

Regulation of two highly similar genes, *omcB* and *omcC*, in a 10 kb chromosomal duplication in *Geobacter sulfurreducens*

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The Fe(III)-reducing micro-organism *Geobacter sulfurreducens* requires an outer-membrane *c*-type cytochrome, OmcB, for Fe(III) reduction, but a related cytochrome, OmcC, which is 73 % identical to OmcB, is not required. The *omcB* and *omcC* genes are part of a tandem chromosomal duplication consisting of two repeated clusters of four genes. The 2.7 kb sequences preceding *omcB* and *omcC* are identical with the exception of a single base pair change. Studies that combined genetic, Northern blotting and primer extension analyses demonstrated that both *omcB* and *omcC* are transcribed as monocistronic and polycistronic (*orf1-orf2-omcB/omcC*) transcripts. All of the promoters for the various transcripts were found to be located within the 2.7 kb identical region upstream of *omcB* and *omcC*. The sequences of the promoter regions for the two monocistronic transcripts are identical and equidistant from the *omcB* or *omcC* start codons. The promoters for the two polycistronic transcripts, in contrast, are distinct. One is specific for transcription of *orf1-orf2-omcB* and the other is associated with transcription of *orf1-orf2-omcC*. Studies with an RpoS-deficient mutant suggested that transcription from all four promoters is RpoS dependent under one or more growth conditions. Deletion of *orfR*, a gene immediately upstream of *orf1-orf2-omcB* that encodes a putative transcriptional regulator, significantly lowered the *omcB* transcription when Fe(III) was the electron acceptor and partially inhibited Fe(III) reduction. In contrast, levels of *omcC* transcripts were unaffected in the *orfR* mutant. These results indicate that *omcB* and *omcC* operons represent a rare instance in which duplicated operons, located in tandem on the chromosome, have different transcriptional regulation.

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INTRODUCTION

Micro-organisms in the family *Geobacteraceae* are the predominant organisms in a wide variety of environments in which dissimilatory metal reduction plays an important role in the degradation of organic matter or the bioremediation of organic or metal contaminants (Lovley, 1991, 2000). Understanding the mechanism of Fe(III) reduction and the regulation of this process in *Geobacteraceae* is necessary in order to model these important environmental processes (Lovley, 2002, 2003). Microbial reducible Fe(III) is primarily present in the form of insoluble Fe(III) oxides in most soils and sediments (Lovley, 1991; Lovley *et al.*, 2004). The mechanisms for electron transfer to Fe(III) appear to be significantly different in phylogenetically distinct Fe(III) reducers (Childers *et al.*, 2002; Nevin & Lovley, 2000,

2002a, b). Present evidence suggests that *Geobacter* species must directly contact Fe(III) oxides in order to reduce them, and that electron transfer to Fe(III) oxides takes place at or near the outer membrane (Lovley *et al.*, 2004). This is consistent with the fact that *Geobacteraceae* have the ability to transfer electrons onto electrodes, which also represent an insoluble, extracellular electron acceptor (Bond *et al.*, 2002; Bond & Lovley, 2003).

Most studies investigating the mechanism of Fe(III) reduction in *Geobacteraceae* have been conducted with *Geobacter sulfurreducens*, because it is closely related to the *Geobacteraceae* that predominate in many subsurface environments and can readily be cultured in the laboratory. Furthermore, the complete genome sequence and a genetic system are available (Coppi *et al.*, 2001; Methe *et al.*, 2003) for this organism. OmcB is an outer-membrane *c*-type cytochrome that is required for Fe(III) reduction in *G. sulfurreducens* (Leang *et al.*, 2003). Deleting *omcB* greatly diminished Fe(III) reduction, and expressing *omcB* *in trans* restored Fe(III) reduction in proportion to the amount of OmcB produced. In contrast, deleting the gene for OmcC,

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are as follows. *orfR*, AAR36113. The *omcB* operon: *orf1*, AAR36111; *orf2*, AAR36110; *omcB*, AAR36109; *orfS*, AAR36107. The *omcC* operon: *orf1*, AAR36105; *orf2*, AAR36104; *omcC*, AAR36103.

