Long-range electron transport to Fe(III) oxide via pili with metallic-like conductivity

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Abstract
The mechanisms for Fe(III) oxide reduction by Geobacter species are of interest because Geobacter species have been shown to play an important role in Fe(III) oxide reduction in a diversity of environments in which Fe(III) reduction is a geochemically significant process. Geobacter species specifically express pili during growth on Fe(III) oxide compared with growth on soluble chelated Fe(III), and mutants that cannot produce pili are unable to effectively reduce Fe(III) oxide. The pili of Geobacter sulfurreducens are electrically conductive along their length under physiologically relevant conditions and exhibit a metallic-like conductivity similar to that observed previously in synthetic organic metals. Metallic-like conductivity in a biological protein filament is a previously unrecognized mechanism for electron transport that differs significantly from the more well-known biological strategy of electron hopping/tunnelling between closely spaced redox-active proteins. The multihelix c-type cytochrome OmcS is specifically associated with pili and is necessary for Fe(III) oxide reduction. However, multiple lines of evidence, including the metallic-like conductivity of the pili and the fact that OmcS molecules are spaced too far apart for electron hopping/tunnelling, indicate that OmcS is not responsible for long-range electron conduction along the pili. The role of OmcS may be to facilitate electron transfer from the pili to Fe(III) oxide. Long-range electron transport via pili with metallic-like conductivity is a paradigm shift that has important implications not only for Fe(III) oxide reduction, but also for interspecies electron exchange in syntrophic microbial communities as well as microbe–electrode interactions and the emerging field of bioelectronics.

Introduction
As reviewed previously [1,2], the search for mechanisms by which micro-organisms might transfer electrons to insoluble extracellular Fe(III) oxide extends back nearly a century. It appears that the capacity for extracellular Fe(III) reduction may have evolved independently multiple times in phylogenetically distinct micro-organisms, and it is clear that there is not one unified mechanism for microbial Fe(III) reduction in all microbes [3].

The mechanisms for Fe(III) oxide reduction in Geobacter species are of particular interest because, of all the micro-organisms known to be capable of dissimilatory Fe(III) reduction in temperate circumneutral pH environments, Geobacter species are often the most abundant [4]. One reason for this is the ability of Geobacter species to use acetate, the central intermediate in anaerobic degradation, as an electron donor [5]. Additional physiological factors may give Geobacter species a competitive advantage over other Fe(III) reducers under some environmental conditions [6–8]. The prevalence of Geobacter species suggests that their mechanisms for Fe(III) oxide reduction may also be an important factor in their ability to outcompete other Fe(III) reducers.

An additional observation which suggests that Geobacter species have evolved highly effective mechanisms for extracellular electron transfer is their enrichment in environments in which extracellular electron acceptors other than Fe(III) are available. For example, when electrodes are introduced as an electron acceptor in anaerobic environments, Geobacter species are typically abundant electrode colonizers [4,9]. Geobacter species were also the most numerous bacteria in electrically conductive aggregates in a digester converting organic wastes into methane [10], which can be attributed to their capacity for direct interspecies electron transfer [11].

The purpose of the present review is to summarize what is known about the mechanisms for Fe(III) oxide reduction in Geobacter species. The emphasis is on the recently discovered metallic-like conductivity of the Geobacter pili [12] and the role that this is likely to play in long-range electron transport. Owing to space limitations, mechanisms for Fe(III) reduction in other Fe(III)-reducing micro-organisms are not discussed, and are summarized in other reviews in this issue of Biochemical Society Transactions.

Initial studies on the role of pili in long-range electron transport
A lack of tools for genetic manipulation stymied early studies on the mechanisms for extracellular electron transfer in Geobacter species. In vitro Fe(III) reductase activity could be
detected in cell components, including in the outer membrane fractions expected to be in contact with Fe(III) oxides [13–16]. However, such studies were inconclusive because of the ease with which reduced redox-active proteins, such as c-type cytochromes, can reduce Fe(III) in vitro. For example, even though periplasmic c-type cytochromes can reduce Fe(III), this is unlikely to be physiologically significant because of a lack of access to insoluble Fe(III) oxides in vivo [17]. It was therefore difficult to have confidence that the in vitro Fe(III) reductase activities that were detected had physiological relevance.

