Identification of Genes Involved in Cytochrome c Biogenesis in *Shewanella oneidensis*, Using a Modified *mariner* Transposon

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A modified *mariner* transposon, miniHimar RB1, was generated to mutagenize cells of the metal-reducing bacterium *Shewanella oneidensis*. The use of this transposon led to the isolation of stable mutants and allowed rapid identification of disrupted genes. Fifty-eight mutants, including BG104 and BG148 with transposon insertions in the cytochrome *c* maturation genes *ccmC* and *ccmF1*, respectively, were analyzed. Both mutants were deficient in anaerobic respiration and cytochrome *c* production.

*Shewanella oneidensis* is a gram-negative metal reducer that belongs to the γ group of the Proteobacteria (15, 33). It is a strict respirer that uses 14 different electron acceptors for respiration. These include oxygen, nitrate, and insoluble Fe(III) and Mn(IV) oxides and oxyhydroxides (9, 12, 15), among others. In addition, *Shewanella* species, including *S. oneidensis*, can reduce toxic metals, such as chromium, arsenate, and uranium (14, 26, 27, 34). The genome sequence of *S. oneidensis* contains 42 cytochrome *c* genes (6, 8). Some of these cytochromes are located in the outer membrane (10, 11), such as MtrC, a decaheme cytochrome that is involved in metal reduction (2).

We and other investigators have previously used Tn5 to generate mutants of *S. oneidensis* (2, 3, 24, 25). However, identification of genes disrupted by Tn5 can be difficult and time-consuming. Additionally, the presence of the transposase within the insertion sequence elements can result in instability of the mutants (personal observations). We have attempted to use miniTn5 to mutagenize *S. oneidensis* but were not successful. To overcome these problems, we modified a minimal *mariner* transposon to isolate mutants of *S. oneidensis*.

**Construction of pMiniHimar RB1 and isolation of *S. oneidensis* mutants.** Derivatives of the *mariner* transposon, Himar1, have been used to generate mutations in diverse organisms, such as *Escherichia coli*, *Mycobacterium smegmatis*, and *Myxococcus xanthus* (7, 23, 35). pMiniHimar1, a derivative of pMyk6K (23), was initially used in an attempt to isolate mutants of *S. oneidensis*, without success. pMiniHimar1 contains a defective Himar1 element (*magellan3*) which includes an R6Kγ origin of replication, a kanamycin resistance gene, and a transposase gene located downstream of a mycobacterial promoter (23). To modify pMiniHimar1, the mycobacterial promoter was removed by digestion with PvuI and NdeI and replaced with a 517-bp oriT-*Pinv* fragment that contains an origin of transfer (oriT) and the *lac* promoter (*Plac*) that we have shown to function in *S. oneidensis* (2). The *(oriT-* *Pinv*) fragment was obtained by crossover PCR using oriT amplified from pJB3CM6 (4) with the primers oriTF (CACATTAAATTGCGCTCACCCCGCTG CATAACCCTGCTT) and Pinv amplific from pBC SK+ (Stratagene) with lacF (GTGAGCGCAAGCATAATAT GTG) and lacR (GTTGATATGTTTCCTGTGTGAA ATT; NdeI site underlined). The resulting plasmid, pMiniHimar RB1 (Fig. 1), was used to generate *S. oneidensis* mutants, with a transposition efficiency of 3 × 10⁻⁵. Transfer of the plasmid by conjugation and isolation of mutants were performed as described previously (1, 2) except that wild-type *S. oneidensis* cells were used in the mating experiments. Analysis of 14 mutants by Southern transfer indicated that all had single transposon insertions (data not shown). To identify disrupted genes, chromosomal DNA was digested with BamHI, self-ligated, and then used to transform *E. coli* EC100D⁺ (Epigenent Technologies). Purified plasmid DNA was sequenced using the primer himar1 (CATTTAATACTAGCGACGCCATCT) and primer 615 (TCGGGTATCGCTCTTGAAGGG). Sites of transposon insertions in 58 mutants deficient in anaerobic respiration or metal reduction were identified. Some of the disrupted genes in these mutants have been previously identified and include genes encoding components of a type II secretion system, menaquinone biosynthesis proteins, and cytochromes, such as *mtrC* and *mtrA* (1, 2, 5, 13, 16, 24).

*Fig. 1.* Map of pMiniHimar RB1. The locations of *oriT-* *Pinv* upstream of the transposase gene, inverted repeats (IR), origin of replication (R6Kγ), and kanamycin (Km) resistance gene are indicated.
Complementation of the mutants restored their ability to use anaerobic growth with nitrate and nitrite (data not shown). They were also deficient in Fe(III) and Mn(IV) reduction and mutants were deficient in anaerobic growth with fumarate, tri- 