another outer-membrane *c*-type cytochrome with 73% amino acid identity to OmcB, had no effect on Fe(III) reduction (Leang *et al.*, 2003). This difference in the function of these very similar *c*-type cytochromes is even more surprising when it is considered that *omcB* and *omcC* are contained within a 10 kb chromosomal duplication. The 2.7 kb sequences upstream of *omcB* and *omcC* are identical with the exception of a single base-pair change, and code for three consecutive open reading frames (*orfR/S*, *orf1* and *orf2*), a putative transcriptional regulator (OrfR/S), a protein of unknown function (Orf1) and a putative *c*-type periplasmic cytochrome (Orf2) (Leang *et al.*, 2003). In order to gain additional insight into the divergent functions of *omcB* and *omcC*, the transcriptional regulation of these genes was investigated. The promoter/operator regions of *omcB* and *omcC* were determined. In addition, the effects of two transcriptional regulators, RpoS and OrfR, on *omcB* and *omcC* expression were evaluated. The results of this study demonstrate that the transcriptional regulation of *omcB* and *omcC* differs substantially, further suggesting that the products of these closely related genes have different functions.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* strain JM109 [*endA1 recA1 gyrA96 thi hsdR17(r_K⁻ m_K⁺) relA1 supE44 Δ(lac-proAB) (F' *traD36 proAB lacI^qZΔM15*)] (Yanisch-Perron *et al.*, 1985) was cultured in LB medium at 37 °C with shaking. Targeted gene disruption experiments were performed on *G. sulfurreducens* strain DL1 (Caccavo *et al.*, 1994) to produce strains DL10 (*orfR::gm*) and DL11 (*orf1-orf2-omcC::kan*). RpoS-deficient mutant DLCN16 (*rpoS::kan*) was obtained from our laboratory culture collection (Nunez *et al.*, 2004). *G. sulfurreducens* strains were cultured anaerobically at 30 °C in either acetate/fumarate (NBAF) or acetate/Fe(III) citrate (FWAFC) medium as previously described (Coppi *et al.*, 2001).*

DNA and RNA manipulations. Genomic DNA was extracted with the MasterPure Complete DNA & RNA Purification kit (Epicentre Technologies). Plasmid DNA and PCR products were purified with the Qiagen mini plasmid purification kits and Qiagen PCR purification kits, respectively. DNA cloning and other manipulations were carried out according to the methods outlined by Sambrook *et al.* (1989). Restriction enzymes and other DNA-modifying enzymes were purchased from New England Biolabs. Probes for Southern or Northern blot analysis were labelled with [α -³²P]dCTP with the NEBlot kit (New England BioLabs). [α -³²P]dCTP was purchased from PerkinElmer Life and Analytical Sciences. Qiagen *Taq* DNA polymerase was used for all PCR amplifications.

Total RNA was isolated from mid-exponential-phase cultures with RNeasy Midi kits (Qiagen) followed by treatment with RNase-free DNase (Ambion). Northern blot analyses were performed with the Northern Max-Gly system (Ambion) using dsDNA probes according to the manufacturer's instructions. The *omcB*-specific probe (435 bp) was amplified with primers 8916 (5'-GGACTGCGCACCATCAAGG-3'), corresponding to +580 to +598 of the *omcB* gene, and 8908-2 (Leang *et al.*, 2003). An *omcC*-specific probe (553 bp) was amplified with primers 8914 (5'-GCCAGAGTGAGGCCCAAGA-3'), corresponding to +285 to +302 of the *omcC* gene, and 8915 (Chin *et al.*, 2004).

Single-step gene replacement (Fig. 1). Genes were deleted by single-step gene replacement as previously described (Lloyd *et al.*, 2003). To disrupt the *orf1-orf2-omcC* cluster, a 2.28 kb linear DNA fragment was generated by recombinant PCR (Lloyd *et al.*, 2003; Murphy *et al.*, 2000) from three primary PCR products: (1) the sequence upstream of *orf1* of the *omcC* cluster [560 bp, -1138 to -578, amplified with primers Triple1 (5'-GCAAGCAGATCAT-GCTTTCC-3') and Triple2 (5'-GTGATCGCTTCTTCGAGAAG-3')]; (2) the 3' end of *omcC* [606 bp, +1471 to +2077, amplified with primers 8901 and 8902 (Leang *et al.*, 2003)]; and (3) a kanamycin resistance (*kan^R*) cassette [1114 bp, amplified with primers Triplekan (5'-CTTCTCGAAGAAGCGATCACACCTGGGATGAATGTCAGC-3') and 8905 (Leang *et al.*, 2003), using plasmid pBBR1MCS-2 (Kovach *et al.*, 1995) as template]. Recombinant PCR was carried out with these three PCR products as templates with distal primers Triple1 and 8901. PCR conditions were as previously described (Lloyd *et al.*, 2003), except that the annealing temperature was 58 °C.

