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# Role of *Geobacter sulfurreducens* Outer Surface *c*-Type Cytochromes in Reduction of Soil Humic Acid and Anthraquinone-2,6-Disulfonate▽

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**Deleting individual genes for outer surface *c*-type cytochromes in *Geobacter sulfurreducens* partially inhibited the reduction of humic substances and anthraquinone-2,6,-disulfonate. Complete inhibition was obtained only when five of these genes were simultaneously deleted, suggesting that diverse outer surface cytochromes can contribute to the reduction of humic substances and other extracellular quinones.**

Humic substances can play an important role in the reduction of Fe(III), and possibly other metals, in sedimentary environments (6, 34). Diverse dissimilatory Fe(III)-reducing microorganisms (3, 5, 7, 9, 11, 19–22, 25) can transfer electrons onto the quinone moieties of humic substances (38) or the model compound anthraquinone-2,6-disulfonate (AQDS). Reduced humic substances or AQDS abiotically reduces Fe(III) to Fe(II), regenerating the quinone. Electron shuttling in this manner can greatly increase the rate of electron transfer to insoluble Fe(III) oxides, presumably because soluble quinone-containing molecules are more accessible for microbial reduction than insoluble Fe(III) oxides (19, 22). Thus, catalytic amounts of humic substances have the potential to dramatically influence rates of Fe(III) reduction in soils and sediments and can promote more rapid degradation of organic contaminants coupled to Fe(III) reduction (1, 2, 4, 10, 24).

To our knowledge, the mechanisms by which Fe(III)-reducing microorganisms transfer electrons to humic substances have not been investigated previously for any microorganism. However, reduction of AQDS has been studied using *Shewanella oneidensis* (17, 40). Disruption of the gene for MtrB, an outer membrane protein required for proper localization of outer membrane cytochromes (31), inhibited reduction of AQDS, as did disruption of the gene for the outer membrane *c*-type cytochrome, MtrC (17). However, in each case inhibition was incomplete, and it was suggested that there was a possibility of some periplasmic reduction (17), which would be consistent with the ability of AQDS to enter the cell (40).

The mechanisms for electron transfer to humic substances in *Geobacter* species are of interest because molecular studies have frequently demonstrated that *Geobacter* species are the predominant Fe(III)-reducing microorganisms in sedimentary environments in which Fe(III) reduction is an important process (references 20, 32, and 42 and references therein). *Geobacter sulfurreducens* has routinely been used for investigations of the physiology of *Geobacter* species because of the availability of its genome sequence (29), a genetic system (8), and a genome-scale metabolic model (26) has made it possible to take a systems biology approach to understanding the growth of this organism in sedimentary environments (23).

**AQDS and humic substance reduction in various *G. sulfurreducens* mutants.** In order to obtain insight into the mechanisms for electron transfer to humic substances in *G. sulfurreducens*, the impact of various deletions in outer membrane proteins on the reduction of AQDS and soil humic acids was investigated. The focus in this study was on the redox-active proteins that have previously been shown to be most abundant on the outer surface of cells. Cells were cultured as previously described (8) with acetate as the electron donor and fumarate as the electron acceptor. Cell suspensions were prepared from 200-ml cultures under anoxic conditions as previously described (39) and suspended in an osmotically balanced solution (OBS) (2.5 g/liter $NaHCO_3$, 0.25 g/liter $NH_4Cl$, 0.006 g/liter $NaH_2PO_4 \cdot H_2O$, 0.1 g/liter KCl, 1.75 g/liter NaCl) (39).

Washed cell suspensions were added to 10 ml of 5 mM AQDS in OBS with or without 10 mM acetate as the electron donor, and the preparations were incubated at 37°C. Production of anthrahydroquinone-2,6-disulfonate was monitored at 450 nm as previously described (19), and the amount was normalized to the amount of protein determined by the bicinchoninic acid method (41). Studies with soil humic acids were conducted in a similar manner with the AQDS omitted and 2 mg/ml Elliott soil humic acid standard (International Humic Substances Society) added. The electrons transferred to humic substances after 2 h were determined as previously described (19).

