



# Direct involvement of *ombB*, *omaB*, and *omcB* genes in extracellular reduction of Fe(III) by *Geobacter sulfurreducens* PCA

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The tandem gene clusters *orfR-ombB-omaB-omcB* and *orfS-ombC-omaC-omcC* of the metal-reducing bacterium *Geobacter sulfurreducens* PCA are responsible for trans-outer membrane electron transfer during extracellular reduction of Fe(III)-citrate and ferrihydrite [a poorly crystalline Fe(III) oxide]. Each gene cluster encodes a putative transcriptional factor (OrfR/OrfS), a porin-like outer-membrane protein (OmbB/OmbC), a periplasmic c-type cytochrome (c-Cyt, OmaB/OmaC) and an outer-membrane c-Cyt (OmcB/OmcC). The individual roles of OmbB, OmaB and OmcB in extracellular reduction of Fe(III), however, have remained either uninvestigated or controversial. Here, we showed that replacements of *ombB*, *omaB*, *omcB*, and *ombB-omaB* with an antibiotic gene in the presence of *ombC-omaC-omcC* had no impact on reduction of Fe(III)-citrate by *G. sulfurreducens* PCA. Disruption of *ombB*, *omaB*, *omcB*, and *ombB-omaB* in the absence of *ombC-omaC-omcC*, however, severely impaired the bacterial ability to reduce Fe(III)-citrate as well as ferrihydrite. These results unequivocally demonstrate an overlapping role of *ombB-omaB-omcB* and *ombC-omaC-omcC* in extracellular Fe(III) reduction by *G. sulfurreducens* PCA. Involvement of both *ombB-omaB-omcB* and *ombC-omaC-omcC* in extracellular Fe(III) reduction reflects the importance of these trans-outer membrane protein complexes in the physiology of this bacterium. Moreover, the kinetics of Fe(III)-citrate and ferrihydrite reduction by these mutants in the absence of *ombC-omaC-omcC* were nearly identical, which suggests that absence of any protein subunit eliminates function of OmaB/OmbB/OmcB protein complex. Finally, *orfS* was found to have a negative impact on the extracellular reduction of Fe(III)-citrate and ferrihydrite in *G. sulfurreducens* PCA probably by serving as a transcriptional repressor.

**Keywords:** Fe(III) reduction, *Geobacter*, porin-cytochrome, trans-outer membrane protein complex, extracellular electron transfer

## Introduction

*Geobacter* sp. play critical roles in global cycling of carbon, iron (Fe), manganese (Mn), and other elements. They can oxidize organic matter intracellularly and then transfer the released electrons to the terminal electron acceptors, such as Fe(III) and Mn(IV) oxides that are external to the bacterial cells. Because of their extracellular electron transfer capability, *Geobacter* sp. have been harnessed

for the bioremediation of metal and organic contaminants in the subsurface sediments and for bioenergy production (Lovley et al., 2004, 2011).

To reduce extracellular Fe(III) and Mn(IV) oxides, *Geobacter* sp. transfer electrons from the quinone/quinol pool in the cytoplasmic or inner membrane, through the periplasm and across the outer membrane to the oxide surfaces directly via *Geobacter* nanowires and/or redox proteins, such as *c*-type cytochromes (*c*-Cyt; Weber et al., 2006; Shi et al., 2007, 2009; Bird et al., 2011; Strycharz-Glaven et al., 2011; Bond et al., 2012; Snider et al., 2012). The outer membrane of Gram-negative bacteria, however, is a physical barrier for electron conductance (Shi et al., 2012a). To overcome this barrier, *Geobacter sulfurreducens* PCA employ the porin-cytochrome (Pcc) trans-outer membrane protein complexes for electron conductance across the outer membrane (Liu et al., 2014; Shi et al., 2014). The characterized Pcc protein complexes consist of a porin-like outer-membrane protein (OmbB or OmbC), a periplasmic 8-heme *c*-Cyt (OmaB or OmaC) and an outer-membrane 12-heme *c*-Cyt (OmcB or OmcC). The Pcc protein complexes have been isolated from the membrane fraction of *G. sulfurreducens* PCA cultured with Fe(III)-citrate as the terminal electron acceptor. After they were reconstituted in proteoliposomes, the isolated Pcc protein complexes transferred electrons from methyl viologen inside the proteoliposomes, across the lipid-bilayer to the external Fe(III)-citrate and ferrihydrite [a poorly crystalline Fe(III) oxide] (Liu et al., 2014). It is proposed that OmbB or OmbC serves as a scaffold through which OmaB or OmaC and OmcB or OmcC are inserted to form a heme-based electron conduit of sufficient length to span the entire width of outer membrane. This model is similar to that proposed for MtrABC trans-outer membrane protein complex of the metal-reducing bacterium *Shewanella oneidensis* MR-1 (Hartshorne et al., 2009; Richardson et al., 2012; White et al., 2013; Liu et al., 2014). In *S. oneidensis* MR-1, MtrABC complex is responsible for trans-outer membrane electron transfer during extracellular reduction of Fe(III) oxides and can transfer electrons directly to Fe(III) oxides at rates sufficient to support *in vivo* anaerobic respiration of *S. oneidensis* MR-1 (Hartshorne et al., 2009; White et al., 2013). It should be pointed out that the Pcc proteins and Mtr proteins are phylogenetically unrelated and they appear to have evolved independently to the similar functions (Liu et al., 2014; Shi et al., 2014).

