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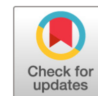
MINIREVIEW



Cytochromes in Extracellular Electron Transfer in *Geobacter*

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ABSTRACT Extracellular electron transfer (EET) is an important biological process in microbial physiology as found in dissimilatory metal oxidation/reduction and interspecies electron transfer in syntrophy in natural environments. EET also plays a critical role in microorganisms relevant to environmental biotechnology in metal-contaminated areas, metal corrosion, bioelectrochemical systems, and anaerobic digesters. *Geobacter* species exist in a diversity of natural and artificial environments. One of the outstanding features of *Geobacter* species is the capability of direct EET with solid electron donors and acceptors, including metals, electrodes, and other cells. Therefore, *Geobacter* species are pivotal in environmental biogeochemical cycles and biotechnology applications. *Geobacter sulfurreducens*, a representative *Geobacter* species, has been studied for direct EET as a model microorganism. *G. sulfurreducens* employs electrically conductive pili (e-pili) and *c*-type cytochromes for the direct EET. The biological function and electronics applications of the e-pili have been reviewed recently, and this review focuses on the cytochromes. *Geobacter* species have an unusually large number of cytochromes encoded in their genomes. Unlike most other microorganisms, *Geobacter* species localize multiple cytochromes in each subcellular fraction, outer membrane, periplasm, and inner membrane, as well as in the extracellular space, and differentially utilize these cytochromes for EET with various electron donors and acceptors. Some of the cytochromes are functionally redundant. Thus, the EET in *Geobacter* is complicated. *Geobacter* coordinates the cytochromes with other cellular components in the elaborate EET system to flourish in the environment.

KEYWORDS *Geobacter*, cytochrome, extracellular electron transfer

Extracellular electron transfer (EET) is a biological activity found in various microorganisms in a diversity of environments (1, 2). Recently it was shown that EET occurs in the mammalian gut (3). In EET, cells export electrons generated in the cytoplasm through the cellular membrane onto an extracellular electron acceptor or import electrons drawn from an extracellular electron donor through the cellular membrane into the cytoplasm. Microorganisms can gain energy for growth by drawing electrons from (oxidizing) and donating electrons to (reducing) extracellular metals (4, 5). Microorganisms can grow in syntrophy, in which different microorganisms exchange electrons with each other (6, 7). EET is crucial for environmental biotechnology, such as bioremediation, metal corrosion, bioelectrochemical systems, and anaerobic digesters (8–11). These EET processes can be direct or indirect. In the indirect way, microorganisms synthesize and secrete redox-active compounds or make use of active redox compounds present in their surrounding environments as electron carriers between the microorganisms and electron donors/acceptors (12, 13). In the direct way, microorganisms physically interact with solid electron donors/acceptors (4–7). The direct transfer is considered to be more advantageous because the synthesis and secretion of redox-active compounds may be costly, and these compounds diffuse and may not be available when needed.

Geobacter species are present in a variety of environments, such as the subsurface where metal reduction is an important biogeochemical process (14) and methanogenic soils and marine sediments where their syntrophic growth occurs with methanogens

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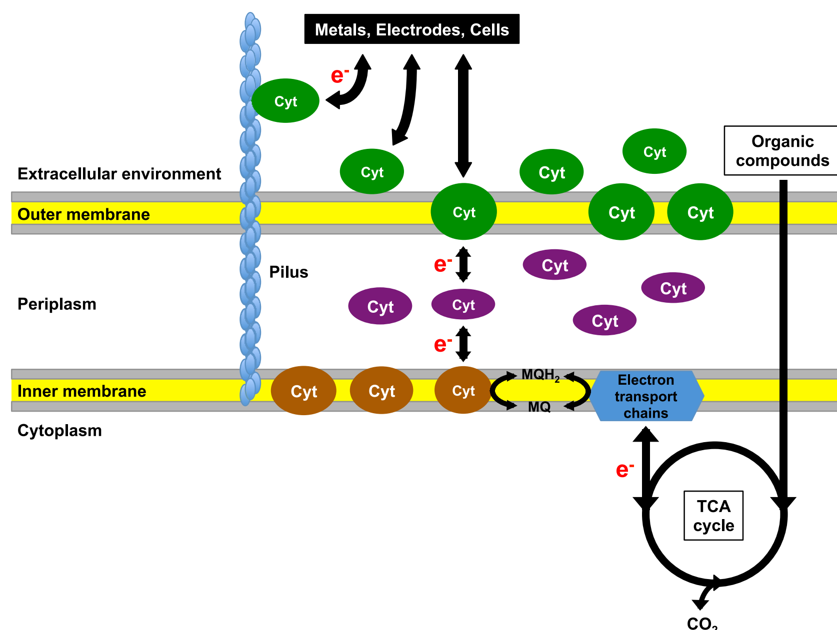

FIG 1 Extracellular electron transfer in *Geobacter*. Cyt, cytochrome; MQ, menaquinone; MQH_2, menaquinol.

(6, 7). They also play roles in environmental biotechnology for bioremediation, current production, electrosynthesis, iron corrosion, and anaerobic digesters (6, 7, 14–17). Therefore, they are of ecological significance in the environment and of great value in biotechnology. One of the superb characteristics of *Geobacter* species is the capability of direct EET with solid metals, electrodes, and other cells (Fig. 1) (18). *Geobacter* species oxidize organic compounds via the tricarboxylic acid (TCA) cycle (19, 20), which generates electrons that are transported through inner and outer membranes to extracellular electron acceptors, or take up electrons from extracellular electron donors that are transported inside cells. *Geobacter sulfurreducens*, which possesses the hallmarks of *Geobacter* species, has been investigated for EET as a model microorganism. *G. sulfurreducens* reduces metal oxides via direct electron transfer (21); consumes and produces currents via direct electron uptake from the cathode and direct electron donation to the anode, respectively (22, 23); directly accepts electrons from Fe(0) (17); and performs direct interspecies electron transfer (DIET) with other cells in syntrophic cocultures as the electron accepting (24) or donating partner (25).

