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**Deciphering the Electron Transport Pathway for Graphene Oxide Reduction by  
*Shewanella oneidensis* MR-1**

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Running title: GO reduction by MR-1.

1 **Abstract**

2 We determined that graphene oxide reduction by *Shewanella oneidensis* MR-1  
3 requires the Mtr respiratory pathway by analyzing a range of mutants lacking these  
4 proteins. Electron shuttling compounds accelerated graphene oxide reduction rate by 3 to  
5 5 fold. These results have important implication in large-scale graphene production in an  
6 environmental benign manner.

7  
8 **Body**

9 The dissimilatory metal-reducing bacterium *Shewanella oneidensis* strain MR-1  
10 (MR-1) has been extensively investigated for its ability to use insoluble substrates such as  
11 iron and manganese oxide minerals (8). MR-1 has developed a pathway to transfer  
12 electrons from the interior of the cell to these external terminal electron acceptors, called  
13 the Mtr respiratory pathway (2, 5, 11, 15). In addition to naturally occurring insoluble  
14 electron acceptors, it has also been reported that MR-1 is able to reduce synthetic  
15 graphene oxide (GO), forming layered stacks of graphene (14, 18). Since current  
16 approach for large-scale graphene production is through chemical reduction of GO (9),  
17 microbial reduction of GO by bacteria such as MR-1 could offer an alternative approach  
18 for graphene production, which is rapid, cheap and environmental friendly. However,  
19 applying microbial extracellular respiration for graphene production requires a thorough  
20 understanding of the molecular dynamics and cellular physiology of electron source  
21 utilization and the reduction of insoluble electron acceptor.

22 It has been proposed that both direct contact and electron shuttling are involved in  
23 the reduction of insoluble substrates by MR-1 (10, 13). Particularly, the Mtr pathway is

24 required for the reduction of metals and electrodes (4, 5). At least six primary protein  
25 components have been identified in this pathway: OmcA, MtrC, MtrF, MtrA, MtrB and  
26 CymA. Current models of electron transfer in MR-1 assume that electrons from carbon  
27 source oxidation are passed via the menaquinone pool to the inner membrane-anchored *c*-  
28 type cytochrome CymA. These electrons are then transferred to a periplasmic *c*-type  
29 cytochrome, MtrA and eventually to outer membrane-anchored *c*-type cytochrome MtrC,  
30 which interact with each other via an integral OM scaffolding protein, MtrB (6). Two  
31 homologs of MtrC, OmcA and MtrF, can compensate for the loss of MtrC to reduce a  
32 variety of substrates (3, 5). Beside direct electron transfer through outer membrane MtrC,  
33 electron shuttles produced *in situ* by MR-1, flavins (riboflavin and flavin  
34 mononucleotide) (12, 17) and added exogenously such as 9,10-anthraquinone-2,6-  
35 disulfonic acid (AQDS) have been shown to facilitate electron transfer to insoluble  
36 terminal electron acceptors and electrodes, also through the Mtr pathway (4, 16).

37 We describe a comprehensive genetic analysis of the role of Mtr respiratory  
38 pathway and test the role of electron shuttles in GO reduction. The results presented here  
39 extend our knowledge of how *Shewanella* catalyze the reduction of insoluble substrates  
40 and may have broader implications regarding the biotechnology application of graphene  
41 production.

42 Strains used in this study have been previously described (5). Strains were grown  
43 in *Shewanella* basal medium (SBM) (7) anaerobically at 30°C with shaking at 220 rpm.  
44 Balch anaerobic tubes (1) were prepared in the anaerobic chamber and sealed with butyl  
45 rubber stoppers. Lactate (15 mM) and GO (0.8 mg/ml) were added as the sole electron  
46 donor and electron acceptor, respectively. GO sheets were synthesized from graphite

47 powder using a modified Hummers and Offenman's method (9). For the GO reduction  
48 assay, overnight aerobic LB cultures were washed in SBM and used to inoculate with a  
49 dilution rate of 1:1000 after normalizing by OD600. Solutions of riboflavin and AQDS,  
50 were prepared in distilled water, filter sterilized and added to a final concentration of 12  
51  $\mu\text{M}$  when appropriate. Cell growth was monitored by colony forming unit (CFU) by  
52 plating onto LB agar plates and incubating aerobically.

53 GO reduction was inferred by the change in optical density (OD, 600 nm) after  
54 correcting for bacterial cells, which was extrapolated based on a standard curve of  
55 OD600 and CFU. In order to confirm the corrected OD600 is a faithful quantification of  
56 GO reduction, we followed GO reduction by MR-1 over time by both X-ray  
57 photoelectron spectroscopy (XPS) and OD600. For XPS sample preparation, cultures at  
58 different time points were collected, washed in 80% ethanol, 1 N HCl and distilled water  
59 as described previously (14), and deposited and dried on silicon wafer. Figure S1 shows a  
60 tight correlation, with a Spearman rank correlation coefficient of 0.94, for the relative  
61 amount of C1s peak by XPS and corrected OD600.