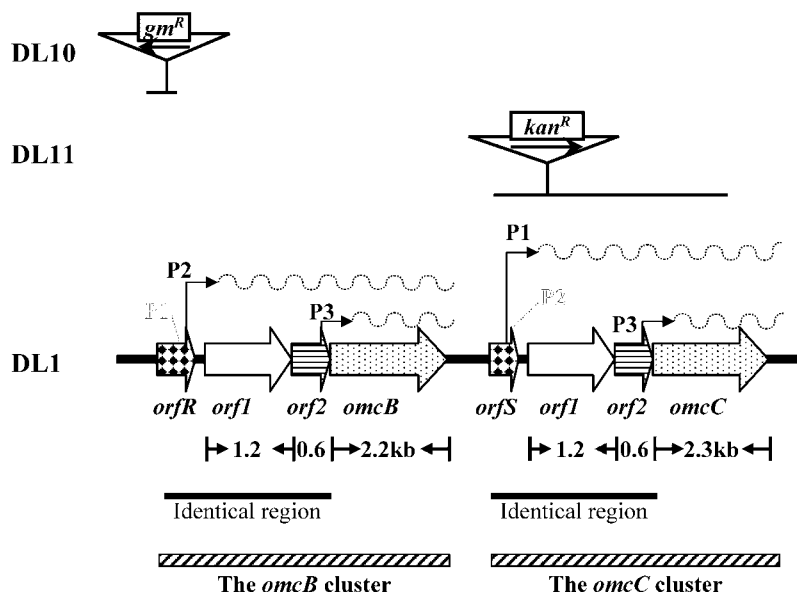


Fig. 1. Organization of the *omcB* and *omcC* clusters, mutation schemes, and predicted promoters for transcription of *omcB* and *omcC*. The *omcB* and *omcC* clusters are indicated by boxes with diagonal lines. Identical 2.7 kb sequences upstream of both the *omcB* and *omcC* genes are represented by a thick black bar. Gene replacement is indicated by a horizontal bar. The transcriptional direction of the *gm^R* or *kan^R* cassettes is indicated by a bold arrow. The 5' of *orfR* was deleted and replaced with a *gm^R* cassette, resulting in strain DL10 (*orfR::gm*), and the *orf1-orf2-omcC* cluster was replaced with a *kan^R* cassette, resulting in strain DL11 (*orf1-orf2-omcC::kan*). The predicted promoters for the *omcB* (P2 and P3) and *omcC* (P1 and P3) clusters are indicated with arrows followed by dotted wavy lines representing the *omcB* or *omcC* transcripts.

To disrupt the *orfR* gene, a similar strategy was used. Three primary PCR reactions were performed to amplify a 1.8 kb linear DNA fragment: (1) the upstream sequence of the *orfR* gene [507 bp, -522 to -16, amplified with primers OP1-for (5'-GGGAGTACAACCTCTC-GG-3') and OrfR-a (5'-GCTGACTATGTAAGCGCTTGC-3')]; (2) the 3' end of the *orfR* gene [517 bp, +284 of the *orfR* gene to +30 of the *orfI* gene, amplified with primers OrfR-b (5'-CGCTGATCCAGCTG-AAGGAC-3') and Orf1-C (5'-GGAGCAGTCGAGTGAACCC-3')]; and (3) the gentamicin resistance (*gm^R*) cassette [802 bp, amplified with primers Gmfor (5'-GTCCTTCAGCTGGATCAGCGATTGACA-TAAGCCTGTTCGG-3') and Gmrev (5'-GCAAGCGCTTACATAGT-CAGCAGCTCGAATTGACGCGTC-3'), using plasmid pBSL141 (Alexeyev *et al.*, 1995) as template]. Recombinant PCR was performed as described above by combining all the three primary products with distal primers OP1-for and Orf1-C. PCR conditions were identical, except that the annealing temperature was 55 °C.

Electroporation, mutant isolation and genotype confirmation were performed as previously described (Coppi *et al.*, 2001; Lloyd *et al.*, 2003). One of each of the mutants, designated DL10 (*orfR::gm*) and DL11 (*orf1-orf2-omcC::kan*), was chosen as the representative strain (Fig. 1).

Expression of *orfR* in trans. The complete *orfR* coding sequence was amplified with primers OrfRexpSphI-F (5'-GGGCATGCCAAG-CGCTTACATAGTACG-3', *SphI* site italicized) and OrfRexpBglIII-R (5'-GGAGATCTCAACCGTTGATCACTTTTGG-3', *BglIII* site underlined) under the following conditions: 96 °C, 40 s followed by 25 cycles of 96 °C, 40 s; 58 °C, 1 min; 72 °C, 1 min; and a final extension at 72 °C for 10 min. The PCR product of the *orfR* coding sequence was digested with *SphI* and *BglIII* and inserted into the *SphI* and *BamHI* sites of the expression vector pCM66 (Marx & Lidstrom, 2001) via ligation; the resulting plasmid was designated pCM-*orfR*. The *orfR* gene was then sequenced to screen for PCR artifacts.

