Reduction of Graphene Oxide *via* Bacterial Respiration

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ABSTRACT Here we present that graphene oxide (GO) can act as a terminal electron acceptor for heterotrophic, metal-reducing, and environmental bacteria. The conductance and physical characteristics of bacterially converted graphene (BCG) are comparable to other forms of chemically converted graphene (CCG). Electron transfer to GO is mediated by cytochromes MtrA, MtrB, and MtrC/OmcA, while mutants lacking CymA, another cytochrome associated with extracellular electron transfer, retain the ability to reduce GO. Our results demonstrate that biodegradation of GO can occur under ambient conditions and at rapid time scales. The capacity of microbes to degrade GO, restoring it to the naturally occurring ubiquitous graphite mineral form, presents a positive prospect for its bioremediation. This capability also provides an opportunity for further investigation into the application of environmental bacteria in the area of green nanochemistries.

KEYWORDS: Graphene · environmental bacteria · biodegradation graphene · electron transfer · bioremediation · *Shewanella*

Graphene oxides (GOs) are layered, oxygenated graphene sheets with epoxide, carboxyl, and hydroxyl groups on their basal planes and edges. Although traditionally seen as a precursor to large-scale graphene synthesis,[1] GO has recently received more attention for its other possible uses.[2,3] GOs are being promoted as useful compounds for incorporation into polymers, ceramics, and metals,[4−9] as novel forms of thin film electronic materials,[10−13] as potential chemotherapeutic delivery vehicles,[14,15] as antibiotics,[16] for hydrogen storage compositions,[17] and for enhanced oil recovery;[18,19] the latter being where larger volumes would enter the environment. Given the wide-ranging applications for this material, it is likely to enter into large-scale production.[18,19] Inevitably, this will lead to the introduction of GO into environmental systems. Thus, it is important to understand whether there is a natural and rapid route for its conversion to graphite, the layered stacks of graphene that can form after the reduction of GO. Graphite is a naturally occurring mineral that is already used abundantly and poses no threat to the environment. Toward this end, we have conducted laboratory studies to assess the interactions between GO and model environmental microbes from the genus *Shewanella*.

Shewanella comprises a group of heterotrophic, facultative anaerobes. They have been found in a wide variety of environments, including lake and marine sediments, estuaries, hydrothermal vents, various fish species, oil brines, ocean water, and spoiled foods.[20] These microbes have the ability to use a large array of organic and inorganic compounds as terminal electron acceptors in their respiratory pathway. In addition to oxygen, other electron acceptors available to *Shewanella* include arsenate, chromium oxides, uranium oxides, dimethylsulfoxide, trimethylsulfoxide, iron oxides, manganese oxides, nitrates, and silver oxide.[21,22] With respect to toxic metals, such as chromium and uranium, *Shewanella* are able to remove them from solution by reducing them to their insoluble forms.[21] These bacteria are known as exoelectrogens[16] because some of these compounds, such as iron oxide, are solids, requiring the bacteria to engage in extracellular electron transfer (EET).[20,21,23] The ability of these organisms to use solids as terminal electron acceptors, their capacity to immobilize toxic metals, and their environmental ubiquity make them good candidates to study how microbes might interact with graphitic nanomaterials.

RESULTS AND DISCUSSION

Five strains from this genus were used, representing a variety of ecological habitats (Table 1). Reduction was evident at 24 h due to the precipitation of graphene from

TABLE 1. Bacterial Strains Used in This Study

strain	origin
Shewanella oneidensis MR-1[a]	Lake Oneida, New York
Shewanella putrefaciens CN32[a]	Uranium Mine, New Mexico
Shewanella amazonensis SB2B[a]	Amazon River Delta
Shewanella putrefaciens W3-18-1[a]	Pacific Ocean Marine Sediments
Shewanella baltica 10735[Tb]	oil brine, Japan

[a]Bacterial strains provided by the *Shewanella* Federation (www.shewanella.org).
[b]Strain provided by Dr. Masataka Satomi, National Research Institute of Fisheries Science (http://nrifs.fra.affrc.go.jp/).
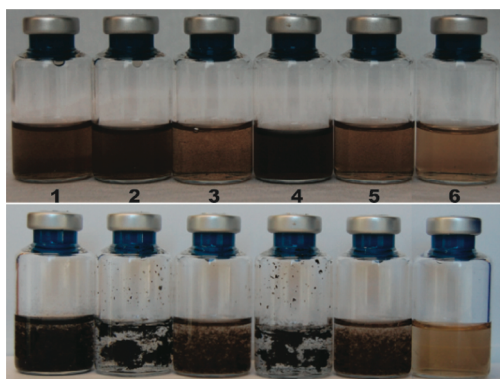