The first evidence for the importance of pili in Fe(III) oxide reduction in Geobacter species [18] was obtained at about the time that methods for genetic manipulation of Geobacter sulfurreducens became available [19]. It was noted that Geobacter metallireducens produced flagella and pili during growth on Fe(III) or Mn(IV) oxides, but not during growth with the soluble electron acceptor Fe(III) citrate [18]. Flagella were hypothesized to provide Geobacter species with motility in order to hunt for Fe(III) oxides [18,20]. Recent studies with both G. metallireducens [21] and G. sulfurreducens [22] have demonstrated that strains that produce functional flagella are more effective in Fe(III) oxide reduction than strains without flagella. Furthermore, subsurface populations of Geobacter species appear to be highly planktonic during active Fe(III) reduction [23].

Further analysis of the appearance of pili in Fe(III) oxide-growing cells revealed that the gene for PilA, the structural pilin protein, was expressed at higher levels during growth on Fe(III) oxide than on Fe(III) citrate [18]. This specific regulation suggested that PilA pilis were required for the reduction of insoluble Fe(III) oxide. In order to follow up on this observation, deletion of PilA was one of the earliest gene knockouts made in G. sulfurreducens [3,24]. Fe(III) oxide reduction was inhibited in the pilA-deficient mutant, but the reduction of Fe(III) citrate was not, which suggested further a specific role for the pili in Fe(III) oxide reduction [25]. The same phenotype has been reported recently for deleting pilA in G. metallireducens [21]. The observation of specific association of nanoparticulate Fe(III) oxide with pili suggested that pili might be an important point of contact between the cells and Fe(III) oxide, and, if so, pili might be involved in electron transfer to Fe(III) oxides [25].

These observations, and the finding that the pili were electrically conductive across their diameter [25], led to the suggestion that the pili were the conduits for long-range electron transport to Fe(III) oxides [5,25,26]. The finding that the conductivity could only be measured across the pili in regions where there were no other proteins associated with the pili suggested that the pili filaments themselves were conductive, rather than redox-active, moieties, such as c-type cytochromes, associated with the pili conferring conductivity [25,26].

Additional circumstantial evidence for the role of G. sulfurreducens pili in long-range electron transport came from adaptive evolution studies and investigations of current-producing biofilms. Strong selective pressure for higher rates of Fe(III) oxide reduction yielded strains of G. sulfurreducens that accumulated mutations in a regulatory gene, which, when deleted, increased expression of pili and doubled the rate of Fe(III) oxide reduction [27]. In a similar manner, selection for the capacity for enhanced current production on graphite electrodes yielded a strain of G. sulfurreducens, designated KN400, that produced more pili than the cells that predominated in the culture used to start the selection experiment [28].

Even with the standard wild-type strain of G. sulfurreducens, the gene for PilA was highly up-regulated in current-producing biofilms compared with biofilms growing with fumarate as the electron acceptor [29]. Deleting pilA greatly reduced biofilm formation and current production [30]. Furthermore, during the initial growth of current-producing biofilms, there was a direct correlation between the thickness of the biofilms and the amount of current generated, suggesting that cells not in contact with the anode were contributing as much to current production as cells near the electrode surface [30]. The cells at a distance from the electrode appeared to be metabolically active [30], and the high metabolic activity of cells in distal portions of the biofilm has subsequently been confirmed [31–33]. These observations led to the suggestion that pili could form a conductive ‘nanopower grid’ to facilitate long-range electron transport through the biofilm [30]. However, direct evidence for conduction along the length of pili networks and a mechanistic explanation for this proposed phenomenon was lacking.

**Metallic-like conductivity of pili under physiologically relevant conditions**

Direct measurements of electron conduction through pili networks were obtained in studies in which aqueous preparations of filaments that were sheared from the outer surface of G. sulfurreducens were placed on electrodes separated by non-conducting gaps [12]. The filaments aggregated as the preparations were dried in air, forming networks connecting the electrodes. The filaments were not treated with fixatives and the air drying left the pili hydrated, thus permitting analysis of conductivity under physiologically relevant conditions. Conductivity that extended over distances of 1 cm was documented [12]. Conductivity was lost when filaments were prepared from cells in which the gene for PilA had been deleted, indicating that the conductivity of filament preparations derived from wild-type cells could be attributed to PilA pili. Electron transport over such long distances demonstrated that pili could conduct electrons along their length and that pili could form conductive networks.