Following electroporation of strain DL10 with pCM-*orfR*, a kanamycin-resistant transformant was isolated and designated DL10/pCM-*orfR*. The simultaneous presence of both the plasmid pCM-*orfR* and the *orfR::gm* mutation in this strain were confirmed by PCR.

Primer extension analysis. Total RNA was isolated from *G. sulfurreducens* as described above. For identification of the P1 and P2 promoters, primer extension experiments were performed at 55 °C using ThermoScript reverse transcriptase (Invitrogen) with primer Orf1-C (complementary to +13 to +31 of *orfI* in both the *omcC* and *omcB* clusters; sequence described above). To define the P3 promoters, primer extension experiments were carried out at 42 °C using RAV2 reverse transcriptase (Amersham Biosciences) with primer OP2b-rev (5'-CGAAGCTGTACTTCTGACC-3', complementary to nucleotides -256 to -237 of *omcB*). The sequencing ladders presented in Figs 3 and 4 were also generated with these same primers using the Thermo Sequenase Cycle sequencing kit (USB) with either plasmids pTOPO-op1 or pTOPO-op2 as template.

Plasmid pTOPO-op1 was constructed by inserting a 1.2 kb PCR fragment consisting of 800 bp of *orfR* upstream sequence and the first 380 bp of the *orfI* gene [amplified with primers OrfR-for and Orf1-rev (5'-GCGTTGTTCCAGTCTACCTG-3')] into the pCR2.1-TOPO vector (Invitrogen). Plasmid pTOPO-op2 was constructed by inserting a 1.2 kb PCR fragment consisting of the last 374 bp of the *orf2* gene and the first 862 bp of *omcB* [amplified with primers Orf2-for (5'-GCAACTTCCTGAGCATCCACC-3') and 8908 (5'-GCGTTGGT-GCCACTTGCACC-3')] into the pCR2.1-TOPO vector (Invitrogen).

Analytical techniques. Fe(II) concentrations were determined with the ferrozine assay as previously described (Lovley & Phillips, 1986). Cell densities of Fe(III)-grown cultures were determined with

epifluorescence microscopy (Lovley & Phillips, 1988). Protein concentration was determined by the bicinchoninic acid method with BSA as a standard (Smith *et al.*, 1985).

RESULTS

Transcriptional organization and initiation sites of the *omcB* and *omcC* clusters

Northern blot analyses with *omcB*- or *omcC*-specific probes revealed two transcripts of about 5 and 2.5 kb for both the *omcB* and *omcC* genes (Fig. 2a, b; lanes 1 and 2). In accordance with previously reported protein data (Leang *et al.*, 2003), both genes were expressed when either fumarate or Fe(III) was the terminal electron acceptor. The *omcB* transcripts were about six to eight times higher during growth with Fe(III) than they were with fumarate (Fig. 2a, lanes 1 and 2). In contrast, the levels of *omcC* transcripts were nearly equivalent during growth on either electron acceptor (Fig. 2b, lanes 1 and 2).

Primer extension studies were performed to define the transcriptional initiation sites of the *omcB* and *omcC* clusters. The primers used to synthesize cDNA for these studies were complementary to the 2.7 kb identical sequence found in both the *omcB* and *omcC* clusters, and were designed to amplify both the long (primer Orf1-C, 5'-GGAGCAGTCGAGTGAACCC-3', complementary to the 5' end of *orfI*) and short (primer OP2b-rev, 5'-CGAAGCTGTACTTCTGACC-3') transcripts of the *omcB* and *omcC* clusters. Two primer extension products were

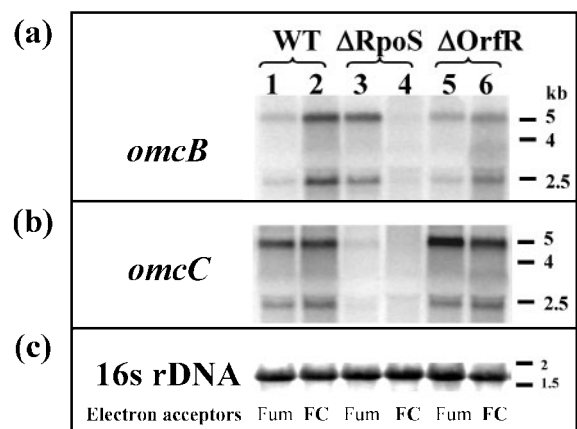


Fig. 2. Northern blot analyses comparing *omcB* and *omcC* expression profiles in wild-type, RpoS-deficient (DLN16) (Nunez *et al.*, 2004) and OrfR-deficient (DL10) mutants. Total RNA (5 µg) was isolated from mid-exponential-phase cells grown in medium with acetate as the electron donor and either fumarate (odd lanes) or Fe(III) (even lanes) as the electron acceptor, and was hybridized with ³²P-labelled probes for (a) *omcB*, (b) *omcC* or (c) 16S rDNA as indicated. Lanes 1 and 2, wild-type. Lanes 3 and 4, DLN16 (*rpoS::kan*). Lanes 5 and 6, DL10 (*orfR::gm*).