Figure 1. Microbial reduction of GO occurs under ambient conditions. Digital photographs of anaerobic serum bottles containing different strains of *Shewanella* and GO (10 mL solution). Bottles 1 = MR-1; 2 = CN32; 3 = SB2B; 4 = W3−18−1; 5 = 10735[T]; and 6 = blank control. The top panel shows the condition of GO after 24 h, while the bottom panel shows conditions after 72 h. The changes in color and solubility of the material in bottles 1−5 indicate extensive reduction of GO by bacteria as the initially soluble GO solution forms graphene precipitates upon reduction. Each bottle was photographed individually with a Nikon D40 SLR camera (set to manual focus) under natural lighting and subsequently combined into one figure.

solution (Figure 1); however, the incubations were allowed to continue for approximately 72 h.

X-ray photoelectron spectroscopy (XPS) was used to characterize the GOs before and after incubation with bacteria. The C 1s XPS spectra for GO had peaks

TABLE 2. Fraction of Reduced Carbon in BCG

	%C—C
GO	28
CCG[a]	83
S. oneidensis MR-1	56
S. putrefaciens CN32	91
S. amazonensis SB2B	75
S. sp. W3-18-1	>95
S. baltica 10735[T]	54
S. oneidensis ΔmtrA	31
S. oneidensis ΔmtrB	40
S. oneidensis ΔmtrC/omcA	55
S. oneidensis ΔcymA	81

[a]Chemically converted prepared using hydrazine at room temperature.

at 287 and 288 eV for the C−OH and C=O bonds, respectively. The C−C bond at 284.5 eV was much less prominent. Analysis of these peak intensities indicated that the biologically mediated reduction of GO was extensive (Figure 2). As reported previously,[24] after chemical reduction the C−OH and C=O peaks decrease in intensity, and the strongest signal comes from the C−C bond. After incubation with bacteria, this was also the case; as much as 95% of the carbon in the biologically converted graphene (BCG) was in the reduced state, and there was no indication of any epoxy or carboxyl groups in the reacted samples (Figure 2, Table 2). Not all of the strains tested reduced GO to the same extent (Table 2). This variability may be due to the rate at which the GO was reduced or to the possibility that the test conditions were not optimal for some strains. Work using other solid-phase terminal electron acceptors has shown that *Shewanella* exhibit different rates of respiration,[25] and the rates and extent of reduction can vary as a function of medium composition.[26] Among a number of strains, the peak at 287 eV was reduced to a shoulder in the C−C peak, indicating that, although reduction did occur, some hydroxyl groups still remained in these samples. Observation with transmis-
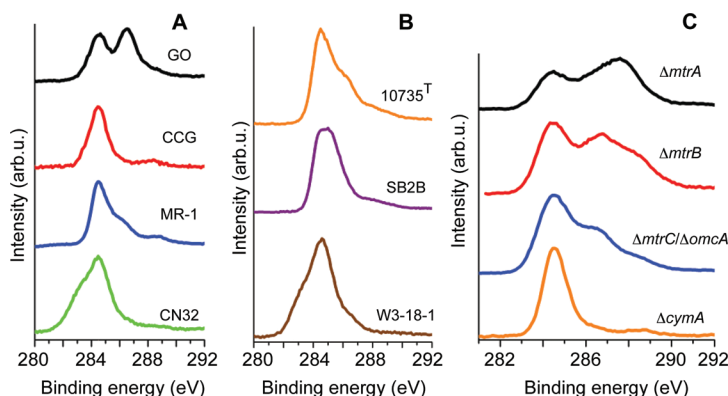