Electrically conductive pili (e-pili) and *c*-type cytochromes play critical roles in the direct EET in *G. sulfurreducens* (Fig. 1) (14, 18, 21). The e-pili are particularly important for long-range direct EET (26–28). The biological function and biotechnology applications of the e-pili have been reviewed recently (26–30), and this review focuses on *c*-type cytochromes. Genomes of *Geobacter* species encode an exceptionally large number of *c*-type cytochromes, but in contrast to high conservation of other energy metabolism proteins, very few cytochromes are well conserved in *Geobacter* species (31). The genome of *G. sulfurreducens* (3.81 Mbp) encodes 111 *c*-type cytochromes (32). Among them, 78 cytochromes have multihemes with an average of 7.5 hemes per cytochrome and are distributed in all subcellular locations (inner membrane, periplasm, outer membrane) (31). The genome of *Shewanella oneidensis* (4.97 Mbp), another model microorganism for EET study (33), codes for 39 *c*-type cytochromes (34). *S. oneidensis* employs indirect EET via flavin as the electron shuttle (35). Substantial redundancy is recognized in each step or subcellular location in the EET in *G. sulfurreducens*. Cytochromes discussed in this review are listed in Table S1 in the supplemental material. EET in *S. oneidensis* has been reviewed recently (33, 36), and the research on the molecular mechanism of EET has been most intensively conducted with *G. sulfurreducens* among

Geobacter species. The cytochromes for the EET in *G. sulfurreducens* are summarized below.

INNER MEMBRANE

Inner membrane cytochromes connect the electron flow between intracellular electron transport chains and periplasmic cytochromes.

Cbc complex. The *G. sulfurreducens* genome encodes four putative cytochrome *bc* (Cbc) complexes (Cbc3 to Cbc6) and the single protein Cbc1 (CbcY, CbcL) containing *b*-type and *c*-type cytochrome domains. The Cbc complexes are considered to provide the electron transfer between the menaquinone/menaquinol pool in the inner membrane and periplasmic cytochromes and generate the proton gradient across the inner membrane that influences ATP synthesis (31). CbcC, a *c*-type cytochrome for Cbc5, was important for reduction of Fe(III) oxide, and CbcV, an iron-sulfur cluster-binding protein for Cbc3, was important for reduction of Fe(III) citrate and Fe(III) oxide (37). Despite downregulation of transcript abundance of *cbc1* during growth on Fe(III) oxide in comparison with Fe(III) citrate (37), deletion of *cbc1* resulted in decreased rates of Fe(III) oxide reduction (38). The deletion also caused slower current production than the parent strain at the poised potential of −100 mV (38).

The Cbc complexes are well conserved in *Geobacter* species (31). Because of the electrical link between the inner membrane and periplasm and the potential influence on ATP synthesis, it is likely that the Cbc complexes take critical part in the respiration of *Geobacter* species in the environment.

ImcH. ImcH was identified by transposon mutagenesis, and insertion mutations in its gene caused an inability to grow on Fe(III) citrate as the electron acceptor (39). Disruption of *imcH* eliminated the ability to reduce Fe(III) citrate, Fe(III)-EDTA, and Mn(IV) oxides (39). The gene disruption also inhibited capability to produce currents at poised potentials greater than +100 mV.

It was suggested that two different electron transfer pathways exist in the inner membrane, one containing Cbc1, which is required for reduction of electron acceptors with reduction potentials at or below −100 mV, and the other including ImcH, which is necessary for reduction of electron acceptors with reduction potentials above −100 mV (38–40). Thus, it was proposed that *G. sulfurreducens* cells could sense the redox potential of extracellular electron acceptors (40). It remains to be seen whether or not disruption of both Cbc1 and ImcH can eliminate the ability to reduce all of these electron acceptors and produce currents at various poised potentials. These suggest that the electron transport between the inner membrane and periplasm may be more elaborate than proposed (31). The Cbc complexes and ImcH have not been purified and biochemically characterized.

MacA. The inner membrane-associated diheme *c*-type cytochrome MacA is important for the reduction of Fe(III) citrate (41), Fe(III) oxide (37), and U(VI) (42). It is proposed that MacA is a regulator for expression of another cytochrome rather than an electron carrier in the EET pathway. Expression of OmcB, an outer membrane *c*-type cytochrome important for Fe(III) reduction, was not detected in a *macA* deletion strain, and in *trans* expression of OmcB in the *macA* deletion strain restored the ability to reduce Fe(III) (43). Recombinant MacA purified from *Escherichia coli* contained two low-spin heme groups with reduction potentials of −237 mV and −138 mV (44). The recombinant MacA transferred electrons to PpcA (44, 45), but physiological relevance of this activity is not clear. Fe(III) reductase activity was not observed with the recombinant MacA (44). The sequence of MacA belongs to the family of the diheme cytochrome *c* peroxidase (46). The recombinant MacA displayed similar electrochemical properties to other bacterial diheme peroxidases and hydrogen peroxide reductase activity (44). However, the deletion of *macA* had no impact on response to oxidative stress (41).

PERIPLASM

Periplasmic cytochromes link the electron transfer between inner and outer membranes.

