Investigation of direct vs. indirect involvement of the c-type cytochrome MacA in Fe(III) reduction by Geobacter sulfurreducens

Byoung-Chan Kim & Derek R. Lovley
Department of Microbiology, 203 Morrill Science IVN, University of Massachusetts, Amherst, MA, USA

Correspondence: Byoung-Chan Kim, Department of Microbiology, 203 Morrill Science IVN, University of Massachusetts, Amherst, MA 01003, USA. Tel.: +1 413 577 0217; fax: +1 413 545 1578; e-mail: bckim@microbio.umass.edu

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Abstract
The electron transfer pathway to Fe(III) reduction in Geobacter sulfurreducens has been hypothesized to consist of a series of c-type cytochromes. Previous genetic studies suggested that the inner membrane-associated, c-type cytochrome, MacA, was a component of the electron transfer chain leading to Fe(III) reduction in the dissimilatory Fe(III)-reducer, G. sulfurreducens. However, investigation of the expression of OmcB, an outer-membrane c-type cytochrome demonstrated previously to be critical for optimal Fe(III) reduction, revealed that both omcB transcript and protein levels were dramatically reduced in the MacA-deficient mutant. Expression of the omcB gene in trans enabled the MacA-deficient mutant to reduce Fe(III) at a rate that was proportional to the level of omcB expression. These results suggest that MacA is not directly involved in electron transfer to Fe(III) and further confirm the importance of OmcB in Fe(III) reduction by G. sulfurreducens.

Introduction
Elucidation of the mechanisms for dissimilatory Fe(III) reduction has been stymied by the fact that many redox-active proteins will nonspecifically reduce Fe(III) in vitro (Petrat et al., 2003; Schröder et al., 2003; Filisetti et al., 2005; Coppi et al., 2007). Thus, although it is possible to purify proteins that can transfer electrons to Fe(III) in vitro (Magnuson et al., 2000; Childers & Lovley, 2001; Kaufmann & Lovley, 2001; Möller & van Heerdern, 2006; Shi et al., 2006), this is no guarantee of in vivo relevance. An alternative is the genetic approach in which genes encoding suspected Fe(III) reductases are deleted and the impact on Fe(III) reduction assessed. However, even when polar effects of making the mutation can be discounted, this approach has the often untested, underlying assumption that removing the putative redox protein under investigation does not negatively impact the expression of other proteins involved in Fe(III) reduction.

For example, the role of the c-type cytochromes of Geobacter sulfurreducens in Fe(III) reduction have been extensively investigated, first with the biochemical (Magnuson et al., 2000, 2001; Kaufmann & Lovley, 2001) and then with the genetic (Leang et al., 2003; Lloyd et al., 2003; Butler et al., 2004; Kim et al., 2005, 2006; Mehta et al., 2005) approach. Geobacter sulfurreducens is of interest because (1) it is closely related to the Geobacter species that predominate in subsurface environments in which Fe(III) reduction is important (Coates et al., 1996; Lonergan et al., 1996; Snoeyenbos-West et al., 2000); (2) its genome sequence is available (Methe et al., 2003); and (3) it is genetically tractable (Coppi et al., 2001). A series of genetic studies identified c-type cytochromes that are required for Fe(III) reduction in the inner membrane [MacA (Butler et al., 2004)], the periplasm [PpcA (Lloyd et al., 2003)], and the outer membrane [OmcB (Leang et al., 2003); OmcS (Mehta et al., 2005); and OmcE (Mehta et al., 2005)]. MacA, PpcA, and OmcB are required for the reduction of soluble, chelated Fe(III) as well as poorly soluble Fe(III) oxide, whereas OmcS and OmcE are only required for Fe(III) oxide reduction. From such results, it is possible to envision that these cytochromes might be part of an electron transport chain moving electrons from the inner membrane to the outer surface of the cell (Lovley, 2006).

More recent studies, however, have demonstrated that deleting genes for some c-type cytochromes in G. sulfurreducens can negatively impact either the transcription or the translation of other cytochromes. Deletion of the gene for the outer membrane cytochrome, OmcF, prevented proper omcB transcription (Kim et al., 2005), while deletion of the outer membrane cytochromes, OmcG and OmcH, inhibit
synthesis or posttranslational modification of OmcB (Kim et al., 2006). The lack of OmcB in these mutants was the likely explanation for the inhibition of Fe(III) reduction. These results suggested that previously reported deletions of c-type cytochromes, shown to have an impact on Fe(III) reduction, should be re-evaluated to determine whether the impact of the deletion on Fe(III) reduction might be less direct than assumed previously. In this study, the major c-type cytochromes mutants that were previously suggested to be directly involved in electron transfer to Fe(III) in *G. sulfurreducens*, based on gene deletion studies, were reinvestigated in order to verify whether they were directly related to electron transfer to Fe(III) reduction.