Multiple lines of evidence suggested that electrons were conducted along pili via mechanisms similar to the metallic-like conductivity in organic metals [12]. Metallic-like
conductivity in biological proteins is a novel concept. It differs significantly from the typical electron transfer in micro-organisms in which electrons hop or tunnel between discrete redox-active proteins, because in metallic-like conductivity, electrons are delocalized rather than being associated with discrete electron carriers [34].

Metallic-like conductivity was apparent from the distinctive pattern of the influence of temperature on conductivity. As the temperature of the pili preparations was decreased from room temperature, there was an initial exponential increase in conductivity, followed by a decrease in conductance at lower temperatures, a response characteristic of organic metals and inconsistent with electron hopping/tunnelling between discrete redox carriers [34]. Furthermore, doping the pili preparations with protons significantly increased conductivity, as would be expected for an organic metal. There is no model in which decreasing pH would significantly enhance conductivity via electron hopping/tunnelling in a similar manner. Furthermore, the highest conductivity was observed at pH 2, a pH at which typical electron carriers such as c-type cytochromes are expected to be denatured. Overlapping π orbitals confer metallic-like conductivity in synthetic organic metals and X-ray diffraction analysis provided evidence for similar overlapping π orbitals in pili preparations [12].

Current-producing biofilms of Geobacter sulfurreducens also exhibited metallic-like conductivity with a strong correlation between the abundance of PilA protein in the biofilms and biofilm conductivity. For example, biofilms grown with fumarate as the electron acceptor were less conductive than biofilms grown with an electrode as the electron acceptor and contained less PilA [12]. A quadruple-mutant strain in which the genes for the outer-surface c-type cytochromes OmcB, OmcE, OmcS and OmcT were deleted had higher PilA levels and higher biofilm conductance than wild-type cells. KN400, which, as noted above, has copious pili [28], also produced biofilms with high conductance [12]. The biofilms of a strain which was genetically engineered for enhanced pili production were significantly more conductive than wild-type biofilms. These findings are consistent with the concept that pili are responsible for long-range electron transport through conductive G. sulfurreducens biofilms.

In addition to the evidence for metallic-like conductivity in pili and conductive biofilms, direct examination of the role of cytochromes as electron carriers along pili or through current-producing biofilms ruled out electron transfer in this more traditional manner. Denaturing c-type cytochromes in filament preparations or in biofilms had no impact on conductivity [12,35]. In contrast with the positive correlation between biofilm conductivity and PilA abundance, there was a negative correlation between biofilm conductivity and the abundance of c-type cytochromes in biofilms [35]. In addition to these considerations, the simplest argument against a role of cytochromes in electron transport along pili is that it is physically impossible because, as discussed below, the cytochromes are not aligned close enough on pili for electron hopping/tunnelling.

Role of cytochromes

Although c-type cytochromes do not appear to play an important role in long-range electron transport along pili of G. sulfurreducens, they are important in Fe(III) oxide reduction. Of particular importance is the multihaem c-type cytochrome OmcS. Expression of omcS is up-regulated during growth on Fe(III) oxide compared with Fe(III) citrate [36], and OmcS is one of the most abundant cytochromes in Fe(III) oxide-grown cells [37]. OmcS was the most abundant c-type cytochrome that could be sheared off the outer surface of cells grown on Mn(IV) oxide [36]. Deleting the gene for OmcS inhibited reduction of Fe(III) and Mn(IV) oxides, but not the reduction of soluble extracellular electron acceptors such as Fe(III) citrate and AQDS (anthraquinone-2,6-disulphonate) [36].

Immunogold labelling demonstrated that OmcS is specifically associated with pili [38]. Although OmcS was abundant on the pili, the spacing between the molecules, ~30 nm, appeared to be too great for OmcS to account for electron transfer along the pili. This has subsequently been confirmed with atomic force microscopy [35].

These observations, and the finding that the low redox potential of OmcS readily facilitates Fe(III) oxide reduction in vitro [39], suggest that the role of OmcS may be to promote electron transfer from pili to Fe(III) oxides [38,40]. Additional evidence for the proposed role of OmcS in facilitating electron exchange with pili is the selection for increased OmcS expression during adaptive evolution for direct interspecies electron transfer between Geobacter species, described below.