The genes encoding Pcc proteins are clustered in the same region (i.e., *pcc* gene cluster) of bacterial genomes. The *pcc* gene clusters are found in the genomes of all sequenced *Geobacter* sp. and 11 other bacteria from six different phyla, which reflect the importance of Pcc protein complexes in trans-outer membrane electrons transfer by the Gram-negative bacteria (Shi et al., 2014). *G. sulfurreducens* PCA possesses four *pcc* gene clusters and two of which, *ombB-omaB-omcB* and *ombC-omaC-omcC*, are directly involved in extracellular reduction of Fe(III) (Liu et al., 2014; Shi et al., 2014). The *ombB-omaB-omcB* and *ombC-omaC-omcC* are part of tandem gene clusters, which also include *orfR* and *orfS* that encode putative transcriptional factors (Figure 1; Leang et al., 2003; Leang and Lovley, 2005; Liu et al., 2014; Shi et al.,

2014). At the amino acid sequence level, OmbB/OmbC and OmaB/OmaC are 100% identical, respectively; while OrfR/OrfS and OmcB/OmcC are 99 and 71% identical, respectively. Thus, *orfR-ombB-omaB-omcB* and *orfS-ombC-omaC-omcC* are a result of gene duplication (Leang et al., 2003; Liu et al., 2014; Shi et al., 2014).

Previously, we found that replacement of *ombB-omaB-omcB* or *ombC-omaC-omcC* with an antibiotic resistant gene had little or no impact on Fe(III)-citrate reduction and limited impacts on ferrihydrite reduction by *G. sulfurreducens* PCA, while disruption of *ombB-omaB-omcB-orfS-ombC-omaC-omcC* significantly impaired the ability of *G. sulfurreducens* PCA to reduce Fe(III)-citrate and ferrihydrite. All these results demonstrate the direct involvement of both *ombB-omaB-omcB* and *ombC-omaC-omcC* in extracellular reduction of Fe(III)-citrate and ferrihydrite (Liu et al., 2014). However, the roles of individual genes of these clusters, such as *ombB* and *omaB*, in extracellular reduction of Fe(III) had remained largely uninvestigated. Moreover, the role of *omcB* in Fe(III) reduction had remained controversial (Leang et al., 2003; Liu et al., 2014). In contrast to our previous results in *G. sulfurreducens* PCA (Liu et al., 2014), disruption of *omcB* in the presence of *omcC* greatly impaired the bacterial ability to reduce Fe(III)-citrate by *G. sulfurreducens* DL-1, which suggests that OmcC is not involved in Fe(III)-citrate reduction (Leang et al., 2003). Moreover, OmcB was once believed to mediate electron conductance across the outer membrane by itself because it was deeply embedded in the outer membrane (Qian et al., 2007; Butler et al., 2009). To further clarify their roles, we conducted detailed characterizations of *ombB*, *omaB*, and *omcB* with regards to extracellular reduction of Fe(III)-citrate and ferrihydrite by *G. sulfurreducens* PCA.

## Materials and Methods

### Bacterial Growth, Mutant Construction, and Gene Cloning

*Geobacter sulfurreducens* PCA (ATCC® 51573™) was routinely cultured in the medium with 10 mM acetate as an electron donor and 40 mM fumarate as an electron acceptor prior to construction of gene replacement mutants. The gene replacement mutants and related complement strains were constructed by using established protocols (Coppi et al., 2001; Leang et al., 2003; Lloyd et al., 2003; Rollefson et al., 2009; Liu et al., 2014). Briefly, the genomic DNA of *G. sulfurreducens* PCA was purchased from ATCC (Manassas, VA, USA), which served as a template for PCR amplification of respective PCR fragments that flanked the target genes. The kanamycin and chloramphenicol resistance genes were amplified with pBBR1-MCS2 and pACYC184 as templates, respectively (Chang and Cohen, 1978; Kovach et al., 1995). The PCR fragments that flanked the targeted genes and PCR-amplified kanamycin or chloramphenicol resistance gene were mixed and then served as the templates for the second round PCR amplification of the fragments that contained respective gene replacement mutants. These fragments from the second round PCR were electroporated into the target cells