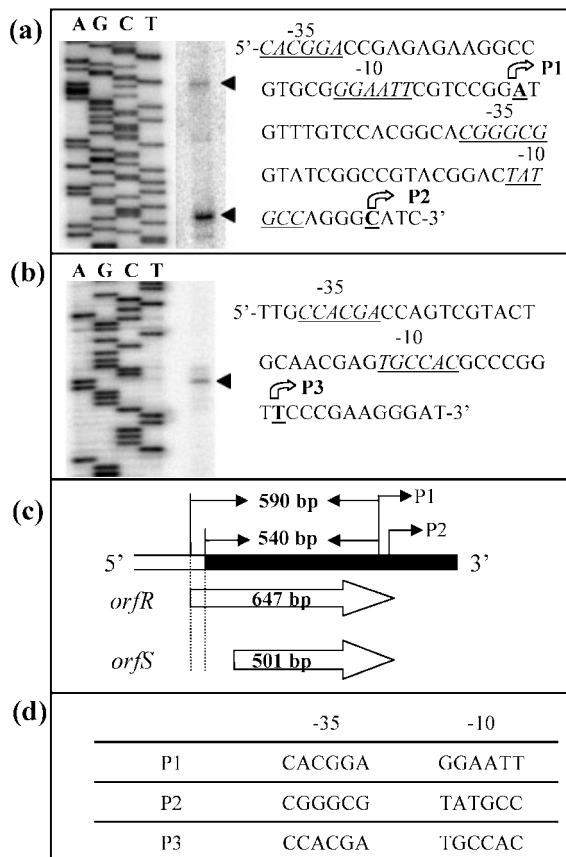


Fig. 3. Mapping of the 5' ends of the long and short transcripts of *omcB* and *omcC* clusters by primer extension analysis. Total RNA (40 μ g) was hybridized with either (a) Orf1-C, complementary to 5' of *orf1*, to investigate the transcription start site of the long transcript, or (b) OP2b-rev, complementary to upstream of *omcB/C* and extended by reverse transcriptase. Precise base mapping was done by comparing the migration of the extended product with a parallel sequencing reaction primed by an identical oligonucleotide. (a) Two transcription start sites were identified for the long transcript (5 kb), designated P1 and P2. (b) One transcription start site was identified for the short transcript (2.5 kb), designated P3. Potential promoter regions, -10 and -35, are italicized and underlined. (c) Relative distance between P1, 5'-end of the identical sequences of the *omcB* and *omcC* clusters and the start codon of either OrfR or OrfS. Open arrows represent the coding regions of *orfR* and *orfS* from the *omcB* and *omcC* clusters, respectively. The filled area represents the beginning of the identical sequences of *omcB* and *omcC* clusters. (d) Potential -35/-10 regions of P1, P2 and P3 are summarized.

identified for the long transcript (Fig. 3a) and one for the short transcript (Fig. 3b). These three promoter regions were designated P1 and P2 for the long transcripts, and P3 for the short transcripts. The mRNA start sites for P1 and P2 were located at the 3' ends of the *orfR* and/or *orfS* genes, the last 57 and 8 nt, respectively, and 200 and 151 nt upstream, respectively, of the two *orf1* start codons. In relation to the

positions of the identical sequences of the *omcB* and *omcC* clusters, the start sites for P1 were located 540 bp downstream of the beginning of the identical sequences, 590 bp downstream of the OrfR start codon and 444 bp downstream of the OrfS start codon (Fig. 3c). The mRNA start site for P3 was within the *orf2* coding regions, 307 nt upstream of the *omcB* and *omcC* start codons. These results, combined with results based on Northern blot analyses, indicated that the long transcript (5 kb) resulted from the co-transcription of the *orf1*, *orf2* and *omcB/omcC* genes, but not the *orfR* or *orfS* genes. The short transcripts, in contrast, were monocistronic (2.5 kb), consisting solely of *omcB* or *omcC*.

