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Biofilm and Nanowire Production Leads to Increased Current in *Geobacter sulfurreducens* Fuel Cells[▽]

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Geobacter sulfurreducens **developed highly structured, multilayer biofilms on the anode surface of a microbial fuel cell converting acetate to electricity. Cells at a distance from the anode remained viable, and there was no decrease in the efficiency of current production as the thickness of the biofilm increased. Genetic studies demonstrated that efficient electron transfer through the biofilm required the presence of electrically conductive pili. These pili may represent an electronic network permeating the biofilm that can promote long-range electrical transfer in an energy-efficient manner, increasing electricity production more than 10-fold.**

Electricigens, microorganisms that can completely oxidize organic compounds to carbon dioxide with an electrode serving as the sole electron acceptor, show promise as catalysts for efficient conversion of a variety of organic wastes and renewable biomass to electricity (11). However, the requirement for electricigens to establish contact with the fuel cell anode in order to produce electricity could potentially be a limiting factor in power production. Electricigens, such as *Geobacter* and *Rhodoferax* species, have been shown to grow in virtual monolayers of cells on anode surfaces (2, 3). If intimate contact with the anode were an absolute requirement for electron transfer from electricigens to electrodes, then sophisticated engineering of anodes to provide large surface areas would be necessary in order to increase the power output of microbial fuel cells.

Although many organisms are capable of donating electrons to the anode of microbial fuel cells (11), in our studies we have focused on *Geobacter sulfurreducens* because it is closely related to the members of the *Geobacteraceae* that are the predominant organisms on anodes harvesting electricity from diverse aquatic sediments (1, 6, 17) and organic wastes (5) and because it is the only genetically tractable (4, 14) electricigen available in pure culture.

Biofilm formation. In order to better understand how *G. sulfurreducens* functions on the anode surface, inocula for microbial fuel cells were prepared by culturing as previously described (13) in freshwater acetate (20 mM)–Fe(III) citrate (50 mM) medium and harvesting organisms in the late exponential phase by centrifugation under anaerobic conditions. The cell pellets were suspended in freshwater medium without an electron donor or acceptor and inoculated into the anode chambers of previously described (2) anoxic, dual-chamber, H-type microbial fuel cells equipped with graphite electrodes, with the anode poised at a constant potential of 300 mV. This anoxic, poised system maintained consistency between different fuel cells, removed any potential limitations resulting from electron transfer at the cathode, and eliminated the possibility of oxygen intrusion into the anode chamber (2) that might support aerobic growth (10). When 10 mM acetate was added to the inoculated freshwater medium in the anode chamber incubated at 25°C, the current rapidly increased to ca. 6 mA and then declined as the acetate was depleted (Fig. 1A). If at the point of maximum current production in batch mode a continual medium feed into the anode chamber was initiated at a flow rate of 0.5 ml/min, the current increased to 12 mA (Fig. 1B).

In order to examine growth on the anode by confocal scanning laser microscopy (CSLM), concave wells (diameter, 1.5 cm; depth, 2 mm) were made in the anode prior to deployment. At different levels of current production, the anodes were removed from the fuel cells, washed with phosphate-saline buffer, fluorescently stained using a BacLight viability kit (Molecular Probes), and examined with a Zeiss LSM510 inverted microscope. CSLM analyses revealed that as the current increased, there was increased coverage of the anode surface with cells, with complete coverage as the current neared 2 mA, and the formation of complex, multilayer biofilms (Fig. 2A to C) composed of cell clusters whose height increased (Fig. 3A). At currents nearing the maximum current outputs in batch mode (Fig. 2C), the average height of the biofilm pillars was 40 μm (± 6 μm), and some cell clusters were as high as 50 μm. The biofilms from fuel cells run with a continuous acetate feed were visually apparent but were too thick to accurately measure the biofilm thickness by CSLM. Protein measurements of the biofilm biomass at different points during current production indicated that there was a direct linear increase in the amount of biomass on the anodes as the current increased and the biofilms developed. Thus, the efficiency of current production per cell did not decrease as current increased even though more cells were at increasingly significant distances from the anode surface. Viability staining indicated that there was no decrease in overall cell viability as current increased (Fig. 3C), and the cells at a distance from the electrode surface remained viable during biofilm development, further suggesting that they were metabolically active and involved in electron transfer to the anode (Fig. 2).