**FIGURE 1 | The *orfR* gene clusters of *Geobacter sulfurreducens* PCA.** The genes encoding transcriptional factors, porin-like outer-membrane proteins, the periplasmic c-type cytochromes and the outer-membrane c-type cytochromes are labeled in black, green, red, and purple, respectively.

separately to make individual gene replacement mutant via double-homologous recombination between the flanking regions of the PCR fragments and the flanking regions of the target genes on the chromosome (Coppi et al., 2001; Lloyd et al., 2003).

For cloning, the target genes were separately amplified by PCR with their respective primers. After treatment with restriction enzymes, such as BamHI, EcoRI, HindIII, SpeI, and XhoI (New England Biolabs, Ipswich, MA, USA), the PCR fragments were cloned into pBBR1-MCS5 by using the Fast-link DNA ligation kit (Epicenter, Madison, WI, USA; Kovach et al., 1995). After verification by sequencing, the cloned genes were introduced to their respective mutants by conjugation via *Escherichia coli* strain WM3064 (Coppi et al., 2001). All gene replacement mutants and complement strains were confirmed by PCR amplifications of the disrupted regions of bacterial genome and the cloned genes in a plasmid, respectively. Bacterial strains, plasmids, and oligonucleotide primers used in this study are listed in Supplementary Table S1. The procedures for SDS-PAGE and heme staining were described previously (Thomas et al., 1976; Shi et al., 2006).

## Fe(III) Reduction

Amorphous 2-line ferrihydrite was synthesized by hydrolysis of  $\text{Fe}(\text{NO}_3)_3$  solution at pH 7 at room temperature. The synthesis was conducted inside an anaerobic chamber to prevent any  $\text{CO}_2$  contamination (100%  $\text{N}_2$ , Innovative Technology, Inc., Amesbury, MA, USA). Briefly, 140 g of  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  was dissolved in 700 mL of ddH<sub>2</sub>O in a 1 L Teflon bottle. The pH of the  $\text{Fe}(\text{NO}_3)_3$  solution was slowly adjusted with 2 M NaOH solution until pH 7, which yielded a dark brown suspension. After overnight equilibration under a  $\text{N}_2$  atmosphere, the suspension pH was still 7. The suspension was centrifuged and resuspended in ddH<sub>2</sub>O, and this washing procedure was repeated for eight times (Schwertman and Cornell, 2000; Shi et al., 2012b). The synthesized two-line ferrihydrite was characterized using transmission electron microscopy (TEM, Jeol JEM 2010 high-resolution TEM, Peabody, MA, USA) and powder X-ray diffraction (XRD, Philips PW 3040/00 X'pert MPD system, Westborough, MA, USA). Fe(III)-citrate was prepared as described previously (Wang et al., 2008). For Fe(III)-reduction assays, all *Geobacter* strains were pre-cultured in the medium with fumarate as an electrons acceptor. Antibiotics were used at 200  $\mu\text{g}/\text{ml}$  for kanamycin and 10  $\mu\text{g}/\text{ml}$  for chloramphenicol and gentamicin. Reduction of 50 mM of Fe(III)-citrate or two-line ferrihydrite was carried out at 30°C with *Geobacter* cells at starting  $\text{OD}_{600}$  of 0.05 in the absence of antibiotic (Leang et al., 2003; Rollefson et al., 2009; Liu et al., 2014). All procedures were performed in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA)

that was filled with 5%  $\text{H}_2$ , 20%  $\text{CO}_2$ , and 75%  $\text{N}_2$ . The reduced Fe(II) was measured with a ferrozine assay (Stookey, 1970), and total Fe was determined with inductively coupled plasma emission spectroscopy (Perkin-Elmer, Waltham, MA, USA).