Single-knockout deletions of genes for the outer membrane *c*-type cytochromes OmcS and OmcE (28) slightly impaired the ability of *G. sulfurreducens* to reduce AQDS and humic substances. Deletion of *omcS* had the greatest impact on AQDS reduction (Fig. 1a), whereas deletion of *omcE* had the most significant impact on humic substance reduction (Fig. 1b). OmcS and OmcE are only loosely bound to the outer surface of *G. sulfurreducens* (28), and previous studies indicated that both of these cytochromes are involved in electron transfer to Fe(III) and Mn(IV) oxides (28) and electrodes (12). Neither protein is required for reduction of soluble, chelated Fe(III)

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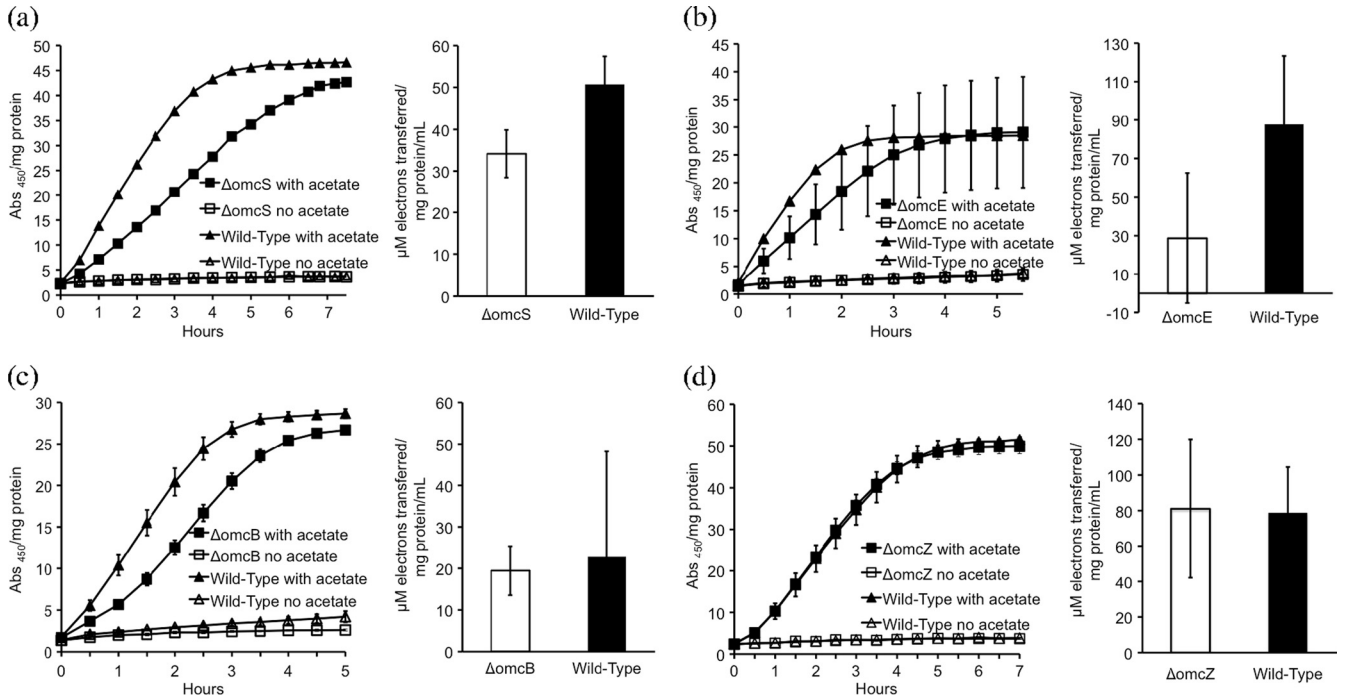


FIG. 1. Impact of deletion of single *c*-type cytochrome genes on reduction of AQDS (left panels) and the Elliott soil humic acid standard (right panels) for strains with the (a) *omcS*, (b) *omcE*, (c) *omcB*, or (d) *omcZ* gene deleted. The rates of reduction for the mutant strains were compared with the rate of reduction for a batch of wild-type cells grown and analyzed simultaneously. Incubation was carried out with acetate as the electron donor and (in controls) without added acetate. The data are the means ± standard errors for triplicate incubations.

(28). The finding that deleting *omcS* or *omcE* did not significantly inhibit the reduction of AQDS is consistent with the finding that addition of AQDS to *omcS* or *omcE* deletion mutants restored the capacity to reduce Fe(III) oxide (28).

Deletion of the gene for OmcB (16) only had a slight impact on the potential for AQDS or humic substance reduction (Fig. 1c). OmcB, which is embedded in the outer membrane (35), is essential for reduction of Fe(III) oxide, although the capacity to reduce some soluble Fe(III) is retained (15, 16). These results suggest that there are additional routes for electron transfer to AQDS and humic substances that are not available for reduction of Fe(III) oxides.