**Materials and methods**

**Bacterial strains and culturing conditions**

*Escherichia coli* strain DH5α [supE44AlacU169 (q80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 recA1] (15, 38) and *E. coli* strain TOP10 [F–mcrA8 (mrr-hsdRMS-mcrBC) q80lacZΔM15AlacX74 recA1 deoR araD139Δ (ara-leu) 7697 galU galK rpsL (Str<sup>R</sup>) endA1 supG] (Invitrogen Co., Carlsbad, CA) were used for DNA manipulations and PCR cloning and other DNA manipulations were carried out using Mini Plasmid purification kits, PCR purification kits, and Qiaquick gel extraction (Qiagen Inc., Beverly, MA). Plasmid DNA purification, PCR product cloning, respectively. *Geobacter sulfurreducens* strains DL1 (Caccavo et al., 1994), DL1-macA (macA::kan) (Butler et al., 2004), DL3 (ppcA::kan) (Lloyd et al., 2003), DL6 (omcB::cam) (Leang et al., 2003), DLM1 (omcS::spec) (Mehta et al., 2005), DLMC8 (omcE::kan) (Mehta et al., 2005), DL1-macA/pJBG-macA (Butler et al., 2004), and DL1-macA/pRG5-omcB (this study) were cultured anaerobically in either acetate–fumarate or acetate–Fe(III) citrate medium as described previously (Coppi et al., 2001).

**DNA manipulations**

Genomic DNA was purified using the MasterPure Complete DNA and RNA purification kit (Epicentre Technologies, Madison, WI). Plasmid DNA purification, PCR product purification, and gel extractions were carried out using Mini Plasmid purification kits, PCR purification kits, and Qiaquick gel extraction kit (Qiagen Inc., Valencia, CA). DNA cloning and other DNA manipulations were carried out according to Sambrook et al., 1989. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). All primers were purchased from Sigma-Genosys (the Woodlands, TX). Taq DNA polymerase (Qiagen Inc.) was used for all PCR amplifications.

**Expression of the omcB gene in trans in the MacA-deficient mutant**

The complete omcB coding sequence was amplified with primers omcBExpF (5′-AATTGAGAGCACACACACC-3′) and omcBExpR (5′-AAGCTTTATTACGGACGGTGTCGTG CC-3′, underline indicates HindIII restriction site). The omcB-coding sequence was cloned into pCR 2.1-TOPO (Invitrogen), excised with EcoRI and HindIII, and inserted into the EcoRI and HindIII sites of pRG5 (Kim et al., 2005) to generate the omcB expression vector, pRG5-omcB, in which omcB was constitutively expressed with tSacI promoters (Kim et al., 2005). Electrotransformation was performed and correct representative strains (DL1-macA/pRG5-omcB) were selected from the spectinomycin-resistant colonies as described previously (Coppi et al., 2001).

**Western blot analysis for detection of OmcB**

Membrane-enriched protein fractions (10 μg lane<sup>−1</sup>) were prepared by sarkosyl extraction (Nikaido, 1994; Kim et al., 2005) and separated by 7.5% Tris-Tricine gel electrophoresis, immunoblotted, and probed with the OmcB-specific antiserum as reported previously (Kim et al., 2006).

