The dissimilatory metal-reducing bacterium *Shewanella oneidensis* strain MR-1 has been extensively investigated for its ability to use insoluble substrates such as iron and manganese oxide minerals as terminal electron acceptors (9). The Mtr respiratory pathway is used by MR-1 to facilitate transfer of electrons from the interior of the cell to these external terminal electron acceptors (2, 6, 12, 18). In addition to naturally occurring insoluble electron acceptors, it has been reported that MR-1 is able to reduce synthetic graphene oxide (GO), forming layered stacks of graphene (17, 21). Because large-scale graphene production typically involves reduction of GO by the use of toxic chemicals (10), microbial reduction of GO to graphene by bacteria could offer an alternative approach that is rapid, cheap, and environmentally friendly. Understanding the molecular mechanisms of GO reduction by microbes may facilitate their use in the commercial-scale production of graphene.

It has been shown that both direct contact and electron shuttling are involved in the reduction of insoluble substrates by MR-1 (11, 16). The Mtr respiratory pathway is required for the reduction of metal oxide minerals and electrodes (5, 6), suggesting that it may also be able to transfer electrons to GO. At least five primary protein components have been identified in this pathway: OmcA, MtrC, MtrA, MtrB, and CymA. Current models of electron transfer in MR-1 assume that electrons from carbon source oxidation are passed via the menaquinone pool and subsequent respiratory pathways in the periplasm and outer membrane (14, 15). Here we describe a comprehensive genetic analysis of the relative importance of proteins in the Mtr respiratory pathway and test the role of electron shuttles in GO reduction, with some contradictory results presented with regard to CymA. The results presented here extend our knowledge of how *Shewanella* catalyzes the reduction of insoluble substrates and may have broader implications for biologically mediated graphene production.

We determined that graphene oxide reduction by *Shewanella oneidensis* MR-1 requires the Mtr respiratory pathway by analyzing a range of mutants lacking these proteins. Electron shuttle compounds increased the graphene oxide reduction rate 3- to 5-fold. These results may help facilitate the use of bacteria for large-scale graphene production.
Strains were grown in Shewanella basal medium (SBM) anaerobically at 30°C with shaking at 220 rpm. Balch anaerobic tubes were prepared in an anaerobic chamber and sealed with butyl rubber stoppers. Lactate (15 mM) and GO (0.8 mg/ml) were added as the sole electron donor and electron acceptor, respectively. Graphite oxide sheets were synthesized from graphite powder by the method of Hummers and Offeman. The aqueous graphite oxide solution was sonicated vigorously for 2 h to facilitate the separation ofGO sheets. For the GO reduction assay, overnight aerobic LB cultures were washed in SBM and used for inoculation at a dilution of 1:1,000 after normalization based on the optical density at 600 nm (OD600). Solutions of riboflavin and AQDS, a synthetic analogue of the redox-active moieties in humic acids, were prepared in distilled water, filter sterilized, and brought to a final concentration of 12 μM. High concentrations of electron shuttles were used to maximize any potential effect on GO reduction. Cell growth was monitored by CFU determination by plating the cells onto LB agar plates and incubating them aerobically.

GO reduction was inferred by the change in OD600 after correcting for bacterial cells (corrected OD600), which was extrapolated based on a standard curve of OD600 and number of CFU. By following GO reduction by MR-1 over time by both X-ray photoelectron spectroscopy (XPS) and OD600 determination, we confirmed that the corrected OD600 is a faithful quantification of GO reduction. Cell growth was monitored by CFU determination by plating the cells onto LB agar plates and incubating them aerobically.

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Impaired GO reduction. To investigate the role of the Mtr respiratory pathway in GO reduction, we tested GO reduction by mutants defective in the Mtr pathway. Because both iron(III) oxide and GO are insoluble at circumneutral pH, we anticipated that the electron transport pathways would be similar. A steady increase in the amount of reduced GO was observed with the wild-type MR-1 strain for at least 40 h, whereas GO reduction was affected by various degrees in the mutant strains (Fig. 1). In contrast to a recent report by Salas et al. (17), we found that CymA is critical for GO reduction, since a strain lacking cymA retained only minimal GO reduction capability (Fig. 1A). Similarly, strains lacking either MtrA or MtrB also showed ~5-fold decrease in GO reduction activity (Fig. 1A), suggesting that MtrA and MtrB are also important for GO reduction (17).