62

63 **Impaired GO reduction.** To investigate whether the electron transport pathway in MR-  
64 1 is similar to that for iron oxide reduction, we tested the capability of GO reduction by  
65 mutants defective in the Mtr pathway. Because both iron oxide and GO have relatively  
66 low solubility at circumneutral pH, we suspect the electron transport pathway would be  
67 similar. Linear reduction of GO was observed with wild type MR-1 for at least 40 hr,  
68 whereas GO reduction was affected by varying degrees in the mutant strains (Figure 1).  
69 In contrast to a recent report (14), we found that CymA is essential for GO reduction

70 (Figure 1A). A strain lacking *cymA* only retained minimal GO reduction capability.  
71 Similar to *cymA* mutant, strains lacking either MtrA or MtrB also showed ~ 5 fold or  
72 greater decrease in GO reduction activity (Figure 1A) suggesting that CymA, MtrA and  
73 MtrB are all essential for GO reduction.

74 To test the terminal electron acceptor for GO reduction, we focused on three  
75 possible outer membrane *c*-type cytochromes including MtrC, OmcA and MtrF (Figure  
76 1B). While mutant strain lacking MtrF reduced GO only slightly slower than wild type  
77 (85%), both OmcA and MtrC mutants showed a significant decrease (about 50%) in GO  
78 reduction. In addition, double mutant missing both MtrC and OmcA reduced GO at a  
79 much slower rate (16%), similar to that of the triple mutant lacking MtrC, OmcA and  
80 MtrF. These results imply that MtrC and OmcA are the major terminal electron  
81 reductases for GO reduction by MR-1, whereas MtrF only plays a minor role, if any.  
82 These results indicate that GO reduction in MR-1 shares a similar electron transfer  
83 pathway as that for other insoluble electron acceptors (Figure 2).

84

85 **Acceleration by electron shuttles.** To investigate whether an excess of extracellular  
86 electron shuttles could enhance GO reduction, we carried out the GO reduction  
87 experiment with the addition of riboflavin and AQDS. It should be noted that these  
88 compounds were added in addition to the normal amount of flavins released by the  
89 bacteria. All strains tested natively accumulate flavins to ~ 250 nM under these  
90 conditions (data not shown). We quantified the degree to which these compounds  
91 increased GO reduction rate when added exogenously to wild-type cultures. When using  
92 an excess (12  $\mu$ M) of riboflavin or AQDS we observed increased GO reduction rates by

93 2.7 and 4.7 fold, respectively (Figure 3). Given that AQDS, a synthetic analogue of the  
94 redox active moieties in humic acids, showed greater acceleration of GO reduction  
95 compared to riboflavin, it suggests that AQDS might react better with the GO than  
96 riboflavin, in contrast to their behavior in ferric oxide reduction (4). The significant  
97 acceleration of GO reduction in the presence of AQDS may have biotechnological  
98 implications for microbial graphene production.

99 To determine if the Mtr pathway is used in the reduction of electron shuttles, we  
100 tested GO reduction capability of mutants defective in the Mtr pathway in the presence of  
101 riboflavin or AQDS (Figure 4). Mutants of the Mtr pathway did not reduce GO at the  
102 same rate as the wild type in the presence of riboflavin or AQDS, indicating that this  
103 pathway is involved in reduction of the electron shuttles during GO reduction. While the  
104 relative GO reduction capability of the Mtr mutants with riboflavin or AQDS follows that  
105 in the absence of electron shuttles, it is worth noting that *mtrB* and *mtrA* mutants  
106 recovered significant amount of GO reduction activity in the presence of AQDS or  
107 riboflavin, although these mutants are severely defective in GO reduction in the absence  
108 of electron shuttles. In contrast, *cymA* mutant strains failed to recover any GO reduction  
109 activity even in the presence of electron shuttles, suggesting electron transfer for GO  
110 reduction may be able to bypass some electron transport proteins in the outer membrane  
111 or periplasmic space, but the inner membrane cytochrome CymA is absolutely essential.  
112 In addition, both the double mutant *omcA/mtrC* and the triple deletion mutant  
113 *omcA/mtrC/mtrF* showed very little GO reduction activity even in the presence of AQDS  
114 or riboflavin, suggesting that the recycling of electron shuttles relies on these outer

115 membrane cytochromes in MR-1, and OmcA and MtrC are the critical terminal  
116 reductases.