**Quantitative reverse transcription PCR (qRT-PCR) and Northern blot analysis for measuring omcB expression**

qRT-PCR analysis was performed to measure the expressed amount of omcB transcripts. Total RNA was purified from mid-log cultures using RNeasy Mini kits (Qiagen Inc.) with an on column DNAse digestion. The DuraScript enhanced avian RT single-strand synthesis kit (Sigma-Aldrich Co., St Louis, MO) was used to generate cDNA with random primers, and the cDNA generated by RT-PCR was used for qRT-PCR amplification as described previously (Holmes et al., 2006). Detection of amplified qRT-PCR products was performed with the GeneAmp 5700 sequence detection system (PE Biosystems, Foster City, CA) according to the manufacturer’s instructions. To verify amplification and correct amplicon size, aliquots from qRT-PCR were examined on an ethidium bromide-stained 2% agarose gel. Equivalent expression of recA, which was shown previously to be constitutively expressed in *G. sulfurreducens* (Holmes et al., 2004, 2005), was confirmed in both the wild-type and the MacA-deficient mutant by qRT-PCR analysis (data not shown). As a result, it was used as an endogenous control for relative qRT-PCR analysis for measuring the abundance of omcB transcripts. Primers 8912 (Chin et al., 2004) and 8908-2 (Leang et al., 2003) were used for qRT-PCR analysis (Methé et al., 2003) for measuring expression level of omcB. Primers 8908-2 and 8916 (Chin et al., 2004) was used to amplify omcB probe for Northern analysis. The amplified probe were labeled with [α-^32P]dATP using a Strip-EZ DNA probe synthesis and removal kit (Ambion Inc., Austin, TX). The [α-^32P]dATP was purchased from Perkin Elmer Life and Analytical Sciences Inc. (Boston, MA). Northern blot
analyses were carried out with the Northern Max-Gly system (Ambion Inc.) according to the manufacturer’s instructions.

**Analytical techniques**

The cytochrome contents were analyzed by Tris-Tricine denaturing polyacrylamide gel electrophoresis followed by staining with \( \text{N,N,N',N'-tetrathiocarbamide} \) as described previously (Thomas et al., 1976; Francis & Becker, 1984). Fe(II) concentrations were determined with the ferrozine assay (Lovley & Phillips, 1986). Cell suspension experiments were carried out as described previously (Leang et al., 2003). Protein concentration was determined by the bicinchoninic acid method with bovine serum albumin as a standard (Smith et al., 1985).

**Results and discussion**

**Detection of OmcB in the mutant strains**

Subcellular protein fractions of MacA-deficient (DL1-macA) (Butler et al., 2004), PpcA-deficient (DL3) (Lloyd et al., 2003), OmcB-deficient (DL6) (Leang et al., 2003), OmcE-deficient (DLMC8) (Mehta et al., 2005), and OmcS-deficient (DLTM1) (Mehta et al., 2005) *G. sulfurreducens* were prepared as described previously (Nikaido, 1994; Kim et al., 2005). When the expression of \( c \)-type cytochromes were analyzed by heme-staining methods, no significant change in the abundance of \( c \)-type cytochromes could be detected in the soluble protein fraction in any of the mutants examined (data not shown). However, OmcB appeared to be much less abundant in the whole membrane fraction of the MacA-deficient mutant, in contrast to the other mutants that had OmcB levels comparable to wild type (Fig. 1a). When the outer membrane-enriched fractions were prepared by sarkosyl extraction methods from wild-type and MacA-deficient mutant, the lack of OmcB in the MacA-deficient mutant was even more apparent (Fig. 1a). This result was further confirmed by Western blot analysis, which showed no OmcB Western signal in the MacA-deficient mutant (Fig. 1b). Northern blot analysis revealed that the two omcB transcripts (Leang & Lovley, 2005) were readily detected in wild-type cells but not in the MacA-deficient mutant (Fig. 1c), indicating that transcription of omcB was lowered in the mutant.

**Expression of the omcB gene in trans in the MacA-deficient mutant**

Consistent with the possibility that the decreased Fe(III) reduction seen in the MacA-deficient mutant was due to decreased transcription of omcB, Fe(III) reduction in the MacA-deficient mutant was similar to Fe(III) reduction in the OmcB-deficient mutant (DL6) (Fig. 2). An omcB expression vector, pRG5-omcB, was constructed by amplifying the omcB-coding sequence as described in Materials and methods. The omcB-coding sequence was subsequently sequenced to screen for PCR artifacts. Following transformation of the MacA-deficient mutant with the omcB expression vector (pRG5-omcB), spectinomycin-resistant colonies were screened for the simultaneous presence of both plasmid and macA::kan mutation by PCR with primers omcBF and omcBR (for pRG5-omcB) and macA1 and macA6 (for the macA::kan mutation), respectively (Butler et al., 2004). A representative transformant of DL1-macA/pRG5-omcB was selected for phenotypic analysis. When omcB was expressed in trans in the MacA-deficient mutant, the capacity for Fe(III) reduction was restored (Fig. 2).