TABLE 1 Key cytochromes in extracellular electron transfer in *G. sulfurreducens*

Name	Location	Reduction potential[b]		Substrate[b]	
		MP	W	*In vitro*	*In vivo*
Cbc[a]	Inner membrane	ND	ND	ND	An, FC, FO
ImcH	Inner membrane	ND	ND	ND	An, FC, FE, Mn
PpcA[c]	Periplasm	−167	210	Aq, FC, FO, FN, H, Mn, U, Cr, S	Aq, FC, U
OmcB	Outer membrane, porin complex	−190	ND	FC, FN, FO	FC, FO
OmcS	Outer membrane, e-pili	−212	320	Aq, Au, Cr, FC, FO, H, Mn, U	An, D, Fe(0), FO, Mn
OmcZ	Anode-biofilm interface	−220	360	Aq, Au, Cr, FC, U, Mn	An, Fe(0), U

[a]Cbc includes Cbc1, Cbc3, and Cbc5 complexes.
[b]ND, not determined; MP, midpoint reduction potential (mV); W, reduction potential window (mV); *In vitro*, reduction by purified cytochrome; *In vivo*, negative impact on growth by gene deletion; An, anode; Aq, AQDS; Au, Au(III); Cr, Cr(VI); D, DIET; FC, Fe(III) citrate; FE, Fe(III)-EDTA; FN, Fe(III)-nitrilotriacetic acid; FO, Fe(III) oxide; H, humic acid; Mn, Mn(IV) oxide; S, elemental sulfur; U, U(VI).
[c]Reduction potentials for PpcA are from reference 52.

PpcA homologs. *G. sulfurreducens* has five homologs of small periplasmic *c*-type cytochromes named PpcA, PpcB, PpcC, PpcD, and PpcE. All of the homologs have three *c*-type heme binding motifs, their protein sizes are 70 to 75 amino acids in length, and their protein sequences share 45 to 77% identities (47, 48). PpcA has been most intensively studied among periplasmic cytochromes, as it has been considered to be a key periplasmic cytochrome in the EET in *G. sulfurreducens* (14, 48). Transcript abundance was highest for *ppcA*, and PpcA was the most abundant protein under several growth conditions among these homologs (49–51).

PpcA was isolated from periplasmic (52) or soluble (53) fractions of *G. sulfurreducens* and purified to homogeneity. PpcA has a signal sequence, and N-terminal sequence of the purified protein indicated cleavage of the signal sequence (53). The purified PpcA contained three hemes, consistent with the predicted three heme binding motifs (53). The midpoint reduction potentials of PpcA purified from *G. sulfurreducens* were −167 mV (53) and −169.5 mV (52) (Table 1). Recombinant PpcA purified from *E. coli* showed a higher midpoint reduction potential of −117 mV (54) than those of PpcA from *G. sulfurreducens*. Reduction potentials of the three hemes, termed hemes I, III, and IV, in the recombinant PpcA were −147, −104, and −111 mV, respectively (54). The region near heme I was identified as the most dynamic segment, which may be involved in interaction with other molecules (55, 56). In addition to the heme-heme redox interactions, the heme-reduction potentials were modulated by the solution pH (55, 56). This was designated a redox-Bohr effect by analogy with the Bohr effect in hemoglobin (55, 56). The redox-Bohr center was located in the vicinity of heme IV (55, 56). The redox-Bohr effect is considered to be a crucial property, which allows PpcA to perform a concerted electron/proton transfer and to contribute to the proton gradient across the cytoplasmic membrane that drives ATP synthesis (55–57). Recombinant PpcA homologs showed similar reduction potentials (48, 55, 56). Structures of the PpcA homologs have the highest degree of conservation around heme IV, and the protein surface around this heme is positively charged in all homologs, suggesting that all PpcA homologs may interact with similar molecules involving this region (47, 55, 56). The structures and surface characteristics near the other two hemes in the homologs differ, and thus each of the homologs may interact with a unique partner via an interface involving the regions of these hemes (47, 55, 56).

The purified PpcA could reduce a variety of substrates (Table 1) (52, 53). Cell suspensions of a *G. sulfurreducens ppcA* deletion strain reduced Fe(III) citrate at a rate 60% of that of the wild-type, and the deletion strain extended a lag period before adapting to growth on Fe(III) citrate as the electron acceptor (53). The cell suspension assays also demonstrated decreased reduction rates for the humic substance analogue anthraquinone-2,6-disulfonate (AQDS) and U(VI) (53). These suggest that PpcA is an important, but not essential, component for the EET.

Potential substitutes for PpcA for facilitating the electron transfer across the periplasm are the PpcA homologs, PpcB to PpcE, because the homologs may interact with

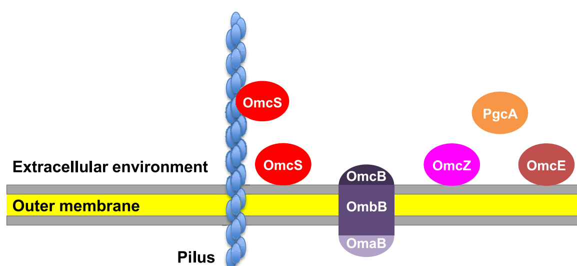

FIG 2 Location of major outer membrane cytochromes. OmcB forms a complex with the porin-like outer membrane protein OmbB and the periplasmic *c*-type cytochrome OmaB.

common redox partners as described above (47, 48). A *G. sulfurreducens* strain possessing PpcA but lacking PpcB to PpcE could reduce Fe(III) citrate, but a *G. sulfurreducens* strain lacking all of the PpcA homologs could not (58). The strain lacking all of the PpcA homologs did not adapt to growth on Fe(III) citrate (58), unlike the strain lacking only PpcA (53), suggesting that one or more of PpcB to PpcE played a role in the adaptation of the strain lacking only PpcA to growth on Fe(III) citrate. It remains to be seen if one of PpcB to PpcE is engineered to be expressed in a level as high as PpcA in the strain lacking all of the PpcA homologs and can confer the ability to reduce Fe(III) citrate similar to the strain possessing only PpcA but lacking PpcB to PpcE.