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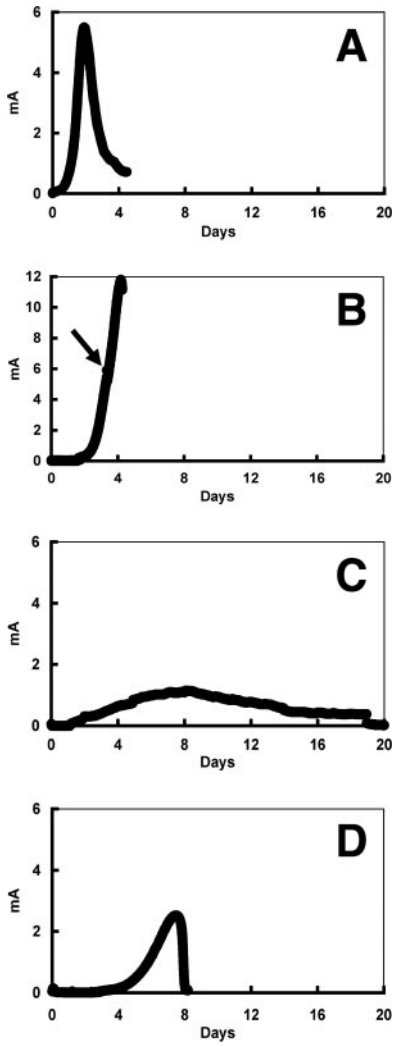

FIG. 1. Current in *G. sulfurreducens* fuel cells. (A and B) Current with wild-type cells growing on a one-time batch feed of acetate (A) or when the system was switched to continuous flowthrough mode at the point indicated by the arrow (B). Note the difference in scale on the *y* axis. (C and D) Current with a pilin-deficient mutant (C) and a strain with the capacity for pilin production restored (D) in batch systems. The data are representative time courses for multiple replicates for each treatment.

Pilus requirement. A potential mechanism for long-range electron transfer in *G. sulfurreducens* is the electrically conductive pili that have been shown to be essential for electron transfer to Fe(III) oxides (16). In previous studies we found that pili were not required for electricity production (7), but in the systems used the maximum current was 0.8 mA, a current at which there would not be substantial stacking of cells on the anode surface. When the previously described pilin-deficient mutant (16) was inoculated into the same system in which wild-type cells produced a maximum current of 6 mA under batch conditions, it produced significantly less current than the wild type produced (Fig. 1C). However, over time the mutant converted approximately as much acetate to electricity as the wild-type cells converted, just at a lower rate. Switching the *pilA* mutant to flowthrough mode did not increase power production. These results indicated that pili are not absolutely required for electron transfer to the anode but are necessary for maximum power.

CSLM revealed that, although the pilin-deficient mutant attached to the anode (Fig. 2D), the biofilm height (3.8 ± 0.8 μm) was much less than that of the wild-type biofilms (Fig. 3A). Furthermore, viability staining indicated that many (ca. 60%) of the cells were dead (Fig. 3C), that the live population was preferentially located in direct contact with the anode surface, and that the dead cells were present primarily in the upper biofilm layers (Fig. 2D). Further evidence that many of the cells were not actively contributing to current production was provided by the finding that current production was low relative to the amount of biomass on the anode (Fig. 3B). The efficiency of current production in the absence of pili (0.5 mA/mg anode protein) was less than one-third of that observed with wild-type cells.

When the *pilA* gene was expressed in *trans* in the pilin-deficient mutant (16), current was produced at a rate intermediate between that of the wild type and that of the mutant (Fig. 1D). This response is similar to the previously reported partial restoration of the ability to reduce Fe(III) oxide in this complemented strain (16). The increase in current production in the complemented strain was associated with corresponding increases in pillar height (Fig. 3A) and biomass and with restoration of the efficiency of power production (1.7 mA/mg anode protein) to levels comparable to those observed with the wild type (Fig. 3B). Furthermore, most (95.5%) of the cells of the complemented strain were viable (Fig. 3C), including the cells that were not in contact with the electrode surface (Fig. 2E).