Another multihaem c-type cytochrome that is important in Fe(III) oxide reduction is OmcB [41], which is embedded in the outer membrane, partially exposed to the extracellular environment [42]. A strain in which the gene for OmcB was deleted could not reduce Fe(III) [41], and, although the OmcB-deficient mutant eventually adapted to reduce Fe(III) citrate, it did not adapt to reduce Fe(III) oxide [43]. As reviewed recently [4] the multihaem c-type cytochrome OmcE and putative multi-copper proteins are also localized on the outer surface of the cell, and gene-deletion studies suggest that they are involved in Fe(III) oxide reduction, but the role of these apparent electron carriers in Fe(III) oxide reduction has yet to be resolved. One possibility is that additional electron carriers are required to transfer electrons to the pili at the outer cell surface, possibly to OmcS attached to pili near the outer membrane surface. However, this model is highly speculative and requires further investigation.

An alternative model that has been proposed [44] is that outer-surface c-type cytochromes, such as OmcZ [45,46], entrapped in an extracellular polymeric matrix are responsible for Fe(III) oxide reduction. However, this model is inconsistent with multiple observations. For example, if OmcZ localized at the outer cell surface was the terminal Fe(III) oxide reductase, then deleting OmcS, which is localized on the pili, well beyond the extracellular matrix, should have no impact on Fe(III) oxide reduction. In a
similar manner, in the OmcZ model, deleting pilA should not inhibit Fe(III) oxide reduction, but this is inconsistent with experimental observation. Unlike Omcs, OmcZ is not highly expressed during growth on Fe(III) oxide [4,37]. Adaptive evolution for enhanced Fe(III) oxide reduction does not increase Omcs expression. Most significantly, purified OmcZ was incapable of Fe(III) oxide reduction [45] and deleting omcZ has no impact on Fe(III) oxide reduction [29]. Thus it is unlikely that OmcZ has an important role in Fe(III) oxide reduction.

As reviewed recently in detail [4], cytochromes are also abundant in the periplasm of Geobacter species and are likely to facilitate electron transfer from the inner membrane to the outer membrane. However, the quantity of c-type cytochromes within cells of Geobacter species seems to be much more than would be required merely for electron-transfer functions. The cytochromes represent a significant amount of electron-storage capacity and may have the additional function of permitting electron transfer across the inner membrane, with associated proton pumping and energy conservation, when cells are not in direct contact with Fe(III) oxides [26,47]. This may allow Geobacter species to use Fe(III) oxide to support cell maintenance and motility as cells move from one insoluble Fe(III) source to another. The ability of abundant c-type cytochromes to store electrons is readily apparent in biofilms of G. sulfurreducens where the cytochromes confer supercapacitor capabilities on the Fe(III) oxide reduction [48].

Conclusions

The evidence currently available indicates that long-range electron transport along the pili of G. sulfurreducens, and presumably other Geobacter species, is feasible via a metallic-like conductivity of the PilA protein filament. This is a novel mechanism for biological electron transport. Electron transfer along pili appears to be a major mechanism of Fe(III) oxide reduction in G. sulfurreducens because of the substantial up-regulation of expression of genes for pili and the pili-associated cytochrome OmcS, coupled with the inability of mutants that cannot produce pili or Omcs to reduce Fe(III) oxide. Other models, such as the suggestion that Fe(III) oxides are reduced by cytochromes in an extracellular matrix, are inconsistent with these as well as other experimental observations noted above. The metallic-like conductivity of the pili is important not only for Fe(III) oxide reduction, but also, as described above, for high-density current production on electrodes. Furthermore, metallic-like conductivity appears to be a factor contributing to DIET (direct interspecies electron transfer) in Geobacter-containing methanogenic aggregates in wastewater digesters [10], consistent with the pil- and Omcs-associated DIET observed in a syntrophic co-culture of G. metallireducens and G. sulfurreducens [11].

However, the development of an integrated model for extracellular electron transfer via pili in Geobacter species is far from complete. Especially needed is additional research into structural features of pili that contribute to metallic-like conductivity, as well as investigations into the mechanisms by which electrons are transferred to the pili.

Funding

Research on conductive pili and mechanisms for Fe(III) oxide reduction in our laboratory is supported by the Office of Naval Research [grant number N00014-10-1-0084], the Office of Science (Biological and Environmental Research), U.S. Department of Energy [award number DE-SC0004114 and Cooperative Agreement number DE-FC02-02ER63446].

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Received 9 May 2012
doi:10.1042/BST20120131