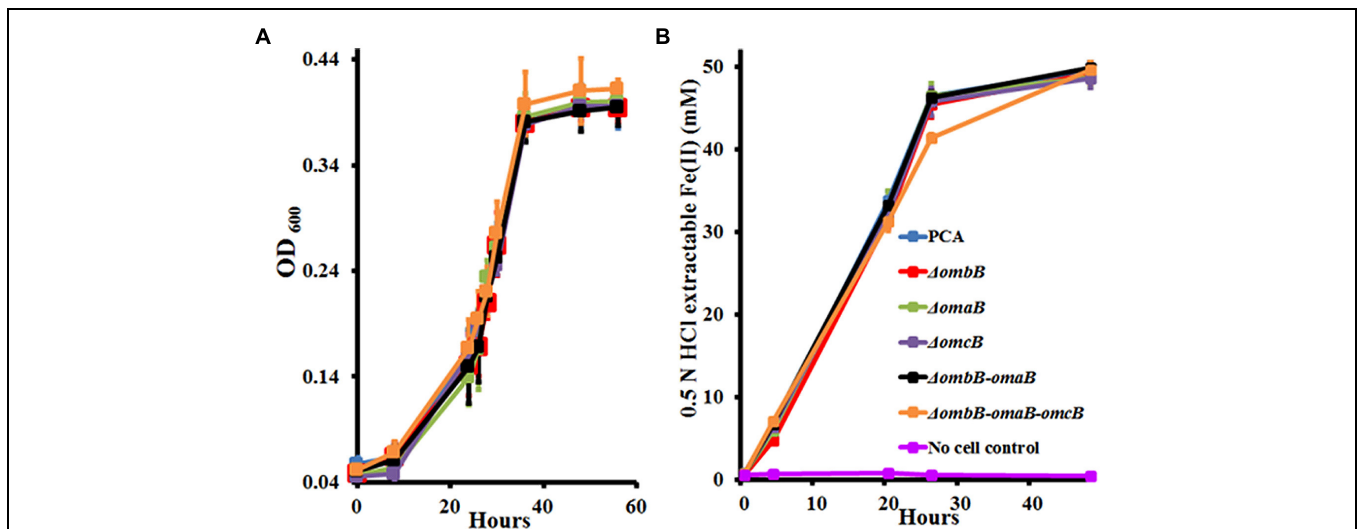
## Results

### Characterization of *ombB*, *omaB*, and *omcB* in the Presence of *ombC-omaC-omcC*

To characterize their roles, we first replaced *ombB*, *omaB*, *omcB*, and *ombB-omaB* with the kanamycin or chloramphenicol resistance gene. Following verification (Supplementary Figure S1A), the resulting mutants were tested for their growth with fumarate or Fe(III)-citrate as the terminal electron acceptor. For comparison, a previously made  $\Delta\text{ombB-omaB-omcB}$  was included. As shown in Figure 2, replacements of *ombB*, *omaB*, *omcB*, and *ombB-omaB* had no impact on bacterial growth with fumarate or reduction of Fe(III)-citrate. Consistent with our previous results, even replacement of the entire *ombB-omaB-omcB* gene cluster only slightly decreased the ability of *G. sulfurreducens* PCA to reduce Fe(III)-citrate at 24 h. Given that both *ombB-omaB-omcB* and *ombC-omaC-omcC* are involved in Fe(III)-citrate reduction (Liu et al., 2014), the lack of apparent phenotype of these mutants in reducing Fe(III)-citrate suggests an overlap function between *ombB-omaB-omcB* and *ombC-omaC-omcC* in Fe(III) reduction, which makes it difficult to accurately evaluate the functions of *ombB*, *omaB*, and *omcB* in the presence of *ombC-omaC-omcC*.

### Characterization of *ombB*, *omaB*, and *omcB* in the Absence of *ombC-omaC-omcC*

To avoid any interference of *ombC-omaC-omcC*, we replaced *ombB*, *omaB*, *omcB*, *ombB-omaB*, and *ombB-omaB-omcB* with a chloramphenicol resistance gene in a previously constructed  $\Delta\text{ombC-omaC-omcC}$  (Supplementary Figure S1B) (Liu et al., 2014). Replacements of these genes had no impact on bacterial growth with fumarate as the terminal electron acceptor (Figure 3A), but significantly lowered the bacterial reduction of Fe(III)-citrate (Figure 3B). At 26 h, *G. sulfurreducens* PCA and  $\Delta\text{ombC-omaC-omcC}$  reduced  $43.3 \pm 1.4$  and  $42.4 \pm 1.5$  mM Fe(III)-citrate ( $n = 3$ ), respectively, while  $\Delta\text{ombB}/\Delta\text{ombC-omaC-omcC}$  (or  $\Delta\text{C}$  cluster),  $\Delta\text{omaB}/\Delta\text{C}$  cluster,  $\Delta\text{omcB}/\Delta\text{C}$  cluster,  $\Delta\text{ombB-omaB}/\Delta\text{C}$  cluster, and  $\Delta\text{B}/\Delta\text{C}$  clusters had a decrease of at least 89% of the wild type's ability to use Fe(III)-citrate as the terminal electron acceptor (Figure 3B). Moreover, the kinetics of Fe(III)-citrate reduction by these newly constructed mutants were nearly identical.