Figure 2. Reduction of GO by bacteria is extensive as observed by XPS. Panels A and B display XPS spectra for BCG produced by various strains of *Shewanella*. GO and CCG are shown in panel A for reference. Panel C displays results from reduction experiments using mutant strains of *S. oneidensis* MR-1. The results in panels A and B demonstrate that, in all cases of GO respiration, loss of oxygen is pronounced. The percentage of C−C bonds increases from approximately 28% in GO to 90−95% in the bacterial products. Panel C shows results for deletion mutants of c-cytochromes associated with metal reduction in *S. oneidensis* MR-1. Spectra indicate that MtrA is essential for GO reduction, while CymA is not.
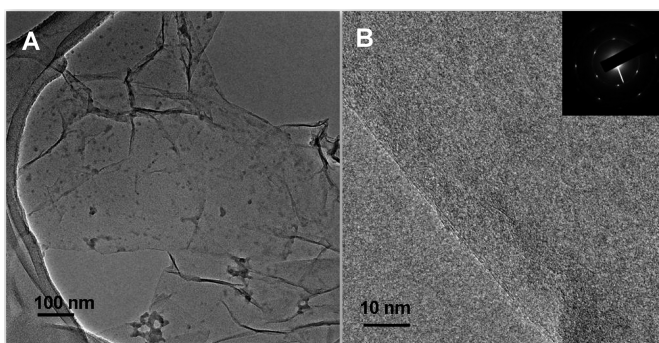
Figure 3. TEM image of BCG. (A) Single-layered BCG flakes; and (B) single-layered BCG edge. The inset in 'B' is the selected area electron diffraction pattern (SAED) of BCG, showing the material's honeycomb crystalline structure.

sion electron microscopy (TEM) indicated that the BCG samples had single-layer thickness (Figure 3).

S. oneidensis MR-1 was incubated with GO thin films in order to test the capacity of bacteria to reduce GO films deposited on a substrate. The conductance of BCG was observed to increase by $10^3 - 10^4$ over the starting GO material (Figure 4). This is comparable to the conductance of chemically converted graphene (CCG) obtained from chemical reduction of GO.[1,27] Although work done with *S. putrefaciens* CN32 showed no significant difference in conductance when compared with MR-1, the variation in conductance of BCG as a function of bacterial strain is currently under investigation.

EET by *S. oneidensis* MR-1 has been attributed to a group of periplasmic (MtrA) outer-membrane (MtrB and MtrC) and inner-membrane (CymA) multihaem c-type cytochromes that are common to all *Shewanella* capable of metal reduction.[23] In order to determine their role in reduction of GO, strains of MR-1 deficient in each of these proteins were incubated with GO as the terminal electron acceptor. While ΔcymA mutants retained the ability to reduce GO, reduction of GO was greatly inhibited by the loss of the *mtrA* gene but not as inhibited in Δ*mtrB* and Δ*mtrC*/Δ*omcA* mutants (Figure 2C, Table 1).

While the CymA protein has been shown to be involved in anaerobic respiration,[28] the present work indicates that it is not required for GO reduction. Taken together, these results suggest that in the case of GO reduction, electrons flow from the inner-membrane quinone pool to the periplasmic MtrA protein *via* a route not involving CymA and that, while MtrB and MtrC/OmcA are involved in GO reduction, other outer-membrane proteins may play a role as well (Figures 2C and 5). Additionally, the extent of GO reduction is variable among the tested strains (Figures 1 and 2). The *Shewanella* genome encodes 42 putative c-type cytochromes located throughout the inner-membrane, periplasm, and outer-membrane.[29] Further work is needed to understand the EET network used by various *Shewanella* in GO reduction.

CONCLUSIONS

This study determined the capability of microbes to process functionalized graphene compounds. These results raise not only the possibility of using environmental bacteria to process graphitic nanomaterials for the purpose of bioremediation but also the potential of using bacteria such as *Shewanella* in green chemistry approaches to materials synthesis. The develop-
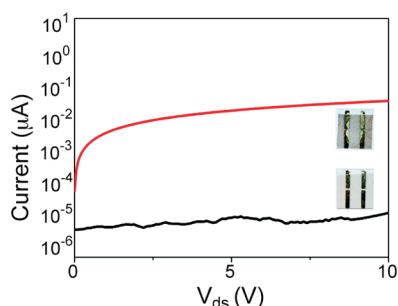