As the PpcA homologs are highly conserved in *Geobacter* species (31), it is likely that they play an essential role in the respiration of *Geobacter* species in the environment. The presence of the multiple homologs may ensure that a route for the electron transfer between the inner and outer membranes is always established in *Geobacter* cells.

PccH. PccH was identified as a highly upregulated gene by comparing the transcriptomes of *G. sulfurreducens* cells grown under current consuming conditions versus current producing conditions (59). Deletion of *pccH* from *G. sulfurreducens* resulted in failure of the current consumption but not production (59). The deletion also caused impaired reduction of Fe(III) oxide (37). PccH is a monoheme *c*-type cytochrome that is predicted to be localized in periplasm, but its actual location has not been verified yet. Recombinant PccH was purified from the periplasmic fraction of *E. coli* (60). PccH presents a unique structure, different from all known *c*-type cytochromes (60–62). PccH has the lowest reduction potential observed to date for a monoheme cytochrome, −24 mV at pH 7, which is about 300 mV more negative than the measurement of horse heart cytochrome *c* (60, 62). In the case of monoheme cytochrome, its reduction potential corresponds to the heme group's reduction potential. These distinctive properties, along with phylogenetic analysis, set PccH apart from the other class I cytochromes and monoheme cytochromes from *G. sulfurreducens*, suggesting that it belongs to a new subfamily (60–62). An electron transfer partner for PccH was proposed to be the cytochrome GSU2515 (63). However, GSU2515 has not been studied previously, and this proposal has not been investigated yet.

OUTER MEMBRANE

Outer membrane cytochromes are the first or final step in the direct EET. Although the term "outer membrane" is used, the actual location of these cytochromes varies significantly (Fig. 2). This variety appears to greatly contribute to the ability of direct EET with diverse extracellular electron donors and acceptors.

Porin-cytochrome complex. Porin-cytochrome (Pcc) complex is important for EET (64, 65). The complex consists of a porin-like outer membrane protein, a periplasmic *c*-type cytochrome, and an outer membrane *c*-type cytochrome (Fig. 2). Four gene clusters for Pcc complexes—GSU2739-2737 (*ombB-omaB-omcB*), GSU2733-2731 (*ombC-omaC-omcC*), GSU2642-2645 (*extABCD*), and GSU2726-2724 (*extEFG*)—were identified in the *G. sulfurreducens* genome (66).

The Pcc complex containing the outer membrane *c*-type cytochrome OmcB is important for Fe(III) reduction in *G. sulfurreducens* (67–69). This complex consists of the porin-like protein OmbB, the periplasmic cytochrome OmaB, and the outer membrane cytochrome OmcB (Fig. 2). The OmaB/OmbB/OmcB complex and MtrABC, a Pcc complex from *S. oneidensis*, appear to be similar in overall structure, subcellular localization, and physiological function, but they share little protein sequence similarity (68). OmcB has a signal sequence similar to those of lipoproteins (70). Electrochemical studies of purified OmcB estimated a midpoint reduction potential of −190 mV (70) (Table 1). For large cytochromes with multiple hemes, the assignment of the reduction potentials to the individual redox centers is not possible with the currently available methodologies, and thus the individual redox properties of the hemes cannot be determined for all cytochromes (48). Purified OmcB reduced Fe(III)-nitrilotriacetic acid and synthetic ferrihydrite (70) (Table 1). The OmaB/OmbB/OmcB complex was isolated, and the average heme content of the isolated complex was 20, corresponding to the sum of *c*-type heme binding motifs in OmcB (12) and OmaB (8) (68). The isolated complex reconstituted in proteoliposomes transferred electrons from reduced methyl viologen across the lipid bilayer of the liposomes to Fe(III) citrate and ferrihydrite (68) (Table 1). Studies by proteinase K treatment and immunogold labeling revealed that OmcB was embedded within the outer membrane, but a portion of OmcB was exposed on the outer surface (71).

Deletion of *omcB* greatly impaired the ability to reduce Fe(III) citrate and Fe(III) oxide (67–69). The deletion slightly decreased the rate of AQDS reduction (72). *G. sulfurreducens* has an OmcB homolog, OmcC (67). In contrast to OmcB, OmcC was dispensable for Fe(III) reduction (67) but might compensate for the lack of OmcB for Fe(III) reduction, as *omcC* transcript was upregulated in the OmcB deletion strain that was adapted to growth on Fe(III) citrate (73). In addition to *omcC*, other genes for outer membrane *c*-type cytochromes were upregulated in the adapted OmcB deletion strain (73). In contrast to Fe(III) citrate, the OmcB deletion strain never adapted to growth on Fe(III) oxide (73). The abundance of *omcB* transcript and OmcB protein was higher during growth on Fe(III) than on fumarate (49, 51). Expression of OmcB appears to be controlled by other outer membrane *c*-type cytochromes, such as OmcF, OmcG, and OmcH, and the inner membrane cytochrome MacA as described above. Deletions of these cytochromes caused defective ability to reduce Fe(III) citrate, but the function of these cytochromes might be regulation of OmcB expression rather than an electron carrier to Fe(III), as OmcB was not expressed in these deletion mutants (41, 43, 74, 75). The regulatory mechanism of these cytochromes in OmcB expression is not elucidated.

GSU2724 (*omcV*, *extG*) encoding an outer membrane *c*-type cytochrome was identified as a gene whose transcript abundance increased during growth on Fe(III) oxide or Mn(IV) oxide in comparison with growth on Fe(III) citrate, and deletion of this gene impaired growth on Fe(III) oxide (37). OmcV could contribute to Fe(III) citrate reduction when genes for other outer membrane cytochromes, *omcBCEST*, were deleted (58).

