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Alignment of the *c*-Type Cytochrome OmcS along Pili of *Geobacter sulfurreducens*▽†

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Immunogold localization revealed that OmcS, a cytochrome that is required for Fe(III) oxide reduction by *Geobacter sulfurreducens*, was localized along the pili. The apparent spacing between OmcS molecules suggests that OmcS facilitates electron transfer from pili to Fe(III) oxides rather than promoting electron conduction along the length of the pili.

There are multiple competing/complementary models for extracellular electron transfer in Fe(III)- and electrode-reducing microorganisms (8, 18, 20, 44). Which mechanisms prevail in different microorganisms or environmental conditions may greatly influence which microorganisms compete most successfully in sedimentary environments or on the surfaces of electrodes and can impact practical decisions on the best strategies to promote Fe(III) reduction for bioremediation applications (18, 19) or to enhance the power output of microbial fuel cells (18, 21).

The three most commonly considered mechanisms for electron transfer to extracellular electron acceptors are (i) direct contact between redox-active proteins on the outer surfaces of the cells and the electron acceptor, (ii) electron transfer via soluble electron shuttling molecules, and (iii) the conduction of electrons along pili or other filamentous structures. Evidence for the first mechanism includes the necessity for direct cell-Fe(III) oxide contact in *Geobacter* species (34) and the finding that intensively studied Fe(III)- and electrode-reducing microorganisms, such as *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1, display redox-active proteins on their outer cell surfaces that could have access to extracellular electron acceptors (1, 2, 12, 15, 27, 28, 31–33). Deletion of the genes for these proteins often inhibits Fe(III) reduction (1, 4, 7, 15, 17, 28, 40) and electron transfer to electrodes (5, 7, 11, 33). In some instances, these proteins have been purified and shown to have the capacity to reduce Fe(III) and other potential electron acceptors *in vitro* (10, 13, 29, 38, 42, 43, 48, 49).

Evidence for the second mechanism includes the ability of some microorganisms to reduce Fe(III) that they cannot directly contact, which can be associated with the accumulation of soluble substances that can promote electron shuttling (17, 22, 26, 35, 36, 47). In microbial fuel cell studies, an abundance of planktonic cells and/or the loss of current-producing capacity when the medium is replaced is consistent with the presence of an electron shuttle (3, 14, 26). Furthermore, a soluble electron shuttle is the most likely explanation for the electrochemical signatures of some microorganisms growing on an electrode surface (26, 46).

Evidence for the third mechanism is more circumstantial (19). Filaments that have conductive properties have been identified in *Shewanella* (7) and *Geobacter* (41) species. To date, conductance has been measured only across the diameter of the filaments, not along the length. The evidence that the conductive filaments were involved in extracellular electron transfer in *Shewanella* was the finding that deletion of the genes for the *c*-type cytochromes OmcA and MtrC, which are necessary for extracellular electron transfer, resulted in nonconductive filaments, suggesting that the cytochromes were associated with the filaments (7). However, subsequent studies specifically designed to localize these cytochromes revealed that, although the cytochromes were extracellular, they were attached to the cells or in the exopolymeric matrix and not aligned along the pili (24, 25, 30, 40, 43). Subsequent reviews of electron transfer to Fe(III) in *Shewanella oneidensis* (44, 45) appear to have dropped the nanowire concept and focused on the first and second mechanisms.

Geobacter sulfurreducens has a number of *c*-type cytochromes (15, 28) and multicopper proteins (12, 27) that have been demonstrated or proposed to be on the outer cell surface and are essential for extracellular electron transfer. Immunolocalization and proteolysis studies demonstrated that the cytochrome OmcB, which is essential for optimal Fe(III) reduction (15) and highly expressed during growth on electrodes (33), is embedded in the outer membrane (39), whereas the multicopper protein OmpB, which is also required for Fe(III) oxide reduction (27), is exposed on the outer cell surface (39).