**FIGURE 2 | Characterization of *ombB*, *omaB*, and *omcB* in the presence of *ombC-omaC-omcC*. (A) Growth on fumarate. (B) Fe(III)-citrate reduction. The curves are labeled in the same way in (A,B), except that no cell control is omitted in (A). The values plotted at each time point are the average OD<sub>600</sub> (A) and 0.5 N HCl extractable Fe(II) (B) measured for each strain from triplicate assays, respectively, and error bars are standard deviations. For points without error bar, the error was smaller than the symbol.**

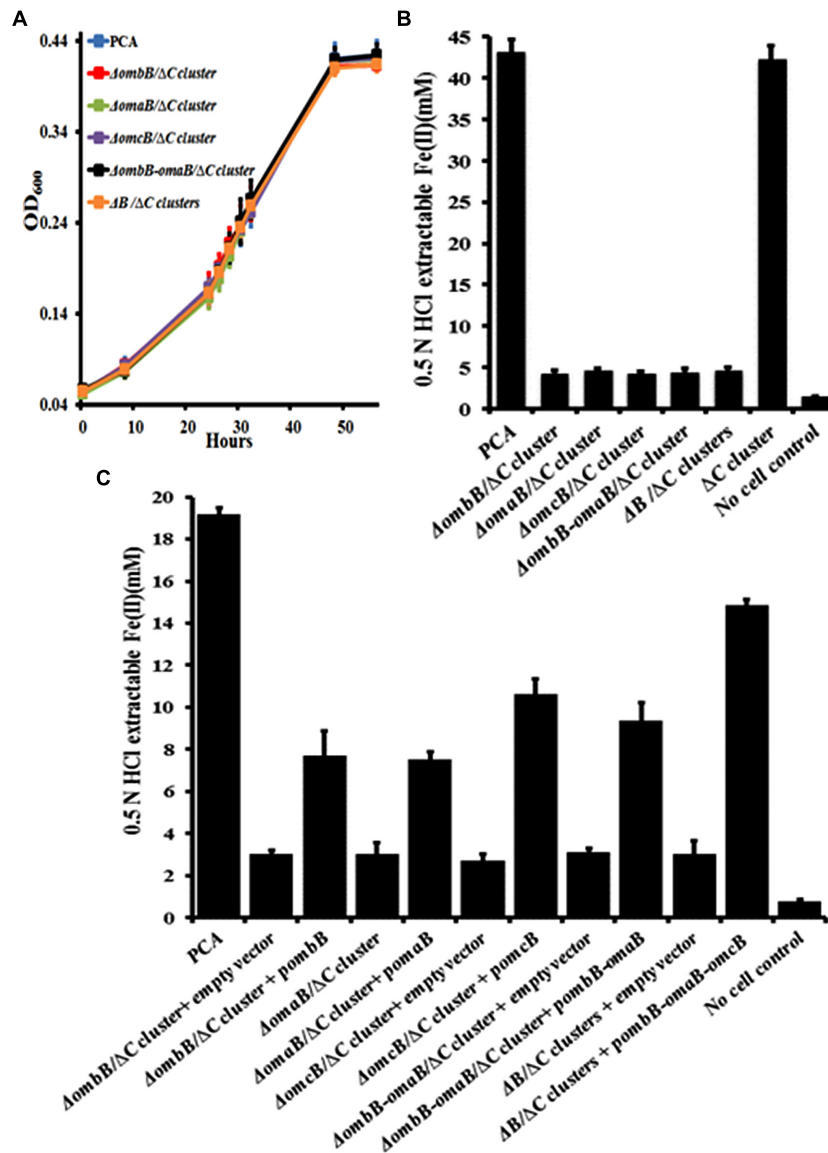
Apparently varied expression levels of heme-containing OmcB or OmaB were observed in different gene disruption mutants (Supplementary Figures S1 and S2). To test any polar effect of gene-replacement, we cloned *ombB*, *omaB*, *omcB*, *ombB-omaB*, and *ombB-omaB-omcB* and introduced the cloned genes into their respective mutants. Empty vector was also introduced into the mutants and resulting strains served as controls. Addition of empty vector had little impact on Fe(III) reduction as the rates for reducing Fe(III)-citrate were nearly identical for those with or without empty vector (Figure 3B; Supplementary Figure S2). Compared to the controls that were introduced with the empty vector, complemented strains exhibited 2- to 5-fold increase in reducing Fe(III)-citrate at 48 h (Supplementary Figure S2). Because complement *omaB* and *omcB* displayed varied levels of expressed proteins (Supplementary Figure S3), the observed difference in Fe(III)-citrate reduction by the complement strains is most likely due to different expression levels of complement genes. All these results consistently show that the phenotypes of Fe(III)-citrate reduction exhibited by these mutants are unlikely attributable to any secondary effect of disrupting these genes. We then tested the impacts of disrupting *ombB*, *omaB*, *omcB*, *ombB-omaB*, and *ombB-omaB-omcB* on the ability of *G. sulfurreducens* PCA to reduce ferrihydrite with these strains.