GSU2642 (*omcW*, *extD*) encoding an outer membrane *c*-type cytochrome was not essential for Fe(III) citrate reduction but could play a role in Fe(III) citrate reduction when genes for other outer membrane cytochromes, *omcBCESTV*, were deleted (58). The GSU2642-2645 (*extABCD*) cluster was also identified by transposon analysis for defective growth on an anode (76). The cluster seems to be required for optimal current production, as a deletion strain of the *extABCD* cluster exhibited delayed initiation and decreased rates of current production at −100 mV (76).

OmcB alone may be sufficient for Fe(III) reduction as the outer membrane *c*-type cytochrome in the Pcc complex homologs in *G. sulfurreducens*. Reintroduction of *omcB* in the septuple deletion strain, which lacked genes for outer membrane cytochromes (*omcBCESTVW*) and could not reduce Fe(III), restored the ability to reduce Fe(III) (58). The OmcB complex appears to be the major route in the electron transfer connecting periplasmic cytochromes and extracellular electron acceptors. Fe(III) reduction is considered to be the vital mode of respiration for *Geobacter* species in the natural environment. Gene

clusters for Pcc complexes were identified in all sequenced *Geobacter* species (66). Therefore, OmcB or its counterpart may play a crucial role in growth of *Geobacter* species in the natural environment.

OmcS. OmcS was identified as one of the most abundant proteins that were easily sheared from the outer surface of *G. sulfurreducens* cells grown on Mn(IV) oxide as the electron acceptor (77). Immunogold study showed that OmcS was localized on the outer surface and along the e-pili (78). Isolated OmcS was shown to form conductive filaments, indicating new possibilities for long-range EET (79, 80). The filaments may increase the cell surface area that is coated with redox active components and provide a route for the electron transfer between outer surface cytochromes and the e-pili for the long-range EET. However, OmcS filaments emanating from *G. sulfurreducens* cells were not observed (78). Further investigation is expected to better understand OmcS filaments. OmcS was predicted to have a signal sequence, and the molecular mass of purified OmcS was closer to the calculated molecular mass without the signal sequence than with the signal sequence (81). The purified OmcS had six hemes, the same as the number of predicted *c*-type heme binding motifs, showed a low midpoint potential (−212 mV) with a redox curve that spans over a broad range of reduction potentials (−360 to −40 mV), and could reduce a diverse range of substrates (81) (Table 1). The broad range of its reduction potential may allow OmcS to accept electrons from and donate electrons to a variety of electron carrier compounds and proteins, consistent with the physiological function.

OmcS was essential for EET to insoluble Mn(IV) oxide and Fe(III) oxide (77). OmcS was also involved in reduction of AQDS and soil humic acid (72). OmcS might also play a role in Fe(III) citrate reduction when OmcB was absent because the OmcB deletion strain adapted to growth on Fe(III) citrate expressed more *omcS* transcripts than the wild type (73). Deletion of OmcS inhibited current production when grown on an anode in an H-cell (see Fig. S1 in the supplemental material) that was not being operated in the flowthrough mode and when the biofilm was very thin (82). However, the OmcS deletion did not have a negative effect on transferring electrons to an anode in the flowthrough mode when the biofilm was thick (83) or on drawing electrons from a cathode (59). OmcS was essential for *G. sulfurreducens* as the electron-accepting partner in syntrophic coculture with *Geobacter metallireducens* via DIET (24). It has been hypothesized that OmcS serves as an electrical contact to promote electron transfer to and from the e-pili (26). Magnetite could serve as a substitute for OmcS (84). Magnetite was associated with the e-pili, and the expression of *omcS* was downregulated in the presence of magnetite (84). The OmcS deletion strain was effective in DIET and Fe(III) oxide reduction in the presence of magnetite (84). OmcS could also play a critical role in drawing electrons from Fe(0), as the *omcS* transcript was upregulated during growth on Fe(0) as the electron donor and deletion of *omcS* resulted in failure to grow on Fe (0) (17). Therefore, OmcS facilitates direct contact with the solid extracellular electron donors and acceptors. However, it was shown that *G. sulfurreducens* strain KN400 did not require OmcS for DIET and Fe(III) oxide reduction, indicating that the requirement of OmcS for EET is strain specific (85).

In the natural environment, insoluble forms of Fe(III) are more prevalent than soluble forms and are likely the source of the electron acceptor for the respiration of *Geobacter* species. OmcS is not conserved in *Geobacter* species (31). However, a cytochrome that was identified by the proteomic analysis of *Geobacter* species in the environment and had no sequence similarity to OmcS could substitute for OmcS in the reduction of Fe(III) oxide (86). Thus, an equivalent of OmcS may be involved in the reduction of insoluble Fe(III) by *Geobacter* species in the environment.

OmcZ. OmcZ was identified by transcriptome analysis comparing *G. sulfurreducens* biofilms grown on an anode as the electron acceptor and fumarate as the electron acceptor and was essential for high-density current production with thick biofilms in the flowthrough H-cell system (83). In contrast, OmcZ was not important for current production with thin biofilms in the H-cell system that was not being operated in the flowthrough mode (82). It was predicted that OmcZ has eight heme binding motifs