OmcZ is a loosely bound outer surface *c*-type cytochrome that is essential for transfer of electrons to electrodes but not for Fe(III) reduction (33). Deleting OmcZ (33) had no impact on AQDS or humic substance reduction (Fig. 1d). In a similar manner, deleting the gene for PilA, the structural pilin protein, did not inhibit the reduction of AQDS or humic substances (data not shown), consistent with the finding that although deletion of *pilA* prevents the production of electrically conductive pili that are required for Fe(III) oxide reduction, these pili are not required for reduction of soluble Fe(III) (36).

OmpB is representative of a class of putative multicopper, outer surface proteins that are required for reduction of Fe(III) oxide but not for reduction of soluble, chelated Fe(III) (13, 27). Surprisingly, deletion of the gene for OmpB (27) enhanced the capacity for AQDS and humic substance reduction (Fig. 2a). Proper localization of OmpB to the outer sur-

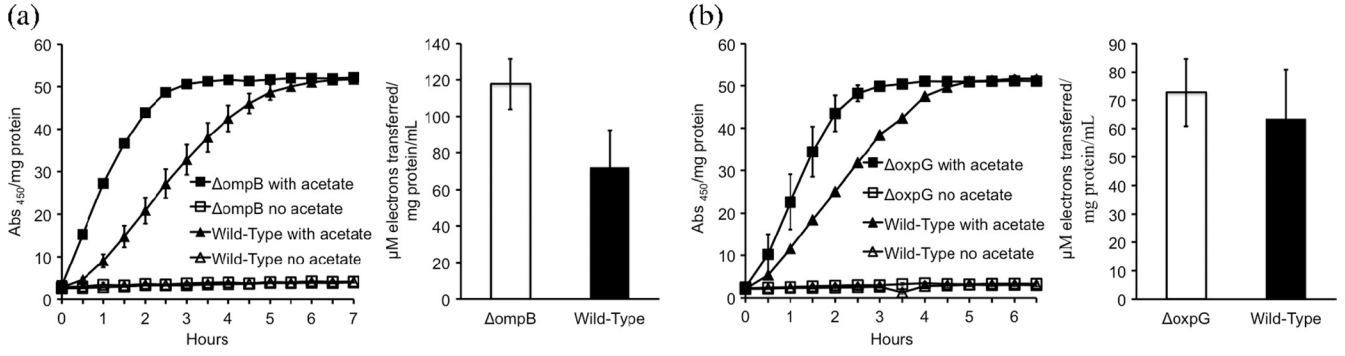


FIG. 2. Impact of deletion of the gene for OmpB (a) or a gene that affects its secretion to the outer surface, *oxpG* (b). Results for AQDS (left panels) and the Elliott soil humic acid standard (right panels) are shown. The data are the means ± standard errors for triplicate incubations.

TABLE 1. Primers used in this study

| Primer | Use(s) | Sequence | Reference |
|---|---|---|---|
| omcS-1 | Recombinant PCR for *omcS* deletion and *omcS-omcT* deletion | CTCCGACAAGCTCAGATGCG | This study |
| omcS-2 | Recombinant PCR for *omcS* deletion and *omcS-omcT* deletion | GCTGCTGCCACGGAAAGACTT | This study |
| omcS-3 | Recombinant PCR for *omcS* deletion and *omcS-omcT* deletion | AAGTCTTTCCGTGGCAGCAGCAGTGCCACCTGGGATGAATG | This study |
| omcS-4 | Recombinant PCR for *omcS* deletion | GTTGCAGAGAGCGCGCTGGTATGGCAGGTTGGGCGTCGC | This study |
| omcS-5 | Recombinant PCR for *omcS* deletion | ACCAGCGCGCTCTCTGCAAC | This study |
| omcS-6 | Recombinant PCR for *omcS* deletion | CTTGAGCCAGCCGAAATCGC | This study |
| omcT-4 | Recombinant PCR for *omcS-omcT* deletion | GTTGCACAGGACCCGTTGATATGGCAGGTTGGGCGTCGC | This study |
| omcT-5 | Recombinant PCR for *omcS-omcT* deletion | ATCAACGGGTCCTGTGCAAC | This study |
| omcT-6 | Recombinant PCR for *omcS-omcT* deletion | CGGGCATCAGGGAATAGAGG | This study |
| omcE-1 | Recombinant PCR for *omcE* deletion | TTGTAGCGAATTGCGGTTGG | This study |
| omcE-2 | Recombinant PCR for *omcE* deletion | GCTTGACCGGCACGTTATTC | This study |
| omcE-3 | Recombinant PCR for *omcE* deletion | GAATAACGTGCCGGTCAAGCGGCCCGGTACCGAGGAC | This study |
| omcE-4 | Recombinant PCR for *omcE* deletion | CATTGCTCAGATCGGTGCCCGCGGTGGAGCTCGAATTG | This study |
| omcE-5 | Recombinant PCR for *omcE* deletion | GGGCACCGATCTGAGCAATG | This study |
| omcE-6 | Recombinant PCR for *omcE* deletion | GCCAAGACCGACACTGACG | This study |
| 2076-1 | Recombinant PCR for *omcZ* deletion | ATGTGATGCGATATCCCGGC | 45 |
| 2076-2 | Recombinant PCR for *omcZ* deletion | CGCTGACGTGACACTCGAGAC | 45 |
| 2076spec-3 | Recombinant PCR for *omcZ* deletion | GTCTCGAGTGTCACGTCAGCGGGAGCACAGGATGACGCCTAAC | This study |
| 2076spec-4 | Recombinant PCR for *omcZ* deletion | GGTGATGCGGAGCTCGTAGCGCATAGTCTCCCCAGCTCTC | This study |
| 2076-5 | Recombinant PCR for *omcZ* deletion | CTACGAGCTCCGCATCACC | 45 |
| 2076-6 | Recombinant PCR for *omcZ* deletion | CACCCAGAGGAGGCAGCAGG | 45 |