As shown in Figure 3C, at 360 h, *G. sulfurreducens* PCA reduced  $19.2 \pm 1.2$  mM ferrihydrite ( $n = 3$ ), however,  $\Delta ombB/\Delta C$  cluster,  $\Delta omaB/\Delta C$  cluster,  $\Delta omcB/\Delta C$  cluster,  $\Delta ombB-omaB/\Delta C$  cluster, and  $\Delta B/\Delta C$  cluster, which all contained the empty vector, only reduced  $2.7 \pm 0.3$  to  $3.1 \pm 0.2$  mM ferrihydrite ( $n = 3$ ). Similar to the results of Fe(III)-citrate reduction, the kinetics of ferrihydrite reduction by these mutants were nearly identical and complements with their respective genes increased the extent of ferrihydrite reduction from 2.5- to 4.9-fold.

### Negative Role of *orfS* in Fe(III) Reduction

During the measurements, we noticed that the  $\Delta B/\Delta C$  clusters constructed in this study reduced much less Fe(III)-citrate than the  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  prepared previously (Figure 4B). The only difference between these two mutants is the presence of *orfS* in the  $\Delta B/\Delta C$  clusters, suggesting that the difference is related to the function of *orfS*.

To further explore the *orfS* function, we complemented  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  with the cloned *orfS*. An empty vector was also introduced into  $\Delta B/\Delta C$  clusters and  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  and resulting strains served as controls. All these strains and *G. sulfurreducens* PCA showed the nearly identical growth pattern with fumarate as the terminal electron acceptor (Figure 4A). However, they reduced Fe(III)-citrate differently. At 48 h, *G. sulfurreducens* PCA and  $\Delta B/\Delta C$  clusters with the empty vector reduced  $50.0 \pm 1.0$  and  $7.8 \pm 0.4$  mM Fe(III)-citrate ( $n = 3$ ), respectively, while  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  with the empty vector and  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  with *orfS* *in trans* reduced  $34.0 \pm 1.3$  and  $26.9 \pm 2.4$  mM Fe(III)-citrate ( $n = 3$ ), respectively. Thus, complement of  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  with *orfS* further decreased Fe(III)-citrate reduction by 23% (Figure 4B). Similarly, complement of  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  with *orfS* further decreased ferrihydrite reduction. At 360 h, *G. sulfurreducens* PCA and  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  with the empty vector reduced  $22.1 \pm 1.4$  and  $5.5 \pm 0.5$  mM ferrihydrite ( $n = 3$ ), respectively. Complement of  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  with *orfS* *in trans* reduced only  $2.7 \pm 0.4$  mM ferrihydrite ( $n = 3$ ), which was very close to  $2.0 \pm 0.3$  mM ferrihydrite ( $n = 3$ ) reduced by  $\Delta B/\Delta C$  clusters with the empty vector (Figure 4C). Thus, the presence



