Running title: Shewanella electron transfer.

Mechanisms of Bacterial Extracellular Electron Exchange.

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ABSTRACT

The biochemical mechanisms by which microbes interact with extracellular soluble metal ions and insoluble redox-active minerals have been the focus of intense research over the last three decades. The process presents two challenges to the microorganism; firstly electrons have to be transported at the cell surface, which in Gram negative bacteria presents an additional problem of electron transfer across the ~ 6 nm of the outer membrane. Secondly the electrons must be transferred to or from the terminal electron acceptors or donors. This review covers the known mechanisms that bacteria use to transport electrons across the cell envelope to external electron donors/acceptors. In Gram negative bacteria electron transfer across the outer membrane involves the use of an outer membrane β-barrel and cytochrome. These can be in the form of a porin-cytochrome protein, such as Cyc2 of *Acidothiobacillus ferrioxydans,* or a multiprotein porin-cytochrome complex like MtrCAB of *Shewanella oneidensis* MR-1. For mineral respiring organisms there is the additional challenge of transferring the electrons from the cell to mineral surface. For the strict anaerobe *Geobacter sulfurreducens* this requires electron transfer through conductive pili to

associated cytochrome OmcS that directly reduces Fe(III)oxides, while the facultative anaerobe *S. oneidensis* MR-1 accomplishes mineral reduction through direct membrane contact, contact through filamentous extentions and soluble flavin shuttles, all of which require the outer membrane cytochromes MtrC and OmcA in addition to secreted flavin.

Keywords

Shewanella, Geobacter, Acidithiobacillus, Mineral oxidising bacteria, Mineral reducing bacteria, cytochrome, nanowire, porin-cytochrome, iron oxide.

INTRODUCTION

For many years it was considered that the redox cycling of metals within the environment occurred through abiotic processes. It was only within the last three decades that the ability of microorganisms to transform subsurface metals and minerals through oxidation or reduction has been accepted as a globally important phenomenon, while metagenomic analysis has found that different microorganisms influence a substantial proportion of mineral cycling within the environment. As iron is the fourth most abundant element in the earth's crust (after oxygen, silicon and aluminium) and the iron cycle is essential to life on earth, bacterial interactions with iron oxide and hydroxide minerals are the most studied processes. However other environmentally important minerals, such as those containing manganese, arsenic, radioactive contaminants *e.g.* uranium and technetium or even rare earth metals such as gold and platinum have been proposed as substrates for these organisms (Lovley, 1993; Nealson & Saffarini, 1994). Both oxidation and reduction of metal substrates are possible. Many microorganisms undergoing anaerobic respiration generate an excess of electrons through the oxidation of organic substrates. These electrons are transported to the surface of the cell where they are discharged into a diverse range of terminal electron acceptors, these can

include soluble electron acceptors such as uranium, technicium, soluble metal chelates, or insoluble acceptors such as iron and manganese oxides or hydroxides (Fredrickson & Zachara, 2008).

In contrast, some microorganisms in environments with limited organic substrate use the oxidation of reduced iron species to liberate electrons that are then transported to oxygen or nitrate species. The ability of microorganisms to utilise metals as electron donors or acceptors are ancient forms of metabolism, and are likely to have evolved on early earth before other anaerobic or aerobic respiratory processes (Ilbert & Bonnefoy, 2013).

These microorganisms have substantial environmental and economic importance. Iron oxidising bacteria are the cause of acid mine drainage but have also been exploited to profitably extract copper from low-grade ore (Johnson, 2014). Microbes in the subsurface are capable of reducing radionuclides, causing them to precipitate and removing them from the groundwater, while in other areas similar processes cause the solublisation of arsenic, leading to increased levels of arsenic in the drinking water (Osborne et al., 2015; Wilkins et al., 2007). There has been substantial interest in the past decade in the ability of these bacteria to interact with electrodes and generate power through biobatteries and microbial fuel cells, with the most recent reported current outputs reaching 6.9 mW/m^2 (Logan et al., 2015). In addition, microbes also can be grown on cathodes allowing for metabolic control and generation of electrosynthetic metabolites (Desloover et al., 2012).

The increase in the ease of genomic and metagenomic sequencing has lead to the putative respiratory chains of many of these metal metabolising organisms being identified. However, despite this wealth of information the mechanistic detail by which microorganisms facilitate the reduction or oxidation of different insoluble substrates is poorly understood. In this chapter we review the current literature underpinning our

understanding of how microorganisms achieve this process, briefly summarising the processes of iron oxidation and iron reduction, before reviewing the literature on the most well understood processes, that of the mineral reducing *Geobacter sulferreducens* and *Shewanella oneidensis.*

1 Diversity of microbe-mineral metabolism.

Over the past two decades many microorganisms have been identified as having the ability to use metal and metal containing minerals as metabolic substrates, either as terminal electron donors or acceptors. During mineral respiration microorganisms oxidise carbon substrates such as lactate or acetate within the cytoplasm, resulting in reduced organic electron carriers such as NADH or $FADH_2$. These carriers are oxidised at the cytoplasmic membrane, resulting in the co-transport of electrons and protons across the membrane. The change in proton gradient generates a proton motive force (PMF) that is used to synthesise ATP, while electrons accumulate within the periplasm or intermembrane space. In gram negative bacteria the electrons are transported across the outer membrane to the cell surface before being transferred into terminal electron acceptors, while in gram positive bacteria the electrons must be transported through the thick cell wall before being transferred into terminal acceptors. Release of these electrons is not thought to contribute to the PMF, but prevents the build-up of charge inside the cell, this process is known as dissimilatory metal respiration (Figure 1).

[Figure 1: Electron transfer pathways of energy production for mineral-respiring bacteria.]

Most metal reducing bacteria are not true lithotrophs as they typically use organic carbon as an electron donor. Metal oxidising bacteria use minerals as electron donors by extracting electrons from reduced metal species such as soluble or insoluble Fe(II) Fe. These microbes couple the oxidation of iron to the reduction of a terminal electron acceptor by oxidising Fe(II) to Fe(III) on the cell surface, and transporting the electrons through the periplasm to the cytoplasmic membrane where they are used to generate both NADH and a PMF that is used to generate ATP. In order to generate a PMF transmembrane reductases at the cytoplasmic membrane couple the reduction of a terminal electron acceptor to the net transport of proton across the cytoplasmic membrane. The most common terminal acceptor is oxygen, and the majority of iron oxidisers fall into this category, however there are examples of anaerobic iron oxidising bacteria, such as *Decholoromonas* spp., which couples the oxidation of iron to the reduction of nitrate.

1.1 Biology of iron metabolising bacteria.

1.1.1 Iron oxidising bacteria

There are a broad range of different bacteria that are capable of oxidising Fe(II) in order to obtain reducing equivalents for the generation of a proton motive force and reduction of NAD^+. These include phototrophic organisms (*Rhodobacter* spp.*),* acidophilic aerobes (*Acidithiobacillus* spp., *Leptospirillium* spp.*)*, neutrophilic aerobes (*Sideroxydans* spp.*, Gallionella* spp.*)* and nitrate respiring anaerobes (*Dechloromonas* spp. Strain UWNR4) *(*Coby et al., 2011; Hedrich et al., 2011). Several of the aerobic iron oxidising bacteria are autotrophic, requiring Fe(II) as the sole source of energy and electrons for carbon fixation, while the phototrophic and anaerobic iron oxidisers are heterotrophic and use electrons from Fe(II) as a supplement .

One of the most important and best studied of the iron oxidising bacteria is the microbe *Acidithiobacillus ferrooxidans* (Roger et al., 2012). This microbe is well known for its environmental impact, and generates energy by coupling the oxidation of Fe(II) to the reduction of O_2. The best known form of this reaction is the oxidation of the Fe(II)S mineral known as pyrite, which plays a central role in acid mine drainage, the cause of acidic lakes and rivers that contain excessive levels of iron. In the presence of water and O_2 the abiotic and biotic reactions below occur. Fe(II) is oxidised by *A. ferrooxidans* to form Fe(II) and the liberated electrons are used by the bacteria to reduce O_2 to H_2O. The resulting Fe(III) then reacts spontaneously with FeS_2 (pyrite) to produce Fe(III), thiosulfate and an excess of protons. This, coupled with the further oxidation of thiosulfate to sulfate by sulfur respiring bacteria, has the accelerative effect of lowering pH, which in turn accelerates the dissolution of pyrite, and increasing the availability of soluble reduced iron (Roger et al., 2012).

$4\ Fe(II) + O_2 + 4\ H^+ \rightarrow 4\ Fe(III) + 2\ H_2O$ (*A. ferrooxidans* biotic reaction*)*

$6\ Fe(III) + FeS_2 + 3\ H_2O \rightarrow 7\ Fe(III) + S_2O_3^{2-} + 6\ H^+$ (Abiotic reaction)

Under neutral pH conditions ferrous iron can also be used as an electron donor. However, at this pH soluble ferrous iron reacts more quickly with oxygen, so under oxygen levels typically found in the environment the spontaneous chemical oxidation of iron outcompetes the biological oxidation of most bacteria (Emerson et al., 2010). This limits the environmental niche of neutrophilic iron-oxidising bacteria to the microoxic zone, where the concentration of oxygen is so low that the rate of chemical iron oxidation is out-competