Title: Geobacter sulfurreducens Strain Expressing Pseudomonas aeruginosa Type IV Pili Localizes OmcS on Pili but is Deficient in Fe(III) Oxide Reduction and Current Production

Running title: Geobacter expression of Pseudomonas Pili

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Abstract

The conductive pili of Geobacter species play an important role in electron transfer to Fe(III) oxides, in long-range electron transport through current-producing biofilms, and in direct interspecies electron transfer. Although multiple lines of evidence have indicated that the pili of Geobacter sulfurreducens have a metal-like conductivity, independent of the presence of c-type cytochromes, this claim is still controversial. In order to further investigate this phenomenon, a strain of G. sulfurreducens, designated strain PA, was constructed in which the gene for the native PilA, the structural pilin protein, was replaced with the PilA gene of Pseudomonas aeruginosa PAO1. Strain PA expressed and properly assembled P. aeruginosa PilA subunits into pili and exhibited a profile of outer surface c-type cytochromes similar to that of a control strain expressing the G. sulfurreducens PilA. Surprisingly, the strain PA pili were decorated with the c-type cytochrome OmcS in a manner similar to the control strain. However, the strain PA pili were 14-fold less conductive than the pili of the control strain and strain PA was severely impaired in Fe(III) oxide reduction and current production. These results demonstrate that the presence of OmcS on pili is not sufficient to confer conductivity to pili and suggest that there are unique structural features of the G. sulfurreducens PilA that are necessary for conductivity.

Introduction

The capacity of Geobacter species for extracellular electron transfer allows them to play an important role in processes such as metal reduction in soils and sediments, bioremediation of organic and metal contaminants in the subsurface, the conversion of organic wastes to electricity, and interspecies electron transfer to promote methane production (1). Geobacter sulfurreducens, and presumably other Geobacter species, produce electrically conductive pili that have been...
proposed to serve as conduit for extracellular electron transfer (2,3), but the mechanisms for
electron transfer along *G. sulfurreducens* pili is a matter of considerable debate (4-6).
Laboratories that have experimentally investigated *G. sulfurreducens* pili have concluded that
electron transport along the pili is an intrinsic function of the pili itself and does not involve
electron hopping/tunneling between traditional electron transfer proteins, such as cytochromes
(2,3,7,8), even though the c-type cytochrome OmcS is associated with the pili (9,10). This
contrasts to the proposed cytochrome-to-cytochrome electron hopping/tunneling along
conductive filaments of *Shewanella oneidensis* (11,12).

A potential explanation for cytochrome-independent electron transport along *G.
sulfurreducens* pili is that the pili possess metal-like conductivity, similar to that observed in
carbon nanotubes and synthetic organic conducting polymers (2). Initial evidence for metal-like
conductivity in pili preparations included: 1) temperature dependence of conductivity
characteristic of metal-like conductivity and inconsistent with electron hopping/tunneling (2); 2)
pH dependence of conductivity characteristic of metal-like conductivity (2); 3) no impact of
denaturing cytochromes on conductivity (2); and 4) spacing of cytochromes on the pili too great
for electron hopping/tunneling (9,10).

The metal-like conductivity was suggested to be due to overlapping pi-pi orbitals of
aromatic amino acids in the carboxy terminus of PilA (2), which modeling studies suggest are
exposed on the outer surface of the pili (13). Replacing the gene for PilA, the structural pilin
protein, with a gene encoding a PilA in which an alanine was substituted for each of five
aromatic amino acids in the carboxyl terminus of PilA, resulted in a strain, designated Aro5,
which produced pili with properly localized OmcS, but with greatly diminished conductivity (7).
The Aro5 strain was unable to effectively reduce Fe(III) oxide or produce high current densities.
Electrostatic force microscopy studies revealed that charge injected into native individual wild-

type pili, still attached to cells, propagated along the pili in a manner comparable to that observed

in carbon nanotubes, even in regions in which no cytochromes were present (N.S. Malvankar et

al., submitted for publication). But there was no such charge propagation in the Aro5 strain.

We considered that an alternative approach to the study of pili function might be to

evaluate the phenotype when pili from other bacteria that are not effective in extracellular

electron transfer, but possess other important pili functions such as attachment, biofilm formation,

and motility (14,15), were expressed in *G. sulfurreducens*. Type IV pili from *Bacteroides

_nodosus* (16), *Moraxella bovis* (17) and *Neisseria gonorrhoeae* (18) were correctly assembled

when the appropriate genes were expressed in *Pseudomonas aeruginosa*. However, in those

instances all the heterologous type IV pili had a structure similar to the *P. aeruginosa* type IV

pili whereas the PilA sequence of *G. sulfurreducens* differs significantly from the PilA of many

bacteria (Figure 1). Most intensively studied PilA have a similar structure with a highly

conserved hydrophobic $\alpha$-helical N-terminal region involved in pilin maturation and assembly

and a globular domain in the carboxy terminus (14). However, after processing of the prepilin by

PilD, the *G. sulfurreducens* PilA is much smaller (61 amino acids) than the processed PilAs of

previously studied gram-negative bacteria (140-161 amino acids), retaining the highly conserved

$\alpha$-helical N-terminal region but not the C-terminal globular domain, which is replaced by a small

random-coiled segment (8). It has been suggested that these differences in sequence (Figure 1)

are a likely explanation for the previously described lack of conductivity of natively expressed *P.

*aeruginosa* pili (3).

Here we report that despite its much larger size and structural differences, the PilA of *P.

*aeruginosa* was not only expressed in *G. sulfurreducens*, but also assembled into pili.
Surprisingly, OmcS attached to these heterologous pili. However, the pili had low conductivity and the strain expressing these pili was deficient in long-range extracellular electron transfer.

**Materials and Methods**

**Bacterial strains, plasmids and culture conditions**

All bacterial strains and plasmids used in this study are summarized in Table S1 in the supplemental material. The *G. sulfurreducens* strains were routinely cultured at 30 °C under strict anaerobic conditions (80/20 N\textsubscript{2}-CO\textsubscript{2}) in mineral based media containing acetate (20 mM) as the electron donor and either Fe(III) citrate (56 mM), fumarate (40 mM), or Fe(III) oxide (100 mM) as electron acceptor, as previously described (19). Fe(II) was determined with the ferrozine assay (20).

Chemically competent *E.coli* TOP10 (Invitrogen, Grand Island, NY, USA) was used routinely for cloning and cultured at 37°C in Luria-Bertani medium with the appropriate antibiotic.

**Construction of strain PA**

Strain PA was constructed from *Geobacter sulfurreducens* strain DL1. Primers used for construction of strain PA and relevant genotypes are listed in Table S2 in the supplemental material. To construct strain PA, three DNA fragments were generated independently by polymerase chain reaction (PCR). Primer pairs GspilAf/GspilAr and GspilACf/GspilACr amplified the 219 bp upstream and 500 bp downstream of the *pilA* gene (locus tag GSU1496), respectively, using *G. sulfurreducens* DL1 genomic DNA as template. Primer pairs PapilAf/PapilAr amplified the *Pseudomonas aeruginosa* PAO1 *pilA* gene (locus tag PA4525)
from *P. aeruginosa* genomic DNA. The three PCR products were combined via recombinant PCR with primer pairs GspilAf/GspilACr as previously described (21). After the sequence was verified, the recombinant PCR product was digested with XhoI and ligated with the vector pPLT173 using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). pPLT173 contains 500 bp upstream of the *Geobacter* pilA gene followed by a gentamicin resistance cassette and an XhoI restriction site. The final plasmid was linearized with Neol (NEB) and electroporated into DL1 competent cells as previously described (19). Transformants were selected on NBAF agar (19) plates containing gentamicin (20 ug/ml) and were verified by PCR using the primers listed in Table S1. Genomic DNA was extracted using the Epicentre MasterPure DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). Plasmids isolation and gel extraction were performed by using QIAprep Spin miniprep Kit and QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) respectively, following manufacturer’s instructions. The high-fidelity JumpStart™ AccuTaq™ LA DNA Polymerase (Sigma-Aldrich, St-Louis, MO, USA) was used routinely for PCR reactions. When needed, the PCR products were cloned into vector pCR2.1-TOPO with TOPO TA Cloning Kit (Invitrogen). The correct sequence was selected after Sanger sequencing verification.

