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Possible Nonconductive Role of *Geobacter sulfurreducens* Pilus Nanowires in Biofilm Formation▽

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Geobacter sulfurreducens **required expression of electrically conductive pili to form biofilms on Fe(III) oxide surfaces, but pili were also essential for biofilm development on plain glass when fumarate was the sole electron acceptor. Furthermore, pili were needed for cell aggregation in agglutination studies. These results suggest that the pili of *G. sulfurreducens* also have a structural role in biofilm formation.**

One of the hallmarks of *Geobacter* species is their ability to conserve energy from the transfer of electrons to a variety of extracellular electron acceptors, such as metals [Fe(III), Mn(IV), and U(VI)], humic acids, and electrodes (5, 6). Establishing an electrical connection with an extracellular electron acceptor poses challenges not faced by microorganisms that reduce soluble electron acceptors within the cell. In contrast to other Fe(III) oxide-reducing bacteria (4, 9–11), such as *Shewanella* and *Geothrix* species, *Geobacter* species do not excrete electron shuttles (8) and require direct contact with the electron-accepting surface (1, 10). Previous studies (14) have demonstrated that the pili of *Geobacter sulfurreducens* are conductive and that expression of pili is required for growth on Fe(III) oxides. These "microbial nanowires" are not required for attachment to the insoluble electron acceptor; rather, they function as electronic conduits to transfer electrons to the Fe(III) oxides, extending the electron transfer capabilities of the cell well beyond the outer surface (14). Pilus "nanowires" also serve as electric conduits to mediate long-range electron transfer across multilayer biofilms formed on anode electrodes, which is required to maximize current production per unit of anode surface area (15).

Biofilms on Fe(III) oxide. When *G. sulfurreducens* (2) was grown under strictly anaerobic conditions at 30°C in freshwater medium (7) with acetate (15 mM) as an electron donor and with Fe(III) oxide coatings [prepared on borosilicate coverslips (17) and providing 4.3 ± 0.7 μmol of Fe(III) per coverslip (mean ± standard deviation; $n = 3$)] as the sole electron acceptor, a biofilm grew on the Fe(III) coating (measured with a crystal violet assay [14]), but planktonic growth was not supported (Fig. 1A). Viability staining with a BacLight viability kit (Molecular Probes) and confocal scanning laser microscopy (CSLM) analyses (14) of 48-h biofilms revealed a structured biofilm composed of cell clusters approximately 18 ± 1 μm high (Fig. 1B). Control coverslips without the Fe(III) oxide coatings did not support biofilm growth (Fig. 1C), suggesting that biofilm growth was not supported by any nutrient carried over in the inoculum. Viability staining suggested that even cells at a substantial distance from the Fe(III) oxide surface remained metabolically active (Fig. 1B). This may be attributed to long-range electron transfer via the electrically conductive pili, as previously proposed for long-range transfer to the anode surface of microbial fuel cells (15). In contrast, a previously described (14) mutant in which the gene coding for PilA, the pilin structural subunit, was deleted grew poorly on the Fe(III) oxide coatings (Fig. 1D) and produced 10-fold less biomass than the wild type produced after 72 h (Fig. 2), and complementation of the mutation in *trans* (14) restored the biofilm phenotype (data not shown). These findings are consistent with the previous finding that pili are required for growth on Fe(III) oxide (14).

Biofilm formation when electron transfer to the Fe(III) oxide surface is not required. Even though pili are not required for growth with fumarate as an electron acceptor (14), addition of fumarate (40 mM) to cultures with Fe(III) oxide-coated coverslips, while having little impact on the biofilm biomass of the wild type, increased the mutant biofilm biomass to approximately one-half of the wild-type biomass (Fig. 2). More wild-type biomass accumulated on glass coverslips when fumarate was provided as the electron acceptor, but the biomass of the pilin-deficient mutant biofilms remained approximately one-half that of the wild-type biofilms (Fig. 2). These results demonstrated that pili are required for optimal biofilm development even when the surface is not the electron acceptor.