The finding that promoters P1 and P2 were located in the duplication region of the *orf1-orf2-omcB* and *orf1-orf2-omcC* clusters led to the question of whether there is a difference in transcriptional start sites for the 5 kb transcripts for the two clusters. In order to evaluate this, an approximately 5 kb chromosomal DNA fragment containing the *orf1-orf2-omcC* cluster was deleted and replaced with a kanamycin-resistance cassette (Fig. 1). The *omcC* cluster-deficient mutant was designated DL11 (*orf1-orf2-omcC::kan*). As expected, the deletion of the *orf1-orf2-omcC* cluster did not affect the abilities of cells to grow in medium containing acetate as the electron donor and either fumarate or Fe(III) citrate as the electron acceptor (data not shown). This result is not surprising given the presence of a second copy of *orf1* and *orf2* upstream of *omcB* and the fact that OmcC is not essential for fumarate or Fe(III) reduction (Leang *et al.*, 2003).

Primer extension analysis indicated that the remaining *orf1-orf2-omcB* cluster in strain DL11 (*orf1-orf2-omcC::kan*) was preferentially transcribed from P2 (Fig. 4a, lanes 3 and 4). Transcriptional initiation from P1 was greatly decreased in strain DL11 with respect to wild-type. These results suggest that expression of the *orf1-orf2-omcB* cluster is initiated from promoter P2, whereas P1 is mainly responsible for expression of the *orf1-orf2-omcC* cluster (summarized in Fig. 1).

The P1, P2 and P3 regulatory regions are RpoS dependent

The -35 sequences of P1, P2 and P3 (Fig. 3) are GC rich, similar to the promoters which are recognized by RNA polymerase containing RpoS in *E. coli* (Wagner, 2000). In order to determine whether transcription from P1, P2 and P3 was RpoS-dependent, Northern blot analyses were carried out on the wild-type strain and an RpoS-deficient mutant (DLCN16) (Nunez *et al.*, 2004). The *omcC* transcripts were hardly detectable (0 to about 7% of wild-type intensity) in the RpoS-deficient mutant supplied with either fumarate or Fe(III) as the electron acceptor (Fig. 2b, lanes 3 and 4). Expression of *omcB* in the RpoS-deficient mutant was also very low (3% of wild-type level) during growth on Fe(III) as the electron acceptor (Fig. 2a, lane 4). However, during growth on fumarate (Fig. 2a, lane 3), the levels of the

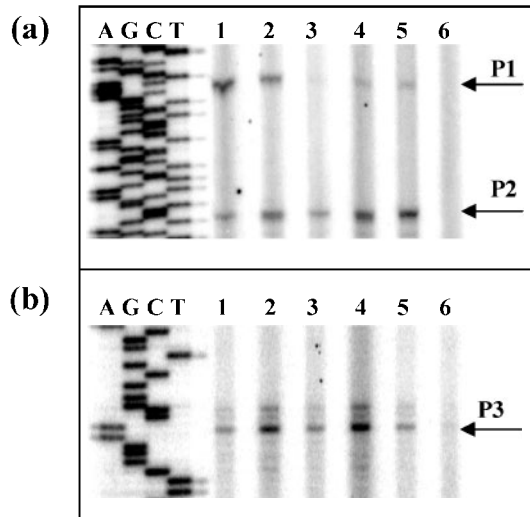


Fig. 4. Primer extension analyses comparing DL11 (*orf1-orf2-omcC::kan*), DLCN16 (RpoS-deficient mutant) (Nunez *et al.*, 2004) and wild-type. Primer extension products were derived from total RNA (40 μ g) isolated from cells grown in medium with either fumarate (odd lanes) or Fe(III) citrate (even lanes) as sole electron acceptor. Lanes 1 and 2, wild-type (DL1); lanes 3 and 4, DL11 (*orf1-orf2-omcC::kan*); lanes 5 and 6, RpoS-deficient mutant. (a) Promoters P1 and P2 from long transcripts; (b) Promoter P3 from short transcripts.

omcB transcripts were much higher (about 500%) in the RpoS-deficient mutant than the wild-type strain.

Similar results were also obtained from primer extension analyses. No products corresponding to P1, P2 or P3 were detectable when total RNA was isolated from the Fe(III)-grown, RpoS-deficient mutant (Fig. 4a, b, lane 6). Primer extension products corresponding to P2 and P3 were detected with the fumarate-grown RpoS-deficient mutant, while P1 was hardly detectable (Fig. 4a, b, lane 5). This result further supports the hypothesis that P1 is the primary promoter for the *omcC* cluster, whereas P2 serves as the promoter for the *omcB* cluster.