**Cell attachment test**

*G. sulfurreducens* strains were cultured anaerobically in NBAF medium (19), which contains acetate as the electron donor and fumarate as the electron acceptor, and incubated at 25 °C for 2 days after they reached the stationary phase. Planktonic cells were decanted and the culture tubes were rinsed twice with Milli-Q water. After decanting the water, 0.1% crystal violet was added to stain the attached cells for 5 min. The stained cells were washed three times.
with Milli-Q water and then destained with 100% ethanol. The absorbance of the chromatic eluate was measured at 580 nm with a Genesys 2 spectrophotometer (Spectronic Instruments).

**Western blots and heme-staining analyses**

Cell lysates were prepared from late-log-phase cells with B-PER Protein Extraction Reagents (Thermo Scientific, Waltham, MA, USA) and outer membrane proteins were mechanically sheared from all the strains and isolated as previously described (22). Protein concentrations were measured with the bicinchoninic acid (BCA) assay (Micro BCA Protein Assay Kit, Thermo Scientific). Equal amounts of protein were separated by SDS-PAGE using 12.5% Tris-Tricine polyacrylamide gel (Amresco, Solon, OH, USA). For heme-staining, reducing reagent was omitted from the SDS loading buffer (23). After electrophoresis, protein bands were semi-dry transferred onto polyvinylidene difluoride (PVDF) membrane (Biorad, Hercules, CA, USA). The blotting membrane was treated with anti-PAO1 PilA rabbit polyclonal antibody (kindly provided by Dr. Lori Burrows) and anti-rabbit secondary antibody tagged with horseradish peroxidase (GE Healthcare, Piscataway, NJ, USA) sequentially as described previously (24). The PilA band was visualized by chemiluminescence (SuperSignal West Pico Kit, Thermo Scientific). C-type cytochromes were heme stained using N,N,N',N'-tetramethylbenzidine, as described previously (23).

**Immunogold labeling and Transmission Electron Microscopy (TEM)**

The presence of *P. aeruginosa* PilA pili was verified with immunogold labeling as previously described (9). Briefly, cultures were grown anaerobically in NBAF medium at 25 °C. Late-log-phase cells were applied on 400-mesh carbon-coated copper grids for 5 min. The grids
were blocked for 30 minutes in 1X phosphate-buffered saline (PBS, pH 7.0) containing 3% bovine serum albumin (BSA), incubated with anti-PAO1 PilA rabbit antibody at a 1:10 dilution for 2 hours and rinsed three times in 1X PBS. The samples were labelled with 10-nm gold conjugated anti-rabbit antibody at a 1:100 dilution (Sigma-Aldrich, Saint Louis, MO, USA) for 1 hour at room temperature, washed three times in 1X PBS and negatively stained with 2% uranyl acetate. Samples were examined using a Tecnai 12 transmission electron microscope operated at 100kV and images were taken with a Teitz TCL camera. The c-type cytochrome OmcS was localized with the same procedure, but substituting the primary antibody with anti-OmcS rabbit polyclonal antibody at a 1:50 dilution.

**Current production**

The capacity for current production was evaluated in dual-chamber microbial fuel cells with 10 mM acetate as electron donor and graphite electrodes poised at +300 mV (Ag/AgCl) as electron acceptor as previously described (25). Once current production began, the anode chamber was switched into flow-through mode at a dilution rate of 0.15/h. Current production was recorded with a Power Lab 4SP and plotted with Chart 5.0 software (ADI instruments, Mountain View, CA).

**Confocal microscopy**

Anode biofilms were imaged with confocal laser scanning microscopy using the LIVE/DEAD BacLight viability stain kit from Molecular Probes (Eugene, OR, USA) as previously described (26,27). Images were processed and analyzed using the Leica LAS software (Leica).
Pili conductivity measurements

The conductivity of pili was determined as previously described (2). Briefly, cells were grown anaerobically in NBAF medium at 25 °C to late-log-phase. Pili were sheared from cells by vortexing at high speed for two minutes in 150 mM ethanolamine buffer (pH 10.5). Cells and debris were separated by centrifugation (Sorvall RC 6 Plus, Thermo Scientific) at 17,000 x g for 30 minutes. The suspended pili were concentrated by ultracentrifugation (Beckman L8-70M) at 100,000 x g for 3 hours. TEM imaging as previously described (2,7) confirmed the presence of abundant pili in the preparations.