This conclusion was consistent with CLSM images of the fumarate-grown biofilms (Fig. 3). In the presence of fumarate, wild-type cells formed pillars that were 19 ± 1.5 and 22 ± 0.5 μm high on Fe(III) oxide-coated surfaces and glass surfaces, respectively. In contrast, the maximum biofilm heights for the pilin-deficient mutant were 7.6 ± 1.5 and 8.5 ± 1.4 μm on the same surfaces. This difference was not apparent in the first 24 h, when both the mutant and wild-type biofilms formed short microcolonies. However, the wild-type microcolonies continued to grow, and both the height and width of the colonies increased to form mature biofilms (Fig. 3). Viability staining indicated that cells in all the layers of the wild-type and mutant biofilms were alive, suggesting that fumarate dif-

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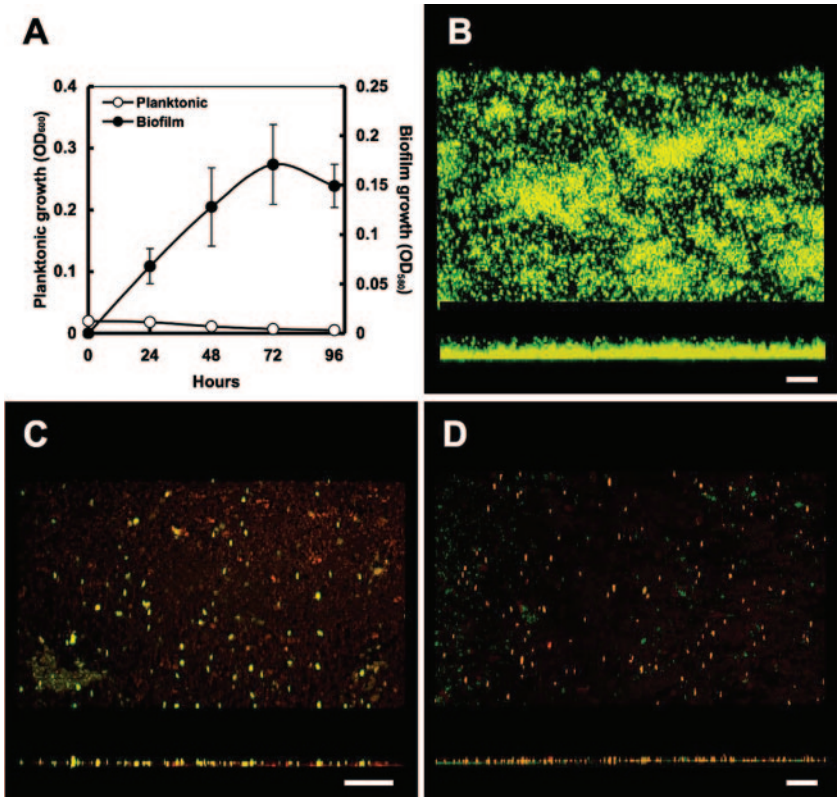

FIG. 1. Biofilm formation on Fe(III) oxide coatings by *G. sulfurreducens*. (A) When provided as a sole electron acceptor for growth, the Fe(III) oxide coating supported the growth of a biofilm, whose biomass increased steadily during the first 72 h, but did not support planktonic growth. OD_{600}, optical density at 600 nm. (B to D) Top view (at a 15° angle) (top panels) and side view (bottom panels) projections generated by CSLM of 48-h wild-type (B) or pilin-deficient mutant (D) biofilms formed on an Fe(III) oxide-coated surface or of a wild-type biofilm on a control coverslip without the Fe(III) oxide coating (C). Green indicates live cells, and red indicates dead cells. Yellow regions are areas where the two dyes overlap. The substratum (coverslip) is at the bottom. Bars, 20 μm.

fusion across the biofilms was not a limiting factor, as previously reported for other bacterial biofilms (16). These results indicate that the pili of *G. sulfurreducens* play a role in the development of the highly structured biofilms of *G. sulfurreducens* that is unlikely to be related to the electrical conductivity of the pili.

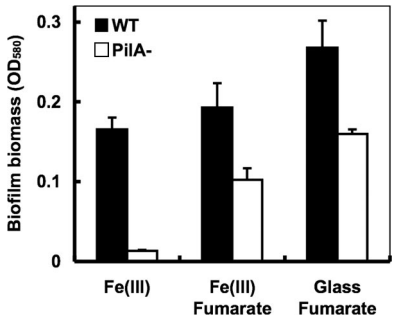

FIG. 2. Average biomasses of mature wild-type (WT) and pilin-deficient mutant (PilA−) biofilms formed on Fe(III) oxide coatings [Fe(III)] or glass surfaces. Where indicated, the soluble electron acceptor fumarate also was present in the growth medium. The results are averages for triplicate samples from two independent experiments. OD_{600}, optical density at 600 nm.