50 ml LB in 500-ml flasks with vigorous shaking for 3 h (early under aerobic and anaerobic conditions. Aerobic growth was in AGCAAGATT), cmfR (CAGTTGGAAAGCCGGAATAGG), and Expand high-fidelity polymerase (Roche Biochemical), CcmF from (32) (GenBank database entry U000008). CcmF is a heme lyase responsible for heme ligation to the apocytochrome (21). ccmF1 lies upstream of ccmG, ccmH, and a putative thioredoxin gene (TIGR locus no. SO0267, SO0268, and SO0269, respectively) (Fig. 2). A 2.58-kb fragment that contains ccmF1 was amplified by using cmfF (CGGCCTGGAAGCAAGATT), cmR (CAGTTGGAAAGCCGGAATAGG), and Expand high-fidelity polymerase (Roche Biochemical), cloned into pJB3Cm6 (4), and used to complement BG104. The ccm mutants, BG104 and BG148, were tested for anaerobic reduction or growth with different electron acceptors that are 50% or more identical to cytochrome c maturation proteins from other bacteria (see reference 31 for a review). CcmA and CcmB are components of an ABC transporter that is required for cytochrome c maturation, whereas CcmC is thought to bind heme and present it to the periplasmic heme chaperone CcmE (20, 22, 28, 29).

BG104 has a transposon insertion in ccmF1. CcmF1 is a protein of 660 amino acids (The Institute for Genomic Research [TIGR] locus number SO0266) that is 44% identical to CcmF from E. coli (32) (GenBank database entry U000008). CcmF is a heme lyase responsible for heme ligation to the apocytochrome (21). ccmF1 lies upstream of ccmG, ccmH, and a putative thioredoxin gene (TIGR locus no. SO0267, SO0268, and SO0269, respectively) (Fig. 2). A 2.58-kb fragment that contains ccmF1 was amplified by using cmfF (CGGCCTGGAAGCAAGATT), cmR (CAGTTGGAAAGCCGGAATAGG), and Expand high-fidelity polymerase (Roche Biochemical), cloned into pJB3Cm6 (4), and used to complement BG104. The ccm mutants, BG104 and BG148, were tested for anaerobic reduction or growth with different electron acceptors used by the wild type as described previously (25). Both mutants were deficient in anaerobic growth with fumarate, trimethylamine oxide, and dimethyl sulfoxide (DMSO) (Table 1). They were also deficient in Fe(III) and Mn(IV) reduction and in anaerobic growth with nitrate and nitrite (data not shown). Complementation of the mutants restored their ability to use these electron acceptors.

BG104 and BG148 were tested for cytochrome c production under aerobic and anaerobic conditions. Aerobic growth was in 50 ml LB in 500-ml flasks with vigorous shaking for 3 h (early log phase). Anaerobic growth was in LB supplemented with 50 mM lactate and 10 mM fumarate in a Coy anaerobic chamber. Because the ccm mutants do not grow anaerobically, the cultures were first grown aerobically, then transferred to an anaerobic chamber, and incubated for 4 h. Heme staining was performed using 3,3′,5,5′-tetramethyl benzidine dihydrochloride as described previously (30). Protein bands that exhibited heme peroxidase activity were detected in cell extracts of wild-type S. oneidensis grown aerobically and anaerobically but were absent from the ccm mutant cell extracts (Fig. 3). In addition to having a loss of c cytochromes, BG104 and BG148 were deficient in cytochrome c oxidase activity. Complementation restored this activity to both mutants (data not shown). Loss of cytochrome c oxidase activity has been observed in Bradyrhizobium japonicum and Paracoccus denitrificans mutants deficient in cytochrome c maturation (17–19).

The genome sequence of S. oneidensis contains two ccmF homologs, ccmF1 (described above) and ccmF2 (TIGR locus no. SO0478). Although CcmF1 and CcmF2 are 50% identical, loss of CcmF1 led to loss of c cytochromes under the growth conditions used in our studies. This finding suggests that CcmF1 may be the major heme lyase in S. oneidensis. The function of CcmF2 remains to be determined.

Concluding remarks. Modification of pMiniHimar1 by the introduction of orit and P_{lac} upstream of the transposase gene led to the isolation of a large number of S. oneidensis mutants. Multiple transposon insertions were not detected in the mutants that we analyzed. Additionally, the transposon insertions in these mutants were stable even in the absence of antibiotic selection. pMiniHimar RB1 has been successfully used to generate mutations in other bacteria, such as Xenorhabdus nematophilus (S. Forst, personal communication), and should be useful for the mutagenesis of other bacteria that are not amenable to electroporation or transformation.

Nucleotide sequence accession number. The HindIII fragment that was sequenced was assigned GenBank accession number AF044582.

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### TABLE 1. Anaerobic growth of wild-type S. oneidensis, ccm mutants (BG104 and BG148), and complemented mutants (BG104C and BG148C)

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Change in OD_{600}</th>
<th>Wild type</th>
<th>BG104</th>
<th>BG148</th>
<th>BG104C</th>
<th>BG148C</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.07</td>
<td>0.04</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.20</td>
<td>0.07</td>
<td>0.06</td>
<td>0.25</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>TMAO</td>
<td>0.23</td>
<td>0.06</td>
<td>0.06</td>
<td>0.25</td>
<td>0.24</td>
<td>0.24</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.29</td>
<td>0.05</td>
<td>0.06</td>
<td>0.29</td>
<td>0.23</td>
<td>0.23</td>
</tr>
</tbody>
</table>

a TMAO, trimethylamine oxide; DMSO, dimethyl sulfoxide.

b Numbers indicate changes in optical densities at 600 nm (OD_{600}) after 24 h incubation from three independent experiments.
REFERENCES