Our cymA mutant result is consistent with how the Mtr respiratory pathway is currently thought to function (18), with CymA providing electrons from the menaquinone pool. An incorrect cymA mutant strain may explain the difference between our observations and those of Salas et al. (17). A recent report by Cordova et al. (4) demonstrated that cymA-null mutants could be suppressed by upregulation of sirCD, which could also explain the discrepancy.

To test potential terminal reductases for GO, we focused...
on three possible outer membrane c-type cytochromes: MtrC, OmcA, and MtrF (Fig. 1B). While a mutant strain lacking MtrF reduced GO only slightly more slowly than the wild type (85%), both the individual omcA and mtrC mutants showed significant decreases (about 50%) in GO reduction rate. A double mutant lacking both MtrC and OmcA reduced GO at a much lower rate (16%), similar to that of the triple mutant lacking MtrC, OmcA, and MtrF. Controls lacking cells or electron donor (lactate) showed only a minimal GO reduction rate (~5%) during the course of incubation (Fig. 1A; and data not shown). These results suggest that MtrC and OmcA are the primary terminal reductases for GO by MR-1 and that another minor GO reduction pathway may exist in parallel to the Mtr pathway.

Acceleration by electron shuttles. To investigate whether an excess of extracellular electron shuttles could enhance GO reduction, we carried out the GO reduction experiment with the addition of riboflavin or AQDS. It should be noted that these compounds were added in addition to the normal level of flavins released by the bacteria. All strains tested naturally secreted flavins to ~1 μM when grown in minimal medium (data not shown). We quantified the degree to which these compounds increased, i.e., accelerated, the GO reduction rate when they were added exogenously to wild-type cultures. When using an excess (12 μM) of riboflavin or AQDS, we observed increases in the GO reduction rate of 2.7- and 4.7-fold, respectively (Fig. 2). AQDS enhanced GO reduction to a greater degree than riboflavin, suggesting that it reacts more quickly with GO. This observation is in contrast to the behavior of these electron shuttles in iron(III) oxide reduction (5, 19). The significant acceleration of GO reduction in the presence of AQDS may have biotechnological implications for microbial graphene production.

We also tested the GO reduction of mutants defective in the Mtr pathway in the presence of additional electron shuttles (Fig. 3). Mutants of the Mtr pathway did not reduce GO at the same rate as the wild type in the presence of riboflavin or AQDS, indicating that this pathway is involved in the reduction of the electron shuttles during GO reduction. We attribute the decreases in OD₆₀₀ at later time points for some strains to the increase of graphene particle aggregation observed in older cultures (data not shown). While the relative GO reduction capability of the Mtr mutants with riboflavin or AQDS follows that of endogenous electron shuttles, it is worth noting that mtrB and mtrA mutants recovered significant levels of GO reduction activity in the presence of excess AQDS or riboflavin. In contrast, cymA mutant strains failed to recover any GO reduction activity in the presence of an electron shuttle, suggesting that the inner membrane cytochrome CymA is a key component in electron transfer to GO. Additionally, both the omcA mtrC double mutant and the omcA mtrC mtrF triple deletion mutant showed very little GO reduction activity even in the presence of AQDS or riboflavin, suggesting that the use of electron shuttles relies on these outer membrane cytochromes in MR-1.

Concluding remarks. We found that GO reduction by S. oneidensis MR-1 is catalyzed primarily by the Mtr respiratory pathway. While MtrA, MtrB, and CymA are important for GO reduction, outer membrane multiheme cytochromes MtrC and OmcA can provide partial compensation in the absence of one another, whereas MtrF is dispensable (see Fig. S1 in the supplemental material). Our CymA result contradicts a previous report concluding that CymA is not required for GO reduction by S. oneidensis (17). Electron shuttles, including riboflavin and AQDS, were able to significantly accelerate the GO reduction rate of MR-1, in a manner that was dependent on the Mtr pathway. This work expands the range of known substrates that can be reduced by S. oneidensis and may lead to engineered strains that can be used in the commercial production of graphene.

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REFERENCES

1. Balch, W. E., and R. S. Wolfe. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of Methanobacterium ruminantium in a pressurized atmosphere. Appl. Environ. Microbiol. 32:781–791.