117

118 **Concluding remarks.** We found that GO reduction in MR-1 shares a similar electron  
119 transfer pathway as that for insoluble electron acceptors (such as ferric oxide). While  
120 MtrA, MtrB and CymA are essential for GO reduction, outer membrane multi-heme  
121 cytochromes MtrC and OmcA can provide partial compensation in the absence of one  
122 another, whereas MtrF is dispensable. Electron shuttles including riboflavin and AQDS  
123 were able to significantly accelerate GO reduction rate in MR-1, in a manner also  
124 dependent on the Mtr pathway.

125

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### **Table and Figure Legends:**

Table S1: The tight correlation of GO reduction by XPS and OD measurements.

Figure 1: Analysis of GO reduction capability of mutants defective in the Mtr pathway.

Error bars indicate standard deviations of measurements from three independent cultures.

Figure 2: A proposed electron flow pathway for GO reduction in *S. oneidensis* MR-1, similar to that used for ferric oxide reduction. Electron flows through CymA in the cytoplasmic membrane to MtrA in the periplasm, before transferred to the terminal GO reductases OmcA and MtrC on the outer membrane that are tethered through MtrB.

Figure 3: Effects of electron shuttles on GO reduction in MR-1. MR-1, wild type MR-1 without exogenously added electron shuttle; MR-1-RF, MR-1 in the presence of riboflavin; MR-1-AQDS, MR-1 in the presence of AQDS. Error bars indicate standard deviations of measurements from three independent cultures.

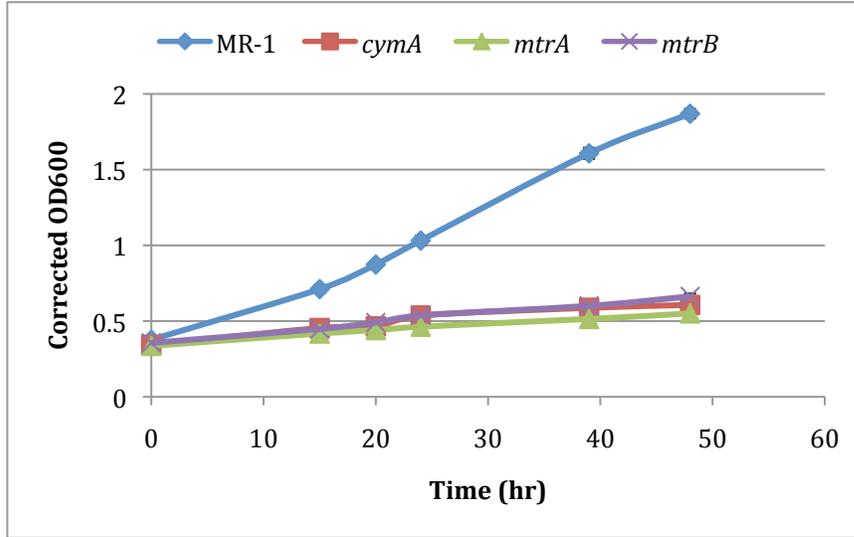
Figure 4. Effects of electron shuttles including riboflavin (A) and AQDS (B) on GO reduction in Mtr mutants. Error bars indicate standard deviations of measurements from three independent cultures.

Table S1:

| Time (hr) | % C1s in XPS | Corrected OD600 |
|-----------|--------------|-----------------|
| 0         | 58.386       | 0.2945          |
| 15        | 70.505       | 0.5312          |
| 20        | 70.811       | 0.6395          |
| 24        | 71.129       | 0.7515          |
| 39        | 74.708       | 1.1883          |
| 61        | 75.646       | 1.14875         |

Figure 1

(A)



(B)