**Correlation between the omcB gene expression and the capacity of Fe(III) reduction**

Western blot analysis revealed that OmcB was more abundant in the MacA-deficient mutant with omcB expressed in trans than in the MacA-deficient mutant with macA expressed in trans (Fig. 3a), consistent with the differences in the capacity for Fe(III) reduction in these strains (Fig. 2). Levels of omcB transcripts were quantified by qRT-PCR methods with previously described primers (Chin et al., 2008).
and were normalized against transcript levels of recA, which is constitutively expressed in *G. sulfurreducens* (Holmes et al., 2004, 2005). The MacA-deficient mutant with omcB expressed in trans had levels of omcB transcripts 47% that of wild type, whereas the level of omcB transcripts was only 20% that of wild type in the MacA-deficient mutant complemented with macA in trans and 4% of wild type in the MacA-deficient mutant (Fig. 3b). The lower level of omcB transcripts in the macA-complemented strain may reflect the fact that macA transcript abundance in this strain is lower than in wild-type cells (Butler et al., 2004).

Resting cell suspensions of the various cell types reduced Fe(III) (Fig. 3c) as follows (mM Fe(II) mg⁻¹ protein h⁻¹): wild type, 10.7; MacA deficient with omcB expressed in trans, 5.3; MacA deficient with macA expressed in trans, 1.87; MacA-deficient mutant, 0.33. Thus, the rate of Fe(III) reduction was directly proportional to the level of omcB transcripts (Fig. 3d). Previous studies demonstrated that the rate of Fe(III) reduction by wild-type cells grown at different dilution rates in chemostats was directly related to levels of omcB transcripts (Chin et al., 2004).

**Implications**

The results presented here suggest that the inhibition of Fe(III) reduction when macA is deleted is the result of a negative impact on the transcription of omcB. In a similar manner, deletion of the outer-membrane c-type cytochrome, omcE, also inhibited omcB transcription (Kim et al., 2005). In neither instance can the unexpected indirect consequences of deleting these genes on transcription of omcB be attributed to polar effects of the gene deletion because the genes deleted and omcB are well separated on the chromosome. Recently, periplasmic sensor domains containing c-type heme from two methyl-accepting chemotaxis proteins (GSU0935 and GSU0582) from *G. sulfurreducens* were crystallized and their molecular structures were determined (Pokkuluri et al., 2008). It has been speculated that the swapped dimerization of these sensor domains and redox-linked ligand switch might be related to the

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**Fig. 2.** Fe(II) production by wild-type, MacA-deficient (DL1-macA), OmcB-deficient (DL6), and the MacA-deficient mutant by expression of either omcB (DL1-macA/pRG5-omcB) or macA (DL1-macA/pJBG-macA) in trans in the MacA-deficient mutant strains during growth in acetate–Fe(III) citrate medium (Coppi et al., 2001). Log phase (A₆₀₀ = c. 0.3) acetate:fumarate grown cultures were inoculated (1% inoculum) into acetate–Fe(III) citrate medium. Fe(II) concentrations were determined by the ferrozine assay as described previously (Lovley & Phillips, 1986). The data are the means for triplicate cultures.

**Fig. 3.** The rate of Fe(III) reduction proportional to the level of omcB expression. (a) Western blot analysis for detection of OmcB in wild-type Geobacter sulfurreducens (WT), MacA-deficient strain with omcB in trans (Δ+ omcB), MacA-deficient strain with macA in trans (Δ+ macA), and MacA-deficient mutant (Δ). (b) Relative quantification of omcB transcripts by qRT-PCR analysis. The data are the means of triplicate reactions. (c) Production of Fe(II) by WT, MacA deficient with omcB in trans, MacA deficient with macA in trans, and MacA-deficient mutant cell suspensions. Acetate and Fe(III) citrate were supplied as the electron donor and acceptor, respectively. Log-phase (A₆₀₀ = c. 0.3) fumarate-grown cultures were harvested and used for this assay. The data are the means for triplicate incubations. Cell suspension experiments were carried out as described previously (Leang et al., 2003). (d) Correlation between the relative expression level of omcB and the rates of Fe(III) reduction based on (b) and (c).
mechanism of signal transduction by these chemotaxis proteins. However, OmcF and MacA do not have moieties that would suggest redox sensors. It is possible that OmcF and/or MacA are other kinds of novel sensors of *G. sulfurreducens* in as yet unknown systems that regulate expression of *omcB*. Alternatively, it may be that disruptions of electron flow and/or improper assembly of protein complexes have more indirect effects on *omcB* transcription. In any event, these results further emphasize that the mechanisms for extracellular electron transfer in *G. sulfurreducens*, and possibly other organisms, are likely to be complex and elucidation of the pathways for electron flow will require multiple and complementary approaches.

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**References**