OmcS is one of the most abundant cytochromes that can readily be sheared from the outer surfaces of *G. sulfurreducens* cells (28). It is essential for the reduction of Fe(III) oxide (28) and for electron transfer to electrodes under some conditions (11). Therefore, the localization of this important protein was further investigated.

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OmcS antibodies. Polyclonal OmcS antibodies were raised against the purified OmcS protein (38) in rabbits (New England Peptide, Gardner, MA). The third-bleed crude antiserum was incubated with membrane-blotted OmcS (100 μg) and washed with TBST buffer (20 mM Tris-HCl [pH 7.5], 0.5 M NaCl, and 0.05% Tween 20). The OmcS antibodies were eluted with 0.1 M glycine-HCl (pH 2.7) buffer and then neutralized with 1 M Tris-HCl (pH 7.5). The purified OmcS antibodies were tested for specificity using Western blot analysis (One-Step Western complete kit with tetramethyl benzidine [TMB]; GenScript) of total proteins prepared from cell lysates of the *G. sulfurreducens* wild type and the mutant strain (28) in which the gene encoding OmcS had been deleted. Total proteins (10 μg) were separated by 10% SDS-PAGE, blotted onto membranes using a semidry transfer cell (Bio-Rad), and incubated with the purified OmcS antibodies. Only one band with a molecular mass corresponding to that of the OmcS protein (i.e., 50 kDa) was detected with Western blot analysis in the wild-type cell lysate, whereas no bands were detected in the cell lysate from the OmcS deletion mutant (data not shown).

Localization of OmcS. In order to localize OmcS, ca. 20 μl of culture was placed on a 400-mesh carbon-coated copper grid and incubated for 5 min. The grids were floated upside down in 1× phosphate-buffered saline (PBS) containing purified OmcS antibodies (diluted 1:50 in PBS with 0.3% bovine serum albumin [BSA]) for an hour at room temperature, washed three times in 1× PBS, and then incubated for 1 h with anti-rabbit IgG conjugated with 10-nm-gold-labeled secondary antibody (Sigma) in PBS with 0.3% BSA. Samples were stained with 2% uranyl acetate and were observed using a JEOL 100 transmission electron microscope at an accelerating voltage of 80 kV. Images were taken digitally using the MaxIm-DL software and analyzed using ImageJ (http://rsbweb.nih.gov/ij/index.html).

During early to mid-log growth in medium containing acetate (15 mM) as the electron donor and fumarate (20 mM) as the electron acceptor, OmcS was in low abundance and localized primarily on the outer surfaces of the cells (Fig. 1a). However, in cultures from late log or stationary phase in the same medium, the gold-labeled antibodies appeared as strands emanating from the cells (Fig. 1b and c and see Fig. SA1 in the supplemental material). At a higher magnification, it was apparent that OmcS was associated with filaments with the same diameter and morphology as those reported (41) for the conductive pili of *G. sulfurreducens* (Fig. 1c). Of 100 randomly sampled cells, 26 had one or more filaments with extensive OmcS coverage. When a strain of *G. sulfurreducens* in which the gene for OmcS had been deleted was examined in the same manner, there was no gold labeling associated with the filaments during mid-log growth (Fig. 1d) or during stationary-phase growth (Fig. 1e). By comparing the wild-type mid-log (Fig. 1a)/stationary phases (Fig. 1b and Fig. SA1) to the OmcS deletion mutant mid-log (Fig. 1d)/stationary phases (Fig. 1e), it was apparent that the labeling increased over time in the wild-type cells, whereas the labeling remained minimal for the mutant cells over time. This suggested that there was a specific association of OmcS with filaments in the wild-type strain.