The pili preparations (5 µg protein) were placed on split-gold electrodes (2), and air-dried overnight in a desiccator, which previous studies (2,3) demonstrated settles pili on the electrodes to form a network, while maintaining a thin water layer on the pili. A voltage ramp of 0-0.05 V was applied across split electrodes in steps of 0.025 V with a source meter (Keithley 2400). After allowing the exponential decay of the transient ionic current, the steady-state electronic current for each voltage was measured every second over a minimum period of 100 seconds with a Labview data acquisition program (National Instruments). Time-averaged current for each applied voltage was calculated to create the current-voltage (I-V) characteristics and to measure conductance ($G$) which is given by the relation $G = I / V$. As previously described (2,7), to calculate, pili conductivity was calculated from measured conductance ($G$) with the formula:

$$\sigma = G \frac{2a}{gL}$$

where $L$ is the length of the electrodes ($L = 2.54 \text{ cm}$); $a$ is the half-spacing between the electrodes ($2a = 50 \mu \text{m}$) and $g$ is the film thickness ($\sim 5 \mu \text{m}$) measured using confocal microscopy.
Results and Discussion

Expression and assembly of *P. aeruginosa* pili in *G. sulfurreducens*

*G. sulfurreducens* strain PA was generated (Figure S1) by deleting the native PilA (Gs-PilA) gene and replacing it on the chromosome with the gene for the PilA of *P. aeruginosa* PAO1 (Pa-PilA). Cell-free lysates of strain PA contained the Pa-PilA monomer (Figure 2A). As expected, no Pa-PilA was detected in the control strain, which, as previously described (7), was constructed in the same manner as strain PA, but retained the PilA sequence of *G. sulfurreducens*.

Transmission electron microscopy coupled with immunogold labeling with antibodies raised against Pa-PilA demonstrated the assembly of mature Pa-PilA pili (Figure 2B-C). Thus, despite significant differences in the size and structure of the mature Pa-PilA and Gs-PilA (Figure 1), Pa-PilA was expressed and properly assembled into pili in *G. sulfurreducens*.

Furthermore, strain PA was highly effective in attachment. The $A_{580}$ of crystal violet that bound to late stationary phase strain PA cells that attached to culture tubes was $6.2 \pm 0.33$ (mean $\pm$ standard deviation; $n=3$) whereas the value for the control strain was $0.6 \pm 0.001$. The ten-fold higher attached biomass of strain PA might be attributed to the larger globular domain of the Pa-PilA pili because the globular domain of type-IV pili are thought to be responsible for pili-mediated cell attachment to surfaces (8,28).

Outer membrane cytochromes profile and alignment of OmcS along Pa-PilA pili

Outer-surface preparations stained for heme revealed similar distributions of $c$-type cytochromes in strain PA and the control strain, with slightly higher intensity staining for some bands in the preparation from strain PA (Figure S2). These included OmcZ, a multi-heme $c$-type...
cytochrome associated with the outer surface of the cell, that is required for optimal current production, but not Fe(III) oxide reduction (25,29). The multi-heme c-type cytochrome, OmcS (30), which is required for Fe(III) oxide reduction (22), was also abundant in strain PA (Figure S2). Immunogold labeling revealed that OmcS was localized along the pili of strain PA (Figure 3A-B), in a manner similar to that previously described for the specific localization of OmcS along the wild-type pili of strain DL1 (9). An association between OmcS and Pa-PilA-pili is surprising because P. aeruginosa does not produce OmcS. One possibility is that OmcS specifically interacts with PilA in the conserved N terminus region, which G. sulfurreducens and P. aeruginosa share. Another possibility is that the highly hydrophobic nature of OmcS (30) leads to a rather non-specific association with hydrophobic regions of the pili.

Long-range electron transfer

Strain PA reduced soluble Fe(III) citrate as well as the control strain (Figure 4A), demonstrating that it had the ability to transfer electrons to the outer cell surface. However, strain PA could not effectively reduce Fe(III) oxide (Figure 4B). The inability to reduce Fe(III) oxides persisted even after prolonged incubation of more than 100 days (data not shown).