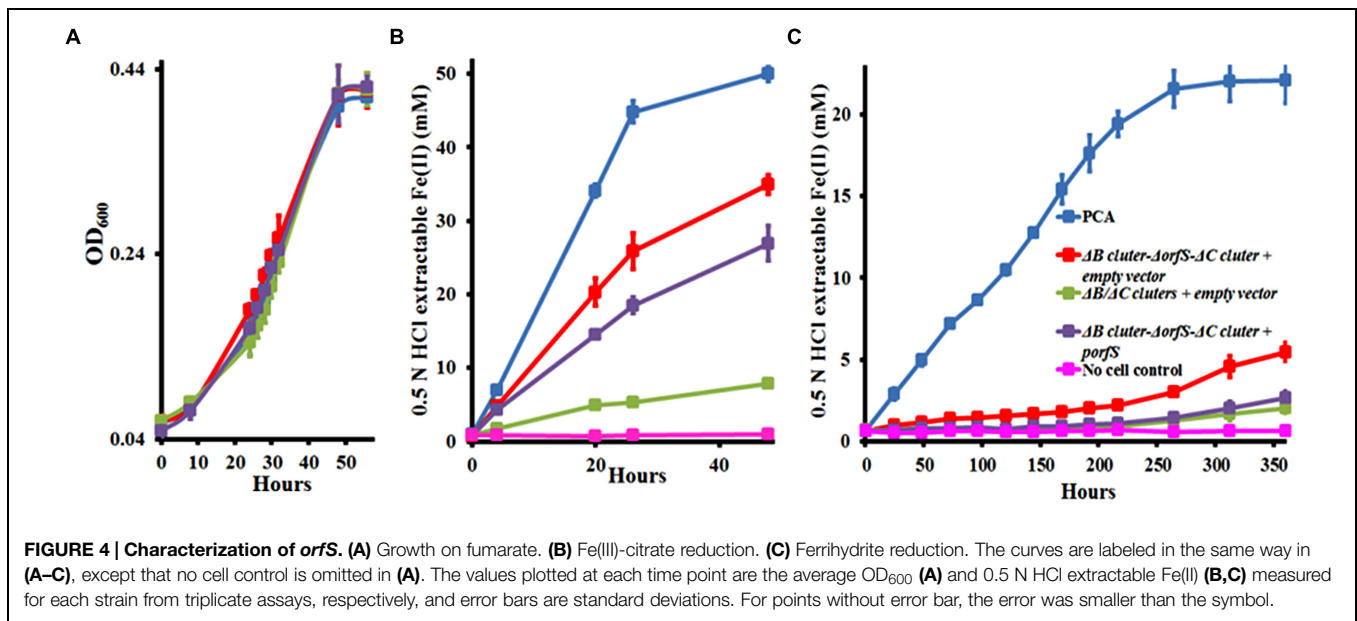
**FIGURE 3 | Characterization of *ombB*, *omaB*, and *omcB* in the absence of *ombC-omaC-omcC*. (A) Growth on fumarate. (B) Fe(III)-citrate reduction at 26 h. (C) Ferrihydrite reduction at 360 h. The values plotted at each time point are the average OD<sub>600</sub> (A) and 0.5 N HCl extractable Fe(II) (B,C) measured for each strain from triplicate assays, respectively, and error bars are standard deviations. For points without error bar, the error was smaller than the symbol (A).**

of *orfS* appears to negatively impact Fe(III) reduction by *G. sulfurreducens*.

## Discussion

In the presence of *ombC-omaC-omcC*, disruptions of *ombB*, *omaB*, *omcB*, or *ombB-omaB* had no impact on Fe(III)-citrate reduction by *G. sulfurreducens* PCA. These results are consistent with our previous finding that replacement of *ombB-omaB-omcB* with an antibiotic gene only slightly decreased Fe(III)-citrate reduction by *G. sulfurreducens* PCA (Liu et al., 2014). However, no observed phenotype for  $\Delta omcB$  in Fe(III)-citrate

reduction by *G. sulfurreducens* PCA contrasts with previous results for *G. sulfurreducens* DL-1, in which replacement of *omcB* significantly impaired this microorganism's ability to reduce Fe(III)-citrate (Leang et al., 2003). This difference between *G. sulfurreducens* PCA and *G. sulfurreducens* DL-1 regarding the role of *omcB* in Fe(III)-citrate reduction was hypothesized to be attributed to the different ability of these two bacterial strains to compensate for the loss of *omcB* (Liu et al., 2014). In sharp contrast to that in the presence of *ombC-omaC-omcC*, disruptions of *ombB*, *omaB*, *omcB*, or *ombB-omaB* in the absence of *ombC-omaC-omcC* greatly impaired the bacterial ability to reduce Fe(III)-citrate as well as ferrihydrite. Together, these results unequivocally demonstrate that *ombC*, *omaC*, and *omcC*



of *G. sulfurreducens* PCA can quickly compensate for the loss of their respective counterparts in *ombB-omaB-omcB*. These results are consistent with our previous findings that both *ombB-omaB-omcB* and *ombC-omaC-omcC* contributed to the extracellular reduction of Fe(III)-citrate and ferrihydrite by *G. sulfurreducens* PCA (Liu et al., 2014). They are also consistent with the findings that both *ombB-omaB-omcB* and *ombC-omaC-omcC* of *G. sulfurreducens* PCA are expressed under the conditions tested and they are highly identical (71–100% identical at the amino acid sequence level; Leang et al., 2003; Liu et al., 2014; Shi et al., 2014).

The observed overlapping role of *ombB-omaB-omcB* and *ombC-omaC-omcC* in Fe(III)-citrate and ferrihydrite reduction by *G. sulfurreducens* PCA is, however, different from that of *mtrD-mtrE-mtrF-omcA-mtrC-mtrA-mtrB* of *S. oneidensis* MR-1. Although *mtrA/mtrD*, *mtrB/mtrD*, and *mtrC/mtrF* are paralogs, respectively, only *mtrC-mtrA-mtrB* have been implicated in extracellular reduction of Fe(III) by *S. oneidensis* MR-1 and the role of *mtrD-mtrE-mtrF* remains unclear (Hartshorne et al., 2009; Coursolle and Gralnick, 2010).