In order to localize OmcS during growth on Fe(III) oxide, cells were grown with acetate (20 mM) and poorly crystalline Fe(III) oxide (100 mmol/liter) as previously described (23). The abundant Fe(III) oxide particles occluded the antibody-labeled OmcS and filaments. Therefore, the culture was fixed anaerobically with paraformaldehyde (2% final concentration) and glutaraldehyde (0.5%) at room temperature for an hour and then treated with oxalate solution (ammonium oxalate, 28 g/liter; oxalic acid, 15 g/liter) in order to dissolve the Fe(III) oxide (23). Cells were concentrated by centrifugation (2,300 × *g*, 5 min) and then placed on the grids to carry out immunogold labeling with anti-OmcS antibodies as described above. OmcS was abundant and associated with filaments in these cultures (Fig. 2 and see Fig. SA2 in the supplemental material). Over half (54) of the 100 randomly selected cells had one or more OmcS-adorned filaments. Analysis of the abundance of gold particles on individual pili (see Fig. SA3 in the supplemental material) revealed that the average distance between two gold particles was 28.6 ± 10.5 nm.

Implications. The results demonstrate that the *c*-type cytochrome OmcS was associated with filaments which had the same diameter and morphology as the previously reported (41) conductive pili of *G. sulfurreducens*. The concept of cytochromes aligning with conductive filaments of *Shewanella oneidensis* was previously proposed but not directly demonstrated (7), and as detailed above, subsequent studies observed that the cytochromes that were proposed to be associated with *Shewanella* nanowires were not on filaments but were in other extracellular locations (24, 25, 30, 40, 43).

Although OmcS was associated with the pili of *G. sulfurreducens*, the apparent spacing between the individual OmcS molecules was much greater than the ca. 2 nm or less that is typically considered necessary for effective electron transfer between cytochromes (16, 37). It is conceivable that steric hindrances (9) or artifacts generated in the sample preparation failed to reveal an actual closer association of the cytochromes or that OmcS is interspersed along the pili with one or more of the other redox-active proteins that are on the outer surfaces of *G. sulfurreducens* cells (27, 28, 33). If so, then OmcS, or a combination of OmcS and other redox-active proteins, could aid in electron transfer along the length of the pili. However, with the data presently available, a more likely explanation is that OmcS facilitates electron transfer from the pili to Fe(III) oxides. OmcS effectively reduces Fe(III) oxide *in vitro*, and the multiple hemes and low redox potential of OmcS (38) may be ideally suited to overcome kinetic barriers to direct electron transfer from the pili to Fe(III) oxides. In this model, electrons are conducted along the pili as the result of the intrinsic conductive properties of the pili, but OmcS is required for electron transfer from the pili to Fe(III) oxides.

In addition to serving as mediators of electron transfer, abundant, multiheme cytochromes, such as OmcS, may function as capacitors, accepting and temporarily storing electrons, when cells are transitioning between Fe(III) oxide sources (6). The display of capacitor cytochromes on filaments would facilitate the rapid discharge of electrons when contact is established with fresh Fe(III) oxide sources or other extracellular electron acceptors.

These studies emphasize that the distribution of outer surface proteins and their interactions are poorly understood in *Geobacter* species. Further investigations are warranted if the

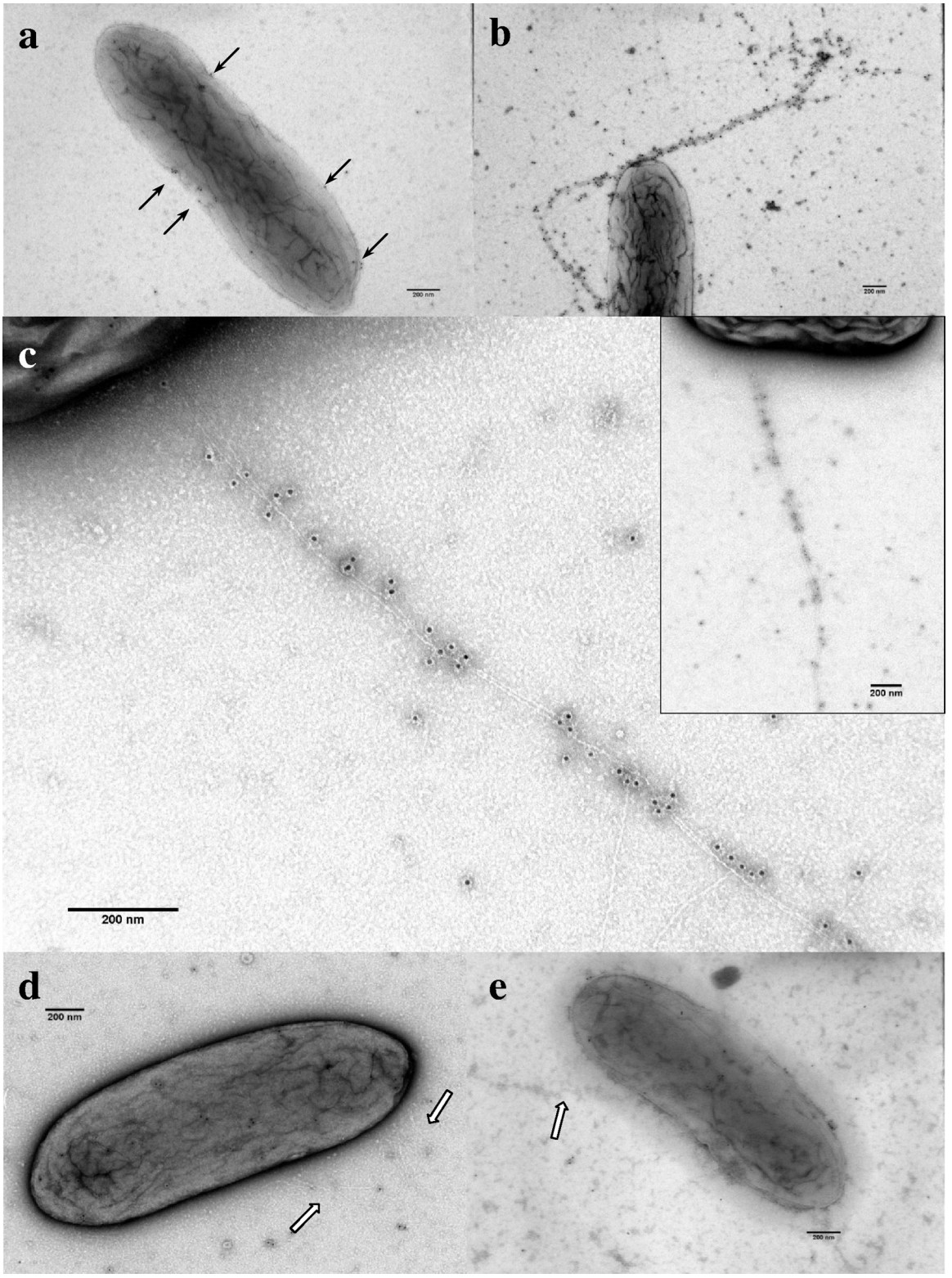

FIG. 1. Transmission electron micrographs of negatively stained *G. sulfurreducens* cells grown in medium with fumarate as the electron acceptor and then successively labeled with anti-OmcS rabbit polyclonal antibodies and anti-rabbit IgG conjugated with 10-nm-gold-labeled secondary antibody. (a) Mid-log-phase cell with arrows indicating the cell surface localization of gold particles associated with OmcS. (b) Late-log-phase cell with filaments labeled with gold particles. (c) A higher magnification of filaments from the late-log-phase cell is shown in the inset. (d and e) Lack of filament labeling in a mid-log-phase (d) and stationary-phase (e) cell of the OmcS deletion mutant. Arrows indicate unlabeled filaments.

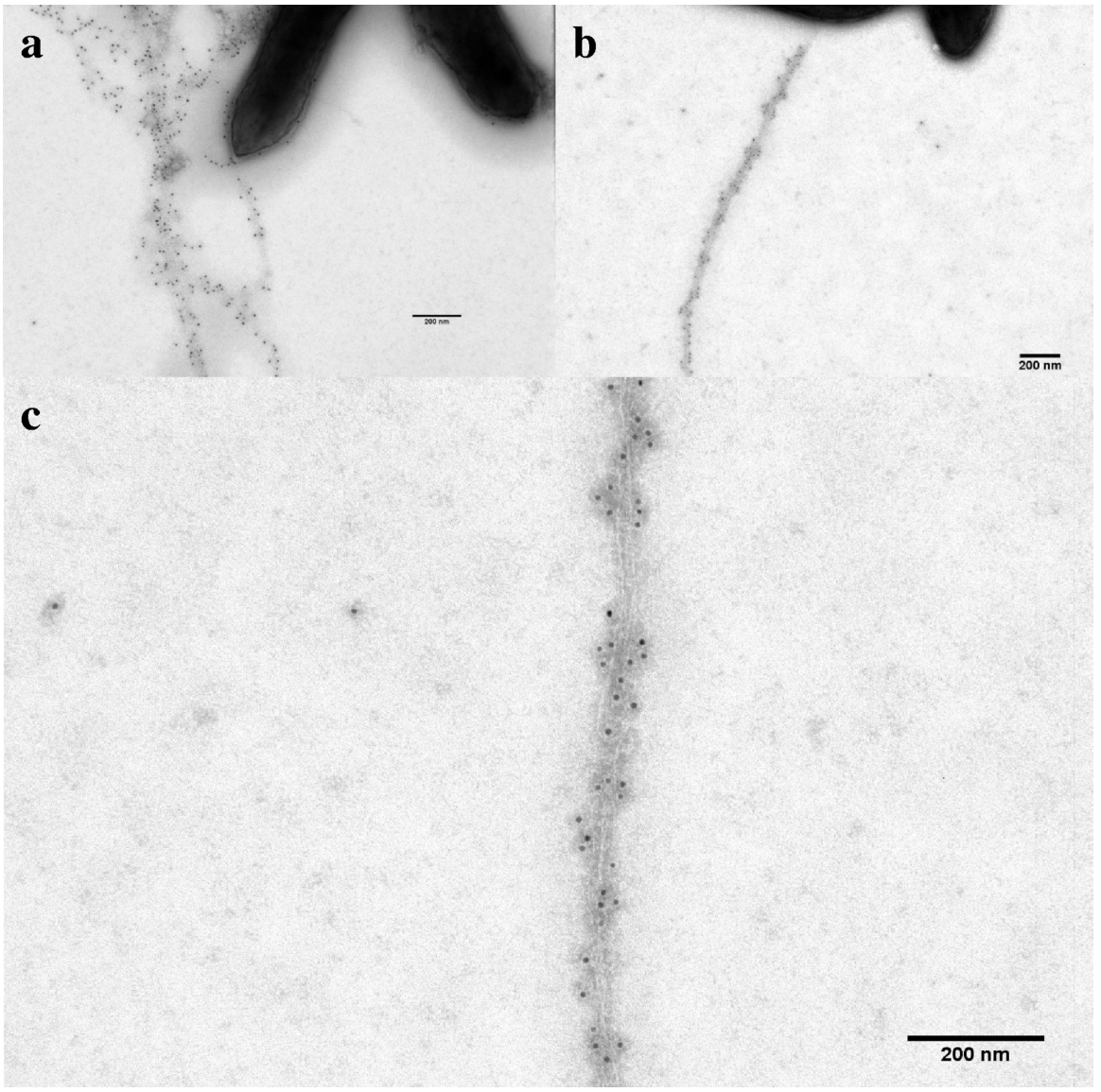

FIG. 2. Transmission electron micrographs of negatively stained *G. sulfurreducens* grown in medium with Fe(III) oxide as the electron acceptor, labeled with anti-OmcS rabbit polyclonal antibodies and with anti-rabbit IgG conjugated with 10-nm-gold-labeled secondary antibody. (a and b) Mid-log-phase cells; (c) higher magnification of the same OmcS-labeled filaments shown in panel b.

full potential of these organisms in practical applications, such as bioremediation and conversion of organic compounds to electricity, are to be realized.

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