Strain PA grew as a biofilm on graphite anodes (Figure 5A) but was deficient in current production compared with the current production of the previously described (7) control strain (Figure 5B). There was a long lag period before strain PA produced current, and current production plateaued at levels much lower than those of the control strain. Even after the extended incubation necessary to observe strain PA current production, the strain PA biofilms (Figure 5A) were much thinner than the ca. 30 µm thick biofilms previously reported (7) for the control strain.
The diminished current production and Fe(III) oxide reduction of strain PA could be attributed to the fact that the *P. aeruginosa* pili had low conductivity. The conductivity of pili preparations sheared from strain PA were 14-fold lower (0.45 ± 0.07 µS/cm; mean ± standard deviation, n=3) than those of the control strain (6.49 ± 3.56 µS/cm), which is consistent with previous studies which suggested that the biofilms and pili of *P. aeruginosa* have poor conductivity (2,3).

**Implications**

The results demonstrate that the structure of PilA is crucial to pili conductivity and the long-range electron transport capabilities of *G. sulfurreducens*. Even though strain PA produced abundant pili with OmcS attached, the pili had low conductivity, and the culture had diminished capacity for reduction of Fe(III) oxides and current production. These results are in accordance with the concept that OmcS does not confer pili conductivity and that pili themselves must have an intrinsic conductivity for electron transfer to Fe(III) oxide and long-range electron transport through current-producing biofilms (31,32). It is speculated that the OmcS localized on pili are required for Fe(III) oxide reduction because the multiple hemes of OmcS facilitate electron transfer from pili to Fe(III) oxides (31,32).

There is not yet sufficient understanding of the structural features of *G. sulfurreducens* pili that are responsible for conductivity to definitively state why the pili of *P. aeruginosa* PAO1 are not conductive. However, of the five aromatic amino acids implicated in contributing to metal-like conductivity of *G. sulfurreducens* pili (7), the PilA of *P. aeruginosa* PAO1 lacks aromatic amino acids 3, 4 and 5 and aromatic amino acid 1 is a tyrosine instead of a
phenylalanine (Figure 1). Furthermore, the large globular domain in the C-terminus of *P. aeruginosa* PAO1 PilA may result in structural differences that prevent conductivity.

The ability of *G. sulfurreducens* to assemble the structurally-divergent type-IV pili of *P. aeruginosa* PAO1 suggests that expression of PilAs of different structure in *G. sulfurreducens* may be a productive approach for screening the pili of other organisms for their capacity for long-range electron transport. Such studies are underway.

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References


reveals outer surface components essential for high density current production in *Geobacter sulfurreducens* fuel cells. PLOS ONE 4:e5628.


**Figure legends**

374 Figure 1. Alignment of mature PilA of *Neisseria gonorrhoeae* MS11, *Moraxella bovis* Epp63, *Pseudomonas aeruginosa* PAO1, *Bacteroides nodosus* H1, and *Geobacter sulfurreducens* DL1. All the sequences start from the PilD cleavage site. Aromatic amino acids of *G. sulfurreducens* PilA essential for pili conductivity are highlighted in red or yellow. The conserved sequences are underlined. The alignment was made with Clustalw2.

381 Figure 2. Expression and assembly of *P. aeruginosa* PilA-pili in *G. sulfurreducens*. (A) Western blot analysis of cell-free lysates of wild-type *P. aeruginosa* PAO1, *G. sulfurreducens* control strain, and strain PA, in which the gene for the wild-type PilA was replaced with the gene for *P. aeruginosa* PilA (B, C) Transmission electron micrographs of negatively stained strain PA, treated with anti-PAO1 PilA rabbit polyclonal antibodies followed by anti-rabbit 10-nm gold
conjugated secondary antibody. Scale bars represent 100 nm (B) and 50 nm (C). The images in B and C are representative of multiple samples.

Figure 3. Transmission electron micrographs of negatively stained cells successively incubated with anti-OmcS rabbit polyclonal antibodies and anti-rabbit IgG conjugated with 10-nm-gold-labeled secondary antibody. (A, B) strain PA. (C) *pilA*-deficient strain of *G. sulfurreducens*. Scale bar represents 100 nM. Images are representative of multiple samples.

Figure 4. Rates of Fe(II) production from the reduction of Fe(III) citrate (A) or Fe(III) oxide (B) by strain PA and the control strain. Results are the means and standard deviations from triplicate cultures.

Figure 5. Current production and anode biofilm formation. (A) Confocal scanning fluorescent microscope image of strain PA biofilm grown on an anode for 38 days and stained with Live/Dead stain. Scale bar is 25 microns. (B) Current production of strain PA and control strain. Results are representative of triplicate replicates.