2. Bretschger, O., et al. 2007. Current production and metal oxide reduction by...
Shewanella oneidensis MR-1 wild type and mutants. Appl. Environ. Microbiol. 73:7003–7012.

3. Bücking, C., F. Popp, S. Kerzenmacher, and J. Gescher. 2010. Involvement and specificity of Shewanella oneidensis outer membrane cytochromes in the reduction of soluble and solid-phase terminal electron acceptors. FEMS Microbiol. Lett. 306:144–151.

4. Cordova, C. D., M. F. R. Schicklberger, Y. Yu, and A. M. Spormann. 2011. Partial functional replacement of CymA by SirCD in Shewanella oneidensis MR-1. J. Bacteriol. 193:2312–2321.

5. Coursolle, D., D. B. Baron, D. R. Bond, and J. A. Gralnick. 2010. The Mtr respiratory pathway is essential for reducing flavins and electrodes in Shewanella oneidensis. J. Bacteriol. 192:467–474.

6. Coursolle, D., and J. A. Gralnick. 2010. Modularity of the Mtr respiratory pathway of Shewanella oneidensis strain MR-1. Mol. Microbiol. 77:995–1008.

7. Hartshorne, R. S., et al. 2009. Characterization of an electron conduit between bacteria and the extracellular environment. Proc. Natl. Acad. Sci. U. S. A. 106:22169–22174.

8. Hau, H. H., A. Gilbert, D. Coursolle, and J. A. Gralnick. 2008. Mechanism and consequences of anaerobic respiration of cobalt by Shewanella oneidensis strain MR-1. Appl. Environ. Microbiol. 74:6880–6886.

9. Hau, H. H., and J. A. Gralnick. 2007. Ecology and biotechnology of the genus Shewanella. Annu. Rev. Microbiol. 61:237–258.

10. Hummers, W. S., and R. E. Offeman. 1958. Preparation of graphitic oxide. J. Am. Chem. Soc. 80:1339.

11. Lies, D. P., et al. 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.

12. Lovley, D. R., D. E. Holmes, and K. P. Nevin. 2004. Dissimilatory Fe(III) and Mn(IV) reduction. Adv. Microb. Physiol. 49:219–286.

13. Marsili, E., et al. 2008. Shewanella secretes flavins that mediate extracellular electron transfer. Proc. Natl. Acad. Sci. U. S. A. 105:3968–3973.

14. Myers, C. R., and J. M. Myers. 1997. Cloning and sequence of cymA, a gene encoding a tetraheme cytochrome c required for reduction of iron(III), fumarate, and nitrate by Shewanella putrefaciens MR-1. J. Bacteriol. 179:1143–1152.

15. Myers, J. M., and C. R. Myers. 2000. Role of the tetraheme cytochrome CymA in anaerobic electron transport in cells of Shewanella putrefaciens MR-1 with normal levels of menaquinone. J. Bacteriol. 182:67–75.

16. Nevin, K. P., and D. R. Lovley. 2002. Mechanisms for accessing insoluble Fe(III) oxide during dissimilatory Fe(III) reduction by Geobacter fermentans. Appl. Environ. Microbiol. 68:2294–2299.

17. Salas, E. C., Z. Sun, A. Lutte, and J. M. Tour. 2010. Reduction of graphene oxide via bacterial respiration. ACS Nano 4:4852–4856.

18. Shi, L., T. C. Squier, J. M. Zachara, and J. K. Fredrickson. 2007. Respiration of metal (hydr)oxides by Shewanella and Geobacter: a key role for multihaem c-type cytochromes. Mol. Microbiol. 65:12–20.

19. Shyu, J. B., D. P. Lies, and D. K. Newman. 2002. Protective role of tolC in efflux of the electron shuttle anthraquinone-2,6-disulfonate. J. Bacteriol. 184:1806–1810.

20. von Canstein, H., J. Ogawa, S. Shimizu, and J. R. Lloyd. 2008. Secretion of flavins by Shewanella species and their role in extracellular electron transfer. Appl. Environ. Microbiol. 74:615–623.

21. Wang, G. M., F. Qian, C. W. Saltikov, Y. Jiao, and Y. Li. 2011. Microbial reduction of graphene oxide by Shewanella. Nano Res. 4:563–570.