face requires a type II secretion system. Deletion of the gene which encodes the pseudopilin protein OxpG results in accumulation of OmpB in the periplasm (27). A strain of *G. sulfurreducens* in which *oxpG* was deleted (27) also reduced AQDS and humic substances faster than wild-type cells (Fig. 2b). These results clearly indicate that, unlike Fe(III) oxide, OmpB is not required for AQDS and humic substance reduction. Why a failure to localize OmpB on the outer surface of the cell enhances AQDS and humic substance reduction requires further study.

The finding that deletion of individual genes for outer surface *c*-type cytochromes only partially inhibited AQDS or humic substance reduction suggested that there are multiple routes for transfer of electrons to these acceptors. Furthermore, the degree of inhibition of AQDS and humic substance reduction associated with deletion of any one outer surface *c*-type cytochrome gene could not necessarily be directly related to the relative flow of electrons to AQDS and humic substances in wild-type cells because of the potential for increased electron flow through alternative routes once a single cytochrome gene was deleted. Therefore, in order to investigate this further, the impact of multiple gene deletions was investigated.

To construct strains deficient in multiple cytochromes, linear DNA fragments for single-step gene replacement were constructed with the primers listed in Table 1 as previously described (14, 18, 30). An *omcB-omcS* double mutant was constructed by replacing the *omcS* gene of strain DL6 (16) with a kanamycin resistance cassette. The genes for OmcS and OmcT are adjacent on the *G. sulfurreducens* chromosome (28), and both of these genes were replaced with a kanamycin resistance cassette in strain DL6 (16) in order to generate an *omcB-omcS-omcT* triple mutant. An *omcB-omcS-omcT-omcE* quadruple mutant was constructed by replacing the *omcE* gene of the *omcB-omcS-omcT* triple mutant (this study) with a gentamicin resistance cassette. An *omcB-omcS-omcT-omcE-omcZ* quintuple mutant was constructed by replacing the *omcZ* gene of the *omcB-omcS-omcT-omcE* quadruple mutant (this study) with a spectinomycin resistance cassette. The orientation of the antibiotic resistance cassettes was the same as that of the disrupted genes. Electroporation, isolation of mutants, and genotype confirmation were performed as previously described (8, 18).

The double mutant in which *omcS* and *omcB* were deleted was impaired in AQDS and humic substance reduction compared to the wild type, but only marginally more than the strain with the single mutation in *omcS* (Fig. 3a). A triple mutant in which the gene for the OmcS homolog OmcT was also deleted had a phenotype similar to that of the *omcB-omcS* double mutant (data not shown). However, the mutations in a quadruple mutant in which the gene for OmcE was also deleted resulted in substantially greater inhibition of AQDS and humic substance reduction (Fig. 3b). Deletion of the genes for all five outer surface *c*-type cytochromes, including *omcZ*, completely inhibited the reduction of AQDS, and there was little, if any, reduction of humic substances (Fig. 3c). This result suggests that although deletion of just *omcZ* did not inhibit AQDS and humic substance reduction, OmcZ can play a minor role in transfer of electrons to these electron acceptors, at least when alternative routes for electron transfer are eliminated.

