Geobacter sulfurreducens* fuel cells. *Appl Environ Microbiol* **72**, 7345–7348.

Rollefson, J. B., Stephen, C. S., Tien, M. & Bond, D. R. (2011). Identification of an extracellular polysaccharide network essential for cytochrome anchoring and biofilm formation in *Geobacter sulfurreducens*. *J Bacteriol* **193**, 1023–1033.

Rotaru, A.-E., Shrestha, P. M., Liu, F., Shrestha, M., Shrestha, D., Embree, M., Zengler, K., Wardman, C., Nevin, K. P. & Lovley, D. R. (2014). A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the reduction of carbon dioxide to methane. *Energy Environ Sci* **7**, 408–415.

Rotaru, A.-E., Woodard, T. L., Nevin, K. P. & Lovley, D. R. (2015). Link between capacity for current production and syntrophic growth in *Geobacter* species. *Front Microbiol* **6**.

Sela, I., Ashkenazy, H., Katoh, K. & Pupko, T. (2015). GUIDANCE2: accurate detection of unreliable alignment regions accounting for the uncertainty of multiple parameters. *Nucleic Acids Res* **43**, W7–W14.

Shrestha, P. M., Rotaru, A. E., Aklujkar, M., Liu, F., Shrestha, M., Summers, Z. M., Malvankar, N., Flores, D. C. & Lovley, D. R. (2013). Syntrophic growth with direct interspecies electron transfer as the primary mechanism for energy exchange. *Environ Microbiol Rep* **5**, 904–910.

Smith, J. A., Nevin, K. P. & Lovley, D. R. (2015). Syntrophic growth via quinone-mediated interspecies electron transfer. *Front Microbiol* **6** 121.

Summers, Z. M., Fogarty, H. E., Leang, C., Franks, A. E., Malvankar, N. S. & Lovley, D. R. (2010). Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. *Science* **330**, 1413–1415.

Sun, H., Zusman, D. R. & Shi, W. (2000). Type IV pilus of *Myxococcus xanthus* is a motility apparatus controlled by the *frz* chemosensory system. *Curr Biol* **10**, 1143–1146.

Szabó, Z., Stahl, A. O., Albers, S. V., Kissinger, J. C., Driessen, A. J. & Pohlschröder, M. (2007). Identification of diverse archaeal proteins with class III signal peptides cleaved by distinct archaeal prepilin peptidases. *J Bacteriol* **189**, 772–778.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585–595.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* **30**, 2725–2729.

Tan, Y., Adhikari, R. Y., Malvankar, N. S., Ward, J. E., Nevin, K. P., Woodard, T. L., Smith, J. A., Snoeyenbos-West, O. L., Franks, A. E. & other authors (2016). The Low Conductivity of Geobacter uraniireducens Pili Suggests a Diversity of Extracellular Electron Transfer Mechanisms in the Genus Geobacter. *Front Microbiol* **7**, 980.

Tomich, M., Planet, P. J. & Figurski, D. H. (2007). The tad locus: postcards from the widespread colonization island. *Nat Rev Microbiol* **5**, 363–375.

Tusnády, G. E. & Simon, I. (2001). The HMMTOP transmembrane topology prediction server. *Bioinformatics* **17**, 849–850.

Vargas, M., Malvankar, N. S., Tremblay, P. L., Leang, C., Smith, J. A., Patel, P., Snoeyenbos-West, O., Synoeyenbos-West, O., Nevin, K. P. & Lovley, D. R. (2013). Aromatic amino acids required for pili conductivity and long-range extracellular electron transport in *Geobacter sulfurreducens*. *MBio* **4**, e00105–00113.

Veazey, J. P., Reguera, G. & Tessmer, S. H. (2011). Electronic properties of conductive pili of the metal-reducing bacterium *Geobacter sulfurreducens* probed by scanning tunneling microscopy. *Physical Review E* **84**.

Venkidusamy, K., Megharaj, M., Schroder, U., Karouta, F., Mohan, S. V. & Naidu, R. (2015). Electron transport through electrically conductive nanofilaments in *Rhodopseudomonas palustris* strain RP2. *RSC Adv.* **5**, 100790–100798.

Voordeckers, J. W., Kim, B. C., Izallalen, M. & Lovley, D. R. (2010). Role of *Geobacter sulfurreducens* outer surface c-type cytochromes in reduction of soil humic acid and anthraquinone-2,6-disulfonate. *Appl Environ Microbiol* **76**, 2371–2375.

Waksman, G. & Hultgren, S. J. (2009). Structural biology of the chaperone-usher pathway of pilus biogenesis. *Nat Rev Microbiol* **7**, 765–774.

Wanger, G., Gorby, Y., El-Naggar, M. Y., Yuzvinsky, T. D., Schaudinn, C., Gorur, A. & Sedghizadeh, P. P. (2013). Electrically conductive bacterial nanowires in biphosphonate-related osteonecrosis of the jaw biofilms. *Oral and Maxillofacial Path* **115**, 71–78.

Yu, N. Y., Wagner, J. R., Laird, M. R., Melli, G., Rey, S., Lo, R., Dao, P., Sahinalp, S. C., Ester, M. & other authors (2010). PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* **26**, 1608–1615.

Zapun, A., Missiakas, D., Raina, S. & Creighton, T. E. (1995). Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli*. *Biochemistry* **34**, 5075–5089.

Zhuang, K., Izallalen, M., Mouser, P., Richter, H., Risso, C., Mahadevan, R. & Lovley, D. R. (2011). Genome-scale dynamic modeling of the competition between *Rhodoferax* and *Geobacter* in anoxic subsurface environments. *ISME J* **5**, 305–316.

Data Bibliography

1. Holmes, Dang, Walker, and Lovley, FigShare https://figshare.com/s/87e875d0c5c97c2e5498 (2016).