The reduction kinetics of  $\Delta ombB/\Delta C$  cluster,  $\Delta omaB/\Delta C$  cluster,  $\Delta omcB/\Delta C$  cluster,  $\Delta ombB-omaB/\Delta C$  cluster and  $\Delta B/\Delta C$  clusters are nearly identical. These results support previous findings that OmbB, OmaB, and OmcB form a functional protein complex, which is a 20-heme trimer, for transferring electrons across the outer membrane (Liu et al., 2014). Thus, loss of any subunit of this protein complex eliminates its function. This is, however, different from the MtrABC protein complex of *S. oneidensis* MR-1 in which MtrAB can still transfer electrons across the outer membrane in the absence of MtrC (Hartshorne et al., 2009; White et al., 2013). This difference between OmbB/OmaB/OmcB of *G. sulfurreducens* PCA and MtrABC of *S. oneidensis* MR-1 may be attributed to the fact that the periplasmic *c*-Cyt OmaB contains eight hemes and the outer-membrane *c*-Cyt OmcB has 12 hemes, while each

of the periplasmic *c*-Cyt MtrA and outer-membrane *c*-Cyt MtrC possesses 10 hemes (Shi et al., 2005, 2006; Hartshorne et al., 2007; Liu et al., 2014). The eight hemes of OmaB may not form a heme-based conduit that is long enough to span the entire width of the outer membrane and may require the two hemes of OmcB for transferring electrons across the outer membrane. Consistent with this suggestion, OmcB is only partially exposed to the bacterial surface (Qian et al., 2007). It is, thus, possible that a substantial portion of OmcB is inserted into the porin-like outer-membrane protein OmbB where it interfaces with OmaB to facilitate transfer of electrons across the outer membrane, which is similar in principle to the MtrABC protein complex of *S. oneidensis* MR-1 (Hartshorne et al., 2009; Richardson et al., 2012). Alternatively, in *S. oneidensis* MR-1, Mtr-associated proteins, such as OmcA and MtrF, can compensate for the loss of MtrC (Coursolle and Gralnick, 2012), while in the absence of *ombC-omaC-omcC*, no additional outer membrane *c*-Cyt is available.

Our previous results showed that  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  could still reduce Fe(III)-citrate and ferrihydrite although at a significantly decreased rate compared to the wild type. Because *G. sulfurreducens* PCA possessed two more *pcc* gene clusters in addition to *ombB-omaB-omcB* and *ombC-omaC-omcC*, we suggested that the residual ability of reducing Fe(III)-citrate and ferrihydrite by  $\Delta ombB-omaB-omcB-orfS-ombC-omaC-omcC$  could be attributed to the functions of remaining *pcc* gene clusters or other redox proteins with trans-outer membrane electron transfer capabilities (Liu et al., 2014; Shi et al., 2014). The observed negative role of *orfS* in reducing Fe(III)-citrate and ferrihydrite by the mutant without *ombB-omaB-omcB* and *ombC-omaC-omcC* indeed supports our previous suggestion. OrfS is predicted to be a transcriptional factor of the TetR family. Previous results indicated that OrfS was not involved in regulating expression of *ombB-omaB-omcB* or *ombC-omaC-omcC* clusters and its functional role was unclear

(Leang and Lovley, 2005). The results reported from this study clearly show that presence of *orfS* decreases Fe(III)-citrate and ferrihydrite reduction. Given that most members of the TetR family are transcriptional repressors (Ramos et al., 2005), we hypothesize that OrfS may also negatively regulate the expression of other genes with functions similar to that of *ombB-omaB-omcB* and *ombC-omaC-omcC* when Fe(III) serves as the terminal electron acceptor.

In summary, the results from this study clearly demonstrate the direct involvements of *ombB*, *ombB*, and *omcB* in extracellular reduction of Fe(III)-citrate and ferrihydrite by *G. sulfurreducens* PCA. They also show a negative role of *orfS* in extracellular reduction of Fe(III) by *G. sulfurreducens* PCA. Moreover, the results from this as well as previous investigations collectively demonstrate the involvement of *ombB-omaB-omcB*, *ombC-omaC-omcC* and probably other proteins in electron conductance across the outer membrane during extracellular reduction of Fe(III) by *G. sulfurreducens* PCA. Existence of multiple and parallel trans-outer membrane extracellular electron transfer pathways critical to extracellular reduction of Fe(III) not only reflects the importance of extracellular reduction of Fe(III) in the physiology of *G. sulfurreducens*, but also is probably one of the main reasons that the results of Fe(III) reduction by this bacterium from different research groups are sometimes not comparable.

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- JF, JZ, and LS designed the study. YL conducted the research. YL, JF, JZ, and LS wrote the paper.
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- Supplementary Material**
- The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.01075>
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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