**Implications.** The results described above suggest that AQDS and humic substances are reduced at the outer surface of *G. sulfurreducens* and that a number of outer surface *c*-type cytochromes previously identified as proteins that are important in transfer of electrons to Fe(III) and/or electrodes contribute to this process. The fact that AQDS reduction was completely inhibited in the quintuple outer surface cytochrome mutant suggests that there is no significant reduction of AQDS in the cells of this mutant. The fact that a number of outer surface cytochromes appear to be capable of transferring electrons to AQDS and humic substances suggests that the reduction of quinone moieties in these molecules is a rather non-

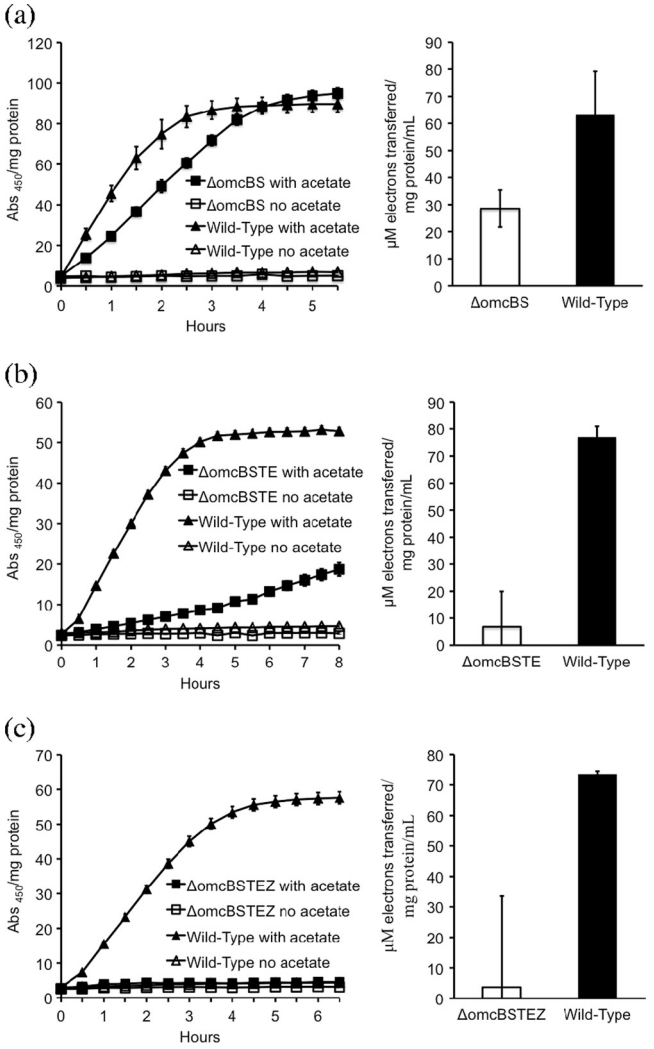


FIG. 3. Impact of deletion of multiple *c*-type cytochromes on reduction of AQDS (left panels) and the Elliott soil humic acid standard (right panels). The strains evaluated were deficient in the genes for (a) OmcB and OmcS; (b) OmcB, OmcE, OmcS, and OmcT; or (c) OmcB, OmcE, OmcS, OmcT, and OmcZ. The data are the means ± standard errors for triplicate incubations.

specific redox reaction. This lack of specificity for AQDS and humic substance reduction contrasts with the specific requirement for the outer surface cytochrome OmcZ for high levels of current production in microbial fuel cells (33, 37) and the requirement for OmcS for Fe(III) oxide reduction (28). However, *G. sulfurreducens* can adapt to the loss of most outer surface cytochromes in order to reduce soluble, chelated Fe(III) (15, 28). It is likely that the similarities between AQDS reduction, humic substance reduction, and soluble Fe(III) reduction and their differences with insoluble electron acceptors, such as Fe(III) oxides and electrodes, are related to access. Electrons can be transferred readily from low-potential hemes to quinones or Fe(III), and mere contact between the two types of molecules is probably sufficient for reduction of soluble quinones or Fe(III). However, accessing insoluble electron acceptors is likely to be more difficult and may require specific arrangements of the outer surface molecules in order to bring hemes and electrodes or Fe(III) oxides into sufficient proximity for electron transfer.

Long-range extracellular electron transfer via quinone-containing electron shuttles can allow microorganisms to reduce not only Fe(III) oxides but also a variety of contaminant metals and organic compounds, as well as electrodes. The finding that diverse outer surface *c*-type cytochromes are capable of extracellular quinone reduction suggests that it may be rather simple to engineer microorganisms which have other desirable properties for this form of extracellular electron transfer.

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