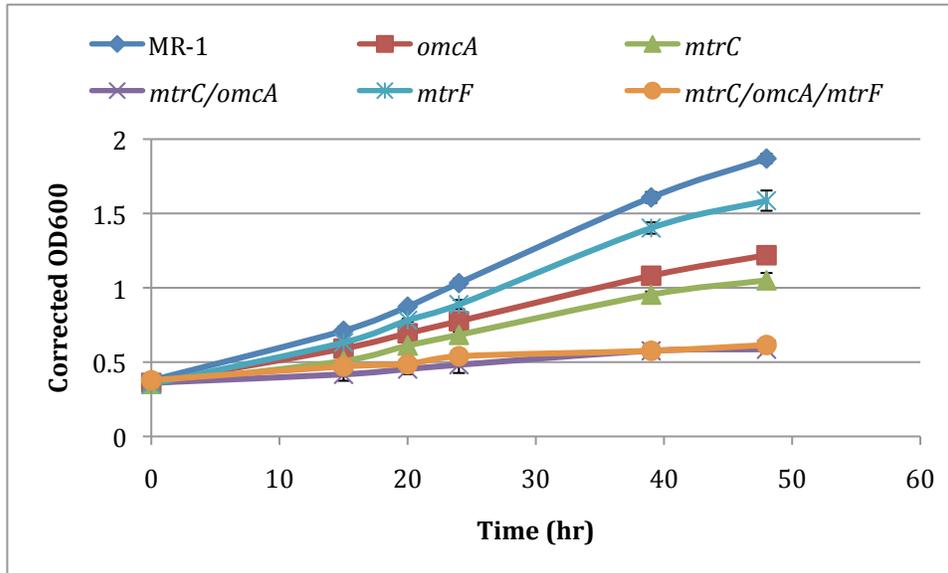


Figure 2

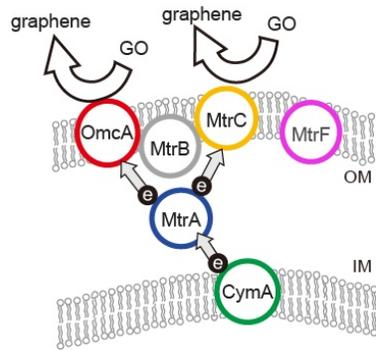


Figure 3

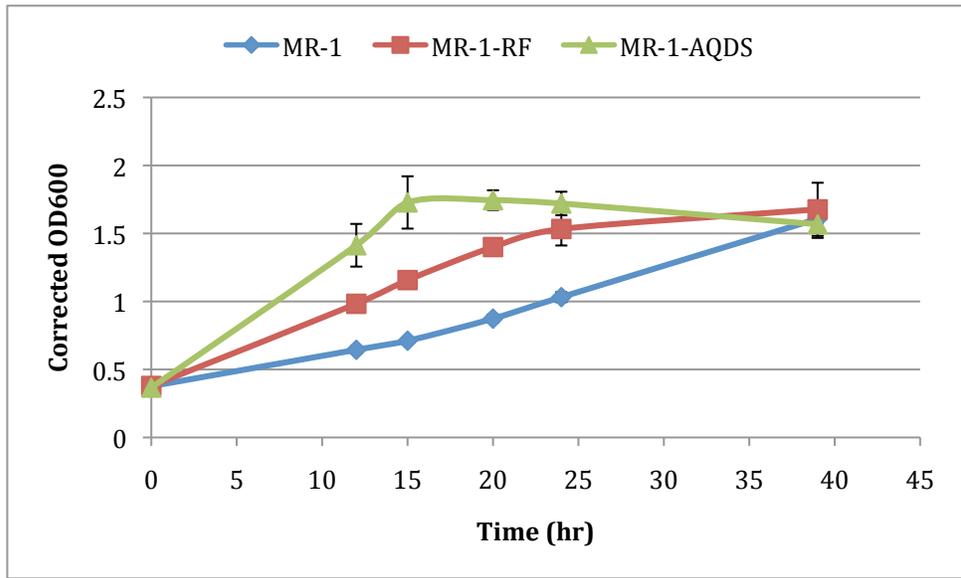
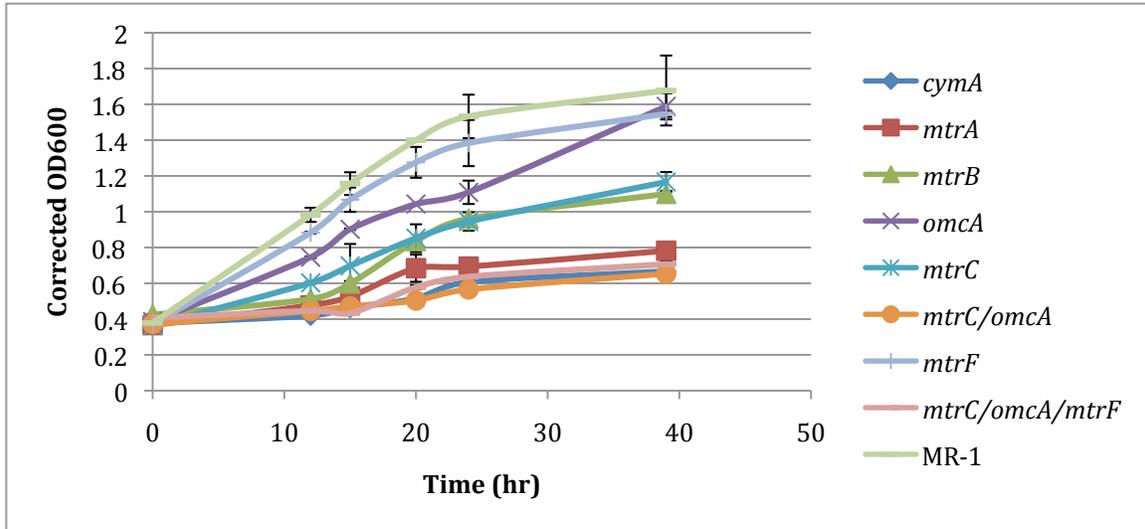


Figure 4

(A)



(B)