Implications. These results demonstrate that the pili of *G. sulfurreducens* permit increased stacking of cells on the anode surface and that there is a corresponding increase in current production. The cells not in direct contact with the anode appear to contribute to current production because they remain viable and the per-cell efficiency of current production does not decrease as the biofilm develops and the height of the pillars increases. This has important implications for the design of microbial fuel cells because it demonstrates that it is possible to enhance current production not only by increasing the surface area of the anode but also by increasing the number of cells contributing to electron flow on a given surface. This facilitates packing more electricity-producing microorganisms into a given volume.

As shown here, and consistent with results of a previous study (7), pili are not required for the lower levels of power production which can be generated from cells in intimate contact with anode surfaces. Under these conditions electron transfer to the anode is likely to be accomplished via outer membrane *c*-type cytochromes, such as OmcS (7). Electron transfer to electrodes via outer membrane *c*-type cytochromes has also been hypothesized for other organisms (8). However, electron transfer via this mechanism would be possible only for cells intimately associated with the anode surface.

It seems likely that pili promote long-range electron transfer across the multilayer biofilms on anodes because the pili are electrically conductive (16). Pili as long as 20 μm have been observed on *G. sulfurreducens*. Therefore, in some instances the pili of cells at a distance from the anode could potentially

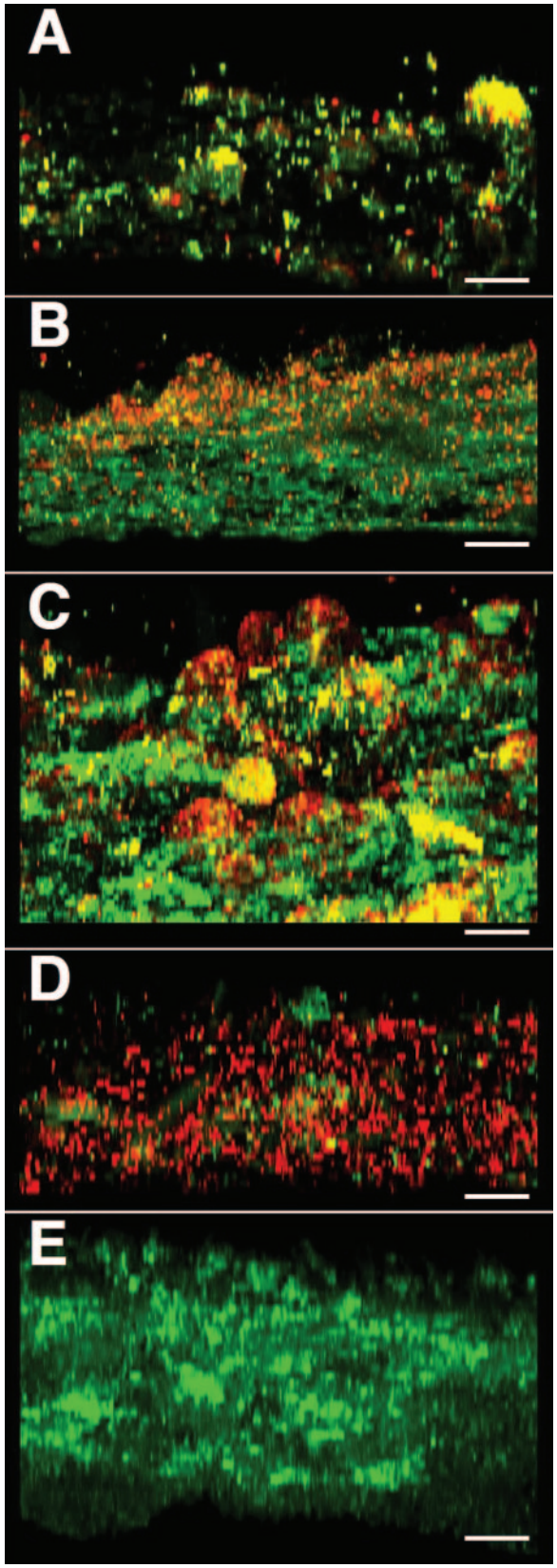

FIG. 2. Confocal scanning laser microscopy of *G. sulfurreducens* on anode surfaces. (A to C) Wild-type biofilms producing 1.4 mA (A), 2.2 mA (B), and 5.2 mA (C). (D and E) Biofilms of a pilin-deficient mutant (D) and the genetically complemented mutant strain (E) when current production was nearing maximum (ca. 1 mA and 3 mA, respectively). Live cells are green, while dead cells are red. The images are three-dimensional side-view images at a 45° angle reconstructed from the fluorescence patterns of a series of two-dimensional optical sections collected by CSLM. Bars, 20 μm.