Identification and characterization of a potential transcriptional regulator of the *omcB* and *omcC* clusters

The ORF (647 bp) upstream of the *orf1-orf2-omcB* cluster, designated *orfR*, encodes a putative TetR family transcriptional regulator, which contains an N-terminal DNA-binding domain (helix–turn–helix, HTH) connected to a non-homologous ligand-binding domain (Grkovic *et al.*, 2002). The N-terminal HTH domain of OrfR is conserved and can be aligned well with N-terminal HTH domains from other TetR family regulators (data not shown). There is also a potential ORF (named *orfS*) upstream of the *orf1* gene of the *omcC* cluster. The ORF of the *omcC* cluster contains only the last third of the DNA-binding HTH domain in its

N-terminus (Wisconsin Package version 10.2; Genetics Computer Group, Madison, WI, USA). Therefore, OrfS was not considered to be a potential transcriptional regulator for *omcB/C* expression and was not studied further.

In order to determine whether OrfR functions as a transcriptional regulator of *omcB* and/or *omcC* expression, an OrfR knockout mutant was constructed (DL10, *orfR::gm*). The OrfR-deficient mutant (DL10) grew as well as the wild-type when fumarate was supplied as the electron acceptor (data not shown). The Fe(III) reduction rate of the OrfR-deficient mutant (DL10) was approximately 55% of that of the wild-type when cells were grown in medium containing Fe(III) as the electron acceptor (Fig. 5). When *orfR* was reintroduced into the OrfR-deficient mutant *in trans*, growth of the complemented strain (DL10/pCM-*orfR*) on Fe(III) was similar to that of the wild-type (Fig. 5).

Associated with the inhibition of Fe(III) reduction in the OrfR-deficient mutant was a decrease in levels of both polycistronic and monocistronic *omcB* mRNA (Fig. 2a, lane 6) to only 42% of wild-type. In contrast, the mutant had levels of *omcC* mRNA that were similar to wild-type during growth on Fe(III) (Fig. 2b, lane 6). During growth on fumarate, the levels of *omcC* transcripts in the mutant were slightly higher than in wild-type (Fig. 2b, lane 5), whereas the levels of *omcB* transcripts in the mutant were similar to wild-type (Fig. 2a, lane 5).

DISCUSSION

Not only are the functions of OmcB and OmcC different (Leang *et al.*, 2003), but also, as shown in this study, the regulation of the *omcB* and *omcC* genes is different. As discussed in detail below, the expression of *omcB* and *omcC*

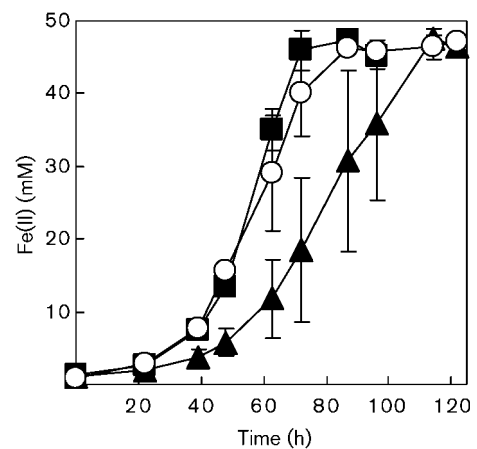


Fig. 5. Fe(III) reduction of wild-type (■), DL10 (*orfR::gm*) (▲) and the complemented strain DL10/pCM-*orfR* (○) in medium containing acetate as the electron donor and Fe(III) citrate as the electron acceptor. Data are means \pm SD of triplicate values.

is under the control of RpoS and possibly a putative transcriptional regulator, OrfR. The results suggest that expression of *omcB* is correlated to Fe(III) respiration whereas the expression of *omcC* is not. In addition, this study aids the further understanding of the controls of dissimilatory metal reduction, as this is believed to be the first report on the regulation of genes whose products are involved in Fe(III) reduction in *G. sulfurreducens*.

Transcription of *omcB* and *omcC*

The results suggest that, under the conditions evaluated, both *omcB* and *omcC* are expressed and are transcribed in a similar manner, as both a monocistronic (*omcB/C*) and a polycistronic (*orf1-orf2-omcB/C*) transcript. The combination of Northern blot and primer extension analyses identified the 2.5 kb transcripts of the *omcB* and *omcC* genes. The results suggest that the transcription initiation site for both of the two monocistronic transcripts is P3, which is found 307 bp upstream of both *omcB* and *omcC* and is located within a 2.7 kb duplication region that is virtually identical in both the *omcB* and *omcC* clusters. The mRNA start sites for the long transcripts, P1 and P2, are also located within the 2.7 kb identical region. The finding that the primer extension product P1 was missing when the *orf1-orf2-omcC* cluster was deleted from the genome suggests that the polycistronic *orf1-orf2-omcC* transcript is initiated from P1, whereas *orf1-orf2-omcB* is initiated from P2. The reasons for this are as yet unknown, but it is possible that there are transcriptional regulators which recognize divergent sequences upstream of the *omcB* or *omcC* cluster but not the other.