and a signal sequence, and its signal-sequence-cleaved form does not have transmembrane signatures (87). OmcZ exists as two forms, 50-kDa and 30-kDa proteins, termed $OmcZ_L$ and $OmcZ_S$, respectively, and $OmcZ_S$ is a proteolytic product of $OmcZ_L$ and contains eight hemes (87). Conductive filaments comprising OmcZ were observed, and this observation may lead to better understanding of the EET via OmcZ (88). Contribution of OmcZ filaments to the conductivity of *G. sulfurreducens* biofilms was reported (88), but earlier studies showed that inactivating cytochromes had no impact on the biofilm conductivity, and the biofilm conductivity was attributed to networks of the e-pili (89). OmcZ filaments associating with *G. sulfurreducens* cells were not found (90). Redox titration analysis revealed that the midpoint reduction potential of $OmcZ_S$ was −220 mV with a large reduction potential range (−420 to −60 mV) (87) (Table 1). Its large reduction potential range was considered to be important for the ability to reduce various extracellular electron acceptors (Table 1) except Fe(III) oxide (87). This is consistent with the phenotype that the *G. sulfurreducens* strain lacking *omcZ* retains the ability to reduce Fe(III) oxide (37). Recombinant $OmcZ_S$ was shown to bind riboflavin (91). Riboflavin was proposed to function as a redox cofactor bound to the outer membrane *c*-type cytochromes, not as an electron shuttle, for current production (92), reduction of Mn(IV) mineral birnessite (93), and syntrophic cocultures (94). It is unlikely that flavin or other small soluble molecules serve as the electron shuttle for *G. sulfurreducens* (95).

Although the deletion of *omcZ* alone did not inhibit reduction of AQDS and soil humic acid, OmcZ could contribute to the reduction of these extracellular electron acceptors when genes for other outer membrane cytochromes, *omcBSTE*, were deleted (72). OmcZ was also necessary for optimal reduction of U(VI) (96). As described above, *G. sulfurreducens* could adapt to the loss of outer membrane cytochromes in order to reduce soluble electron acceptors (58, 72, 73). However, the OmcZ deletion strain has never adapted to regain the ability of high-density current production with biofilms (83). Immunogold study demonstrated that OmcZ was highly concentrated at the biofilm-anode interface when *G. sulfurreducens* was grown on an anode as the electron acceptor (90). This specific localization was not observed when *G. sulfurreducens* was grown on an anode but fumarate was the electron acceptor. Electrochemical analyses suggested higher resistance of electron transfer between the biofilm and the anode in the OmcZ deletion cells than in the wild type (97). OmcZ may serve as an electrochemical gate facilitating electron transfer from *G. sulfurreducens* biofilms to the anode surface. In contrast, deletion of *omcZ* had no negative impact on drawing electrons from a cathode (59). However, OmcZ could play an important role in accepting electrons from Fe(0) because *omcZ* expression was upregulated during growth on Fe(0) as the electron donor and deletion of *omcZ* inhibited growth on Fe(0) (17).

Other outer membrane *c*-type cytochromes. In the *G. sulfurreducens* genome, downstream of *omcS*, there is a gene termed *omcT* that is cotranscribed with *omcS*, and OmcT is a homolog of OmcS with 62% protein sequence identity (77). The transcript of *omcT* was highly upregulated during growth on Fe(III) or Mn(IV) oxide as the electron acceptor (37) and on Fe(0) as the electron donor (17). However, the OmcT protein was not detected among the sheared proteins (77), and proteome analysis did not identify OmcT as an abundant protein during growth on Fe(III) oxide (50). Posttranscriptional regulation may exist for its expression. OmcT was not required for reduction of Fe(III) oxide, Mn (IV) oxide, Fe(III) citrate, and fumarate (77).

OmcE was another abundant *c*-type cytochrome among the sheared proteins (77). It was important for the reduction of Fe(III) oxide but with less impact than OmcS, as an *omcE* deletion strain was still capable of reducing Fe(III) oxide with a long lag period and at a lower reduction rate than the wild type (77). Reduction of Mn(IV) oxide was also affected with a lag period in contrast to the wild type. The ability to reduce AQDS and soil humic acid was slightly impaired by the deletion of OmcE (72). U(VI) reduction was influenced by the OmcE deletion (42). One or more of OmcB, OmcE, OmcS, OmcT, and OmcZ may be involved in reduction of Hg(II), as the deletion strain of these

cytochromes decreased Hg(II) reduction (98). OmcE has not been characterized by biochemical methods.

PgcA is an extracellular *c*-type cytochrome. It was identified by an adaptive evolution study for faster reduction of Fe(III) oxide by the wild-type *G. sulfurreducens* strain (99). The adaptive evolution generated mutations in a GEMM (genes related to the environment, membranes, and motility) riboswitch sequence for expression of *pgcA* that resulted in its increased expression and enhanced ability to reduce Fe(III) oxide. Another adaptive evolution study with *G. sulfurreducens* strain KN400 lacking the gene for the pilin PilA of the e-pili also identified PgcA (95). The PilA deletion strain was severely impaired for Fe(III) oxide reduction, but an adapted PilA deletion strain gained capability to reduce Fe(III) oxide by producing PgcA as the electron shuttle. Addition of purified PgcA to cell suspension assays for Fe(III) oxide reduction increased the reduction rates (100). However, the adapted PilA deletion strain remained unable to produce high levels of currents (95). Producing electron shuttles is costly, and diffusion of electron shuttles is problematic as they may be lost when required. Accordingly, the strain expressing the e-pili capable of direct EET to Fe(III) oxide outcompeted the adapted strain lacking the e-pili and producing PgcA when they were grown on Fe(III) oxide (95). This adapted evolution of the PilA deletion strain also created a mutation in the GEMM riboswitch sequence for *pgcA*, and increased *pgcA* expression was observed (95).

OmcF is important for reduction of Fe(III) citrate (74), Fe(III) oxide (37), and U(VI) (42) and for current production (101). As described above, OmcF appears to influence the expression of OmcB and is considered as a regulator rather than an electron transfer carrier (74). A soluble part of OmcF was purified from *E. coli* (102). Unlike the other outer membrane cytochromes (OmcB, OmcS, OmcZ), the reduction potential of the recombinant OmcF was +180 mV (102), which is much higher than those of OmcB, OmcS, and OmcZ and may be related to the consideration that OmcF is a regulator and not an electron transfer component. Structure of the recombinant OmcF was similar to that of cytochrome c_6 from the green alga *Monoraphidium braunii* (102). The cytochrome c_6 in algae and cyanobacteria mediates electron transfer from cytochrome b_6f to photosystem I (103).

SPECIFICITY OF CYTOCHROMES

It has been thought that the outer membrane cytochromes for the reduction of the soluble extracellular electron acceptors such as Fe(III) citrate and humic substances are less specific while OmcS and OmcZ for the EET with insoluble electron donors and acceptors are highly specific. OmcS and OmcZ are not highly conserved in *Geobacter* species (31). Locations of these cytochromes are considered to be important for their EET ability, as access to the insoluble electron donors/acceptors is more restricted than the soluble ones. When the *gscA* gene encoding a *c*-type cytochrome that is homologous to the *Geobacter* cytochrome identified in the *in situ* uranium bioremediation site was expressed in the OmcS deletion strain, the capacity for Fe(III) oxide reduction was restored (86). Atomic force microscopy showed that GscA was associated with the e-pili like OmcS (86). OmcS and GscA are 432 and 1,027 amino acids in length with 6 and 10 *c*-type heme binding motifs, respectively, and are not similar to each other, with only 24% identity in protein sequence. These support that location of a cytochrome is critical for the Fe(III) oxide reduction. Similar study for OmcZ has not been conducted yet.

MODEL FOR EET

It is likely that the Cbc complexes and ImcH in the inner membrane are involved in redox reactions with the menaquinone/menaquinol pool in *G. sulfurreducens*, but this remains to be verified. The predominant electron transfer bridge between the inner and outer membranes is the periplasmic cytochrome PpcA (Fig. 3). The major electron transfer pathway from the outer membrane to extracellular Fe(III) is mediated by the OmaB/OmbB/OmcB complex (Fig. 3). It is considered that the electron transfer from PpcA to OmcB occurs via the periplasmic cytochrome OmaB in the complex. Although

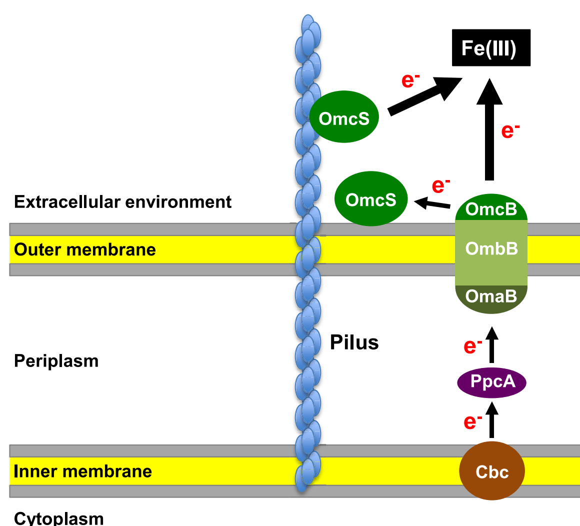

FIG 3 Model for minimal route in extracellular electron transfer to Fe(III) in *G. sulfurreducens*.

the reduction potential of OmaB is not known, the reduction potential window of PpcA overlaps the midpoint reduction potential of OmcB (Table 1). In the case of soluble Fe(III) citrate, OmcB is likely the terminal Fe(III) reductase, and the electron transfer from OmcB to Fe(III) citrate (reduction potential, +372 mV) (104) appears to go downhill. For insoluble Fe(III) oxide, the OmaB/OmbB/OmcB complex transfers electrons to OmcS, which is associated with the e-pili and acts as the terminal reductase (Fig. 3). The reduction potential window of OmcS overlaps the midpoint reduction potential of OmcB (Table 1) and reduction potentials of various Fe(III) oxides (104). In addition to the cytochromes and the e-pili, putative multicopper oxidases are known to be important for the EET to Fe(III) oxide in *G. sulfurreducens* (105, 106). One of the putative multicopper oxidases, OmpB, was very loosely associated with the cell surface, and its majority was present in the culture supernatant (71). The function of these putative multicopper oxidases is not known. Flagella could enhance the ability of *G. sulfurreducens* to reduce Fe(III) oxide (107). Resurrecting the gene for the master regulator of flagella and motility gene expression conferred flagella production and motility in the otherwise nonmotile *G. sulfurreducens*. The motility is considered to be important for growth of *Geobacter* in the environment in order to locate Fe(III) oxide (14).

For the anode as the electron acceptor, OmcZ localized at the biofilm-anode interface functions as the terminal electron carrier from the cell to the anode. The reduction potential window of OmcZ (Table 1) covers various anode potentials poised in *G. sulfurreducens* fuel cells, suggesting that OmcZ is able to directly transfer electrons to the anode. Unlike OmcS, interaction of OmcZ with the e-pili was not observed (90), and it is not clear how electron transfer takes place far from the anode surface within biofilms. *G. sulfurreducens* appears to differentially utilize the Cbc complex and ImcH in the inner membrane with respect to the potential of the anode (40). Periplasmic cytochromes essential for the EET to the anode have not been identified yet.

It appears to be possible that reverse flow of electrons from other cells to *G. sulfurreducens* cells in DIET takes place via OmcS and the e-pili. OmcS as well as OmcZ may also be the interaction point with Fe(0) as the electron donor. However, the electron transfer route from the outer membrane into the cell has not been determined for the EET from the other cell and Fe(0) yet. The periplasmic cytochrome PccH may be an electron carrier from the outer membrane to the inner membrane in a similar manner proposed for the cathode as the electron donor. However, the function of PccH has not been evaluated for DIET and the electron uptake from Fe(0). Cytochromes in the

outer and inner membranes that are important for the direct EET from the cathode have not been identified yet.