***Geobacter* pili promote autoagglutination.** Pili in various bacteria mediate twitching motility during biofilm formation (12). Other pili, such as the toxin-coregulated pili (TCP) of *Vibrio cholerae* (13), and also the pili of *G. sulfurreducens* (14) do not appear to be involved in motility. Rather, TCP are a structural biofilm component that mediate cell interactions leading to microcolony development during colonization of the human intestine (3) or during biofilm formation on chitin surfaces (13). The ability of TCP to promote bacterial interactions also enables TCP-expressing cells to autoagglutinate in vitro (3). Similar agglutination studies were carried out with *G. sulfurreducens* by growing cells with fumarate as the electron acceptor at 25°C to induce pilus formation (14). The degree of agglutination was assayed by measuring the optical density at 600 nm of the cells that remained in suspension and subtracting the value obtained from the optical density of the culture after disruption of the aggregates with agitation. After 72 h of growth the wild-type strain formed large aggregates that settled at the bottom of the culture vessel (Fig. 4). There was no autoagglutination at 30°C, a temperature at which planktonic cells do not express pili (14). The mutant in which *pilA* was deleted did not agglutinate at 25°C (Fig. 4). Complementation of the mutation with a wild-type copy of the *pilA* gene expressed in *trans* produced a strain that agglutinated at levels

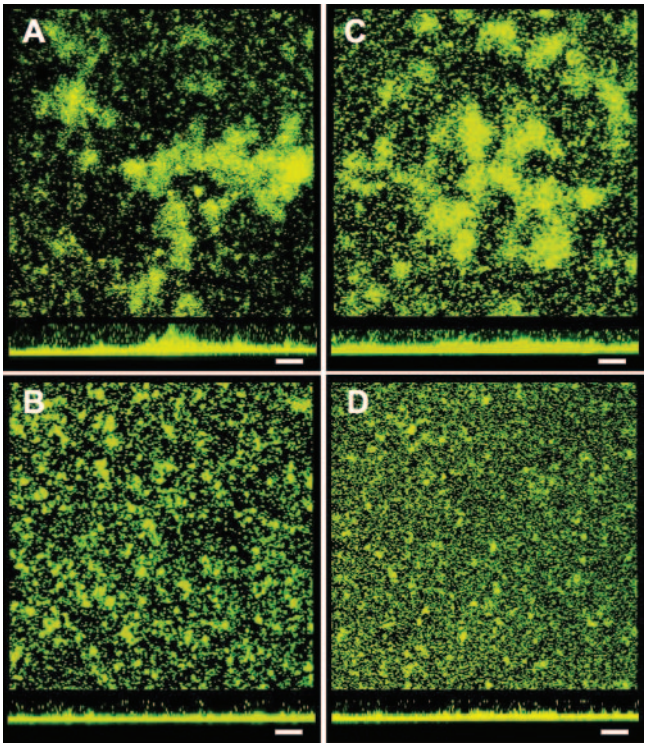

FIG. 3. CSLM analyses of wild-type (A and C) and PilA$^-$ (B and D) biofilms formed on Fe(III) oxide coatings (A and B) or glass coverslips (C and D) in medium with fumarate. Green indicates live cells, and red indicates dead cells. Yellow indicates dye overlap. The images are three-dimensional top views (top panels) and side views (bottom panels) reconstructed from the fluorescence patterns of the series of two-dimensional optical sections collected by CSLM. Bars, 20 μm.

that were much higher than the levels observed for the wild-type strain (Fig. 4), consistent with the fact that genetic complementation leads to overproduction of pili (14). These results suggest that the pili of *G. sulfurreducens* participate in cell-cell aggregation necessary for the development of microcolonies during biofilm differentiation.

Implications. The results presented here demonstrate that in addition to serving as electric conduits for electron transfer to Fe(III) oxides (14) and long-range electron transfer across anode biofilms in *G. sulfurreducens* fuel cells (15), the *G. sulfurreducens* pili also are required for maximum biofilm growth even when electron transfer to an electron-accepting surface is not required. This is an important consideration because the overall rate of electron transfer to an electron-accepting surface is dependent upon the number of metabolically active cells that can stack on the surface. Thus, high rates of electron transfer to an electron-accepting surface require not only the electronic capabilities of the pili but also their structural attributes that permit cells to stack at high densities on a given surface. These considerations make it clear that further evaluation of the contributions of pili and other outer cell components to the biofilm structure is essential in order to better understand, and perhaps optimize, electron transfer to electron-accepting surfaces.