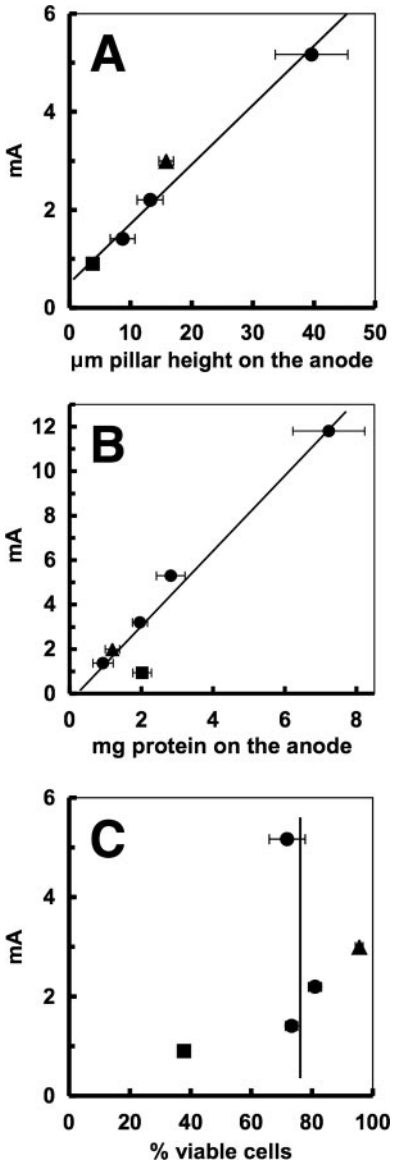

FIG. 3. Pillar height (A), biomass (B), and cell viability (C) of *G. sulfurreducens* anode biofilms at different rates of current production. ●, wild-type cells; ■, pilin-deficient mutant; ▲, genetically complemented mutant strain. The pilin-deficient mutant produced equivalent amounts of power whether it was in batch or flowthrough mode. The error bars for pillar heights indicate standard deviations for all pillars from at least five fields (140 by 140 μm^2). The lines are the regression lines calculated for the wild-type anode biofilms.

make electrical contact either with the anode surface or with the cells coating the anode. Cell-to-cell electron transfer via intertwined pili (16) might establish a "nano power grid" in which the pilus network transfers electrons through the 40- to

50-μm-thick anode biofilm. Furthermore, it is well known that in other organisms pili play an important role in the early stages of biofilm formation (15), as well as cell cluster maturation (9), on a variety of surfaces. Therefore, it is conceivable that *G. sulfurreducens* pili have other, unknown functions in biofilm differentiation on anodes.

The fact that *G. sulfurreducens* and other organisms can directly transfer electrons to electrodes is remarkable, since it is unlikely that there has ever been any evolutionary pressure on these organisms to make electricity in the natural environment. The ability of *G. sulfurreducens* to transfer electrons to electrodes may be attributed to its capacity to transfer electrons to Fe(III) oxides, which are also insoluble, extracellular electron acceptors. However, the stacking of *G. sulfurreducens* on anodes has no known counterpart in Fe(III) oxide reduction in sedimentary environments, where it is not advantageous for the cells to permanently attach to the Fe(III) oxides (12). These considerations suggest that there may be substantial opportunities to increase long-range electron transfer to anodes with genetic engineering and/or adaptive evolution.

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