Other results also suggest that expression of the *omcB* and *omcC* genes is regulated differently. Levels of both *omcB* transcripts are higher when Fe(III) serves as the electron acceptor than during growth on fumarate, whereas those of *omcC* are similar. In addition, the absence of two transcriptional regulators, RpoS and OrfR, had different effects on *omcB* and *omcC* expression under specific conditions. In the presence of fumarate as an electron acceptor, elimination of *rpoS* led to an increase in *omcB* expression but a dramatic decrease in *omcC* expression. During growth on fumarate, deletion of *orfR* increased the expression of the long *omcC* transcript but did not affect *omcB* expression. Likewise, during growth on Fe(III), *omcB* expression decreased whereas *omcC* expression was relatively unaffected.

During growth on Fe(III), *omcB* and *omcC* transcripts were hardly detectable in the RpoS-deficient mutant, suggesting that RpoS regulates transcription of all four promoters, P1_{omcC}, P2_{omcB}, P3_{omcB} and P3_{omcC}. Regulation of *omcB* and *omcC* expression by RpoS is consistent with the fact that the sigma factor RpoS of *G. sulfurreducens* has high similarity to the sigma factor RpoS of *E. coli* and other Gram-negative bacteria (Nunez *et al.*, 2004), and that the -35 regions of P1_{omcC}, P2_{omcB} and P3_{omcB} (P3_{omcC}) are GC rich (4/6, 6/6 and 4/6 respectively), similar to those recognized by RNA

polymerase containing RpoS in *E. coli*. However, a potential alternative explanation for the observed results is that other regulator(s), which might be RpoS dependent, control the transcription initiation of *omcB* and/or *omcC* operons. For example, during growth on fumarate, *omcB* transcript levels in the RpoS mutant were higher than in the wild-type, whereas transcription of *omcC* still appeared to be inhibited. This suggests that at least one other regulator, which may be RpoS dependent, functioned as a repressor of *omcB* transcription in the absence of Fe(III) and further emphasizes that the primary function of OmcB is probably electron transfer to Fe(III).

Other potential global regulators appear to be involved in regulation of the *omcB* and/or *omcC* operons. Whole-genome microarray analyses have revealed that a mutant of *G. sulfurreducens* in which *fur* (ferrous uptake regulator) was deleted had higher levels of *omcB* transcripts, but not *omcC* transcripts, during growth on fumarate with limited iron (R. O'Neil, unpublished results). A potential *fur* box was found 80 bp upstream of the P1 transcriptional start site (J. Krushkal, personal communication). Furthermore, *omcB/omcC* expression may also be related to the level of ppGpp in the cells (L. DiDonato, unpublished results). In the absence of RelA (ppGpp synthetase I) during growth on fumarate, the expression of *omcB* was up-regulated whereas that of *omcC* was not.

Another factor potentially controlling transcription of *omcB*, and hence Fe(III) reduction, appears to be the product of *orfR*. The notable effect of deleting *orfR* was a partial inhibition of Fe(III) reduction associated with lower levels of *omcB* transcripts. Deleting *orfR* did not have a significant effect on levels of *omcC* transcripts during growth on Fe(III). This suggests that the product of *orfR* may be an activator for *omcB* transcription, but only during growth on Fe(III), and further emphasizes that the function of OmcB is related to Fe(III) reduction. The combination of results suggests that OrfR may function as a modulator to fine tune the expression of the *omcB/C* operon under different growth conditions. Clearly, the mechanisms by which OrfR functions in regulating the expression of these genes warrant further study.

The genome of *G. sulfurreducens* contains genes for over 100 *c*-type cytochromes (Methe *et al.*, 2003) and many of these appear to have arisen as the result of gene duplications. The high degree of identity between all of the components of the *omcB* and *omcC* operons (79–100%) suggests that this duplication occurred on a relatively recent evolutionary time scale. However, the regulation of *omcB* and *omcC* is markedly different. The results presented here, as well as functional analysis of other *c*-type cytochrome genes in *G. sulfurreducens* (L. DiDonato, unpublished results), suggest that many of the cytochromes with similar gene sequences have different functions and/or are differentially regulated. Investigation into the function and regulation of these duplicated genes will aid further understanding of the physiology of *G. sulfurreducens*.

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