The majority of the cytochromes in *Geobacter* species have multiple hemes (31). The broad reduction potential windows of the multiheme cytochromes appear to allow direct EET with a variety of electron donors/acceptors in different reduction potentials, permitting *Geobacter* species to grow in a diversity of environments (108, 109).

PERSPECTIVES

The EET from electron donors is less understood. The wild-type *G. sulfurreducens* can consume currents. However, its current consumption rate is low and its current-consuming biofilms are much thinner than its current-producing biofilms. Consequently, investigation of the electron uptake with the cathode-grown cells has not progressed much. In contrast, *G. sulfurreducens* strain ACL consumed currents at a 10-fold higher rate and formed much thicker biofilms on the cathode than the wild type (15). Thus, the ACL strain may guide future investigation of electron uptake. With the thick biofilms grown on the cathode, transcriptome and proteome analyses as conducted with the biofilms grown on the anode will be readily feasible, and genes and proteins that are differentially expressed will be identified. The function of the identified genes and proteins in the EET from the cathode may be elucidated by genetic methods. The proteins can be characterized by biochemical and biophysical methods as employed for those involved in other EET pathways. Although *Geobacter* species have not been isolated as an iron-corroding agent from environments, *G. sulfurreducens* may become a model microorganism for iron corrosion research. *G. sulfurreducens* is the only genetically tractable microorganism that is able to directly draw electrons from iron (17).

Structural study is required for the OmaB/OmbB/OmcB complex. Recently, the atomic structure of the *S. oneidensis* MtrABC complex was reported (110). Similar approaches should be applicable to the structural study in *G. sulfurreducens*. Although structures of isolated OmcS and OmcZ filaments were reported, physiological function of these filaments is elusive. These filaments were polymerization of the cytochromes (79, 80, 88). Structural investigation with biochemically active OmcS (81) and OmcZ (87) as purified previously may provide insights into the structure-function relationship. Mutation analysis as conducted for the e-pili may reveal the mechanism of the conductivity of the cytochrome filaments. Unlike the e-pili, OmcS and OmcZ are not conserved in *Geobacter* species (31). Further research is expected to see if other *Geobacter* species produce filaments of cytochromes. Protein nanowires in *G. sulfurreducens* are under debate (26, 111). Magnetite could substitute for OmcS in the direct EET, but the e-pili were still required (84). The *G. sulfurreducens pilA* deletion strain could not carry out the EET in the presence of OmcS (21, 24). The addition of magnetite did not enable the *pilA* deletion strain to perform the EET (84). In addition, the e-pili were essential for the EET to the anode in thick biofilms in the presence of OmcZ (83, 112). Therefore, OmcS and OmcZ are unable to fulfill the function of the e-pili in the EET and vice versa. The filaments of OmcS (78) and OmcZ (90) emerging from *G. sulfurreducens* cells were not observed while the e-pili emanating from *G. sulfurreducens* cells were shown (113). *G. sulfurreducens* pili assembled from PilA pilin monomers without cytochromes were demonstrated to be electrically conductive (114). Evaluation of electron transfer between the cytochromes and between OmcS and the e-pili is crucial. Structural analysis of the e-pili associated with OmcS will provide new insight for the EET mechanism.

The minimal route for the EET to Fe(III) is proposed (Fig. 3), but to evaluate this, it is necessary to construct *G. sulfurreducens* strains in which the potential routes for EET are clearly defined and controlled at each subcellular location. This construction would be labor-intensive and time-consuming since *G. sulfurreducens* has the substantial redundancy. Alternatively, genetically tractable microorganisms with no or few cytochromes, such as *E. coli*, can be used for engineering heterologous electron transport chains (115). Recently, an *E. coli* strain was constructed in which *G. sulfurreducens* e-pili

were heterologously assembled (116). Thus, *E. coli* may serve as a chassis for building direct EET chains from *G. sulfurreducens*.

There are a number of putative cytochromes that have not been characterized in *G. sulfurreducens*. *G. sulfurreducens* can reduce various metals (14). Uncharacterized cytochromes appear to be involved in reduction of Pd(II) (117). There may be other cytochromes that are indirectly involved in the electron transfer, for example, as a regulator for expression of other cytochromes as described above and electron sink or capacitor to monitor and regulate the redox status of cells and redox proteins in the electron transport chains (118, 119). *G. sulfurreducens* appears to have homeostasis for electron flux by changing Fe(II)/Fe(III) ratios in the multiheme cytochromes (120).

Better understanding of EET is beneficial for advancing environmental biotechnology practices. EET is important for bioremediation of contaminated environments, metal corrosion, microbial current production, microbial electrosynthesis/electrofermentation, and anaerobic digesters. Gaining fundamental knowledge about the molecular mechanism in EET will enable the engineering of EET systems to develop more efficient strategies for these biotechnology practices. The cytochrome filaments would provide a new resource in electronics applications, as the e-pili are utilized as electrically conductive nanowires in various electronics applications, such as electricity generation, biocompatible artificial neurons, and sensors (30). More knowledge of the cytochrome filaments and techniques for engineering the cytochrome filaments are needed for electronics applications.

CONCLUDING REMARKS

The mechanism of the EET in *G. sulfurreducens* is very elaborate. With the myriad genes for cytochromes in its genome, a variety of cytochromes are differentially expressed for the EET with different electron donors and acceptors and are carefully arranged in the inner membrane, periplasm, outer membrane, and extracellular space. *Geobacter* harmonizes the cytochromes with other cellular components to optimize the EET system. This flexibility may make *Geobacter* suitable for growth in diverse environments where EET is advantageous. However, despite intensive studies, a complete picture of the EET mechanism has not yet been achieved. Further research for the EET mechanism is warranted.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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