Figure 4. Conductance plots taken of GO films before and after incubation with bacteria demonstrate a decrease in film resistance of up to 10^4. Black = before; and red = after. Reduction is apparent *via* the darkening in film color (inset where the film is underneath the four electrodes and the before is on the bottom, and the after is on the top). The inset was photographed as described in Figure 1.
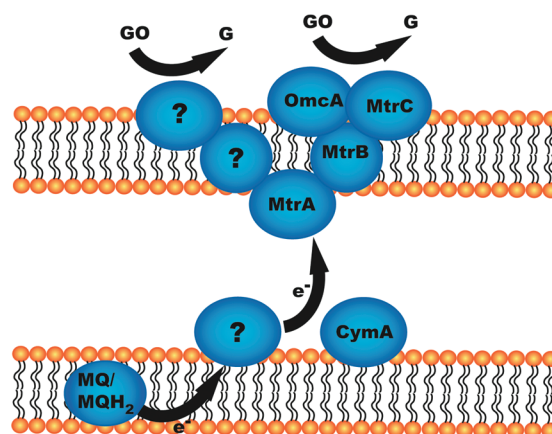

Figure 5. MtrA mediates electron transfer from the inner membrane to the outer membrane in the case of GO reduction. MtrA, MtrB, MtrC, and CymA are known to be involved in the reduction of solid materials, such as iron and manganese oxides. Work presented here indicates that, in the case of GO reduction, *Shewanella* does not use CymA to mediate electron flow from the quinone pool to the periplasmic cytochrome, MtrA. Additionally, other outermembrane cytochromes may be involed in GO reduction.

ment of green chemistries has taken on greater prominence,[30] and recent work highlights the attempt to produce graphene using fewer contaminants.[27,31] Exoelectrogens provide the opportunity to investigate how redox-active enzymes might be employed in these efforts. Finally, the results here show promise for some graphitic nanotechnologies to be environmentally benign.

MATERIALS AND METHODS

Bacterial Cultures. Bacteria were inoculated from frozen stocks into Luria–Bertani (LB) broth and grown overnight at room temperature, shaking at approximately 125 rpm. The overnight cultures were washed in saline solution to remove all traces of LB. Bacteria were inoculated into serum bottles containing 10 mL of *Shewanella* Federation-defined medium, with lactate, and 2 mg of GO. Prior to inoculation, the serum bottles (containing medium and GO) were gassed with ultrapure N_2 to remove traces of oxygen. Final cell concentrations were approximately 10^8 cells/mL. The samples were done in triplicate and incubated in the dark at room temperature. Mutant strains were prepared in the same fashion as the wild-type strains. Negative controls contained all the elements described above but without bacteria.

Graphene Oxide Preparation and Characterization of Bacterially Converted Graphene. GO was prepared from graphite powder using the method of Hummers and Offeman.[32] The samples, including controls, were collected and washed using the following sequence: 18 MΩ water (Millipore Milli-Q Gradient), followed by a 3–5 min wash in 80% ethanol, 18 MΩ water, a 3–5 min wash in 1 N HCl, and 18 MΩ water. The samples were then air-dried and analyzed with XPS. Analysis was carried out on a PHI Quantera SXM scanning X-ray microprobe with a base pressure of 5×10^{-9} Torr. The X-ray source was an Al cathode ray set at 100 W and a pass energy of 140.00 (survey scan) and 26.00 eV (high-resolution scan), takeoff angle was 45°, and beam size was 100 μm. Quantification of carbon functional groups was based on Yang *et al.*[33] and is summarized as follows: The C1s peak was fitted into four peaks. The sp2 C—C peak was assigned at 284.5 eV. Chemical shifts of +1.5, +2.5, and +4.0 eV were assigned to C—OH, C=O, and O=C—OH functional groups, respectively. Additionally, epoxide groups (C—O—C) were assumed to have a C1s binding energy similar to C—OH.[34]

GO Thin Films and Conductivity Measurements. To test the conductivity of BCG, 3 mL of GO_{aq} (0.5 mg/mL H_2O) was sprayed onto a glass slide. Immediately after application to each slide, the material was dried and annealed on a hot plate at 100 °C. Au was then sputtered into the edges of the slide, atop the GO, to a thickness of 10 nm. The conductance of the GO was determined. The slides were then incubated anaerobically with strain MR-1 for approximately 72 h. After incubation, the slides were collected, washed gently with 18 MΩ water to remove as much biomass as possible, and dried thoroughly. The conductance of the BCG was then determined. Conductance was measured with a two-point probe at room temperature using an Agilent 4155C semiconductor parameter analyzer under vacuum (10^{-6} Torr).

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