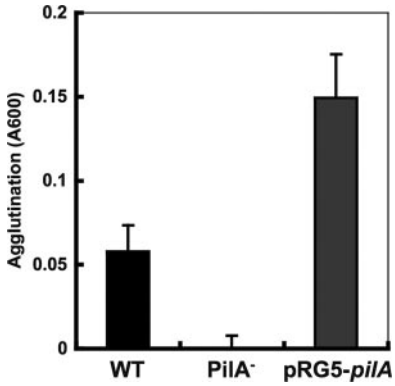

FIG. 4. Autoagglutination phenotypes of a wild-type strain (WT), a pilus-deficient mutant (PilA$^-$), and a genetically complemented mutant (pRG5-*pilA*) grown under pilus-inducing conditions (25°C). The results are the averages for triplicate samples from two independent experiments. A600, absorbance at 600 nm.

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REFERENCES

1. **Bond, D. R., and D. R. Lovley.** 2003. Electricity production by *Geobacter sulfurreducens* attached to electrodes. Appl. Environ. Microbiol. **69:**1548–1555.
2. **Caccavo, F., Jr., D. J. Lonergan, D. R. Lovley, M. Davis, J. F. Stolz, and M. J. McInerney.** 1994. *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl. Environ. Microbiol. **60:**3752–3759.
3. **Kirn, T. J., M. J. Lafferty, C. M. Sandoe, and R. K. Taylor.** 2000. Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by *Vibrio cholerae*. Mol. Microbiol. **35:**896–910.
4. **Lies, D. P., M. E. Hernandez, A. Kappler, R. E. Mielke, J. A. Gralnick, and D. K. Newman.** 2005. *Shewanella oneidensis* MR-1 uses overlapping pathways for iron reduction at a distance and by direct contact under conditions relevant for biofilms. Appl. Environ. Microbiol. **71:**4414–4426.
5. **Lovley, D. R.** 2006. Bug juice: harvesting electricity with microorganisms. Nat. Rev. Microbiol. **4:**497–508.
6. **Lovley, D. R., D. E. Holmes, and K. P. Nevin.** 2004. Dissimilatory Fe(III) and Mn(IV) reduction. Adv. Microb. Physiol.**49:**219–286.
7. **Lovley, D. R., and E. J. P. Phillips.** 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl. Environ. Microbiol. **54:**1472–1480.
8. **Nevin, K. P., and D. R. Lovley.** 2000. Lack of production of electron-shuttling compounds or solubilization of Fe(III) during reduction of insoluble Fe(III) oxide by *Geobacter metallireducens*. Appl. Environ. Microbiol. **66:**2248–2251.
9. **Nevin, K. P., and D. R. Lovley.** 2002. Mechanisms for accessing insoluble Fe(III) oxide during dissimilatory Fe(III) reduction by *Geothrix fermentans*. Appl. Environ. Microbiol. **68:**2294–2299.
10. **Nevin, K. P., and D. R. Lovley.** 2002. Mechanisms for Fe(III) oxide reduction in sedimentary environments. Geomicrobiol. J. **19:**141–159.
11. **Newman, D. K., and R. Kolter.** 2000. A role for excreted quinones in extracellular electron transfer. Nature **405:**94–97.
12. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30:**295–304.
13. **Reguera, G., and R. Kolter.** 2005. Virulence and the environment: a novel role for *Vibrio cholerae* toxin-coregulated pili in biofilm formation on chitin. J. Bacteriol. **187:**3551–3555.
14. **Reguera, G., K. D. McCarthy, T. Mehta, J. S. Nicoll, M. T. Tuominen, and D. R. Lovley.** 2005. Extracellular electron transfer via microbial nanowires. Nature **435:**1098–1101.
15. **Reguera, G., K. P. Nevin, J. S. Nicoll, S. F. Covalla, T. L. Woodard, and D. R. Lovley.** 2006. Biofilm and nanowire production lead to increased current in microbial fuel cells. Appl. Environ. Microbiol. **72:**7345–7348.
16. **Stewart, P. S.** 2003. Diffusion in biofilms. J. Bacteriol. **185:**1485–1491.
17. **van Schie, P. M., and M. Fletcher.** 1999. Adhesion of biodegradative anaerobic bacteria to solid surfaces. Appl. Environ. Microbiol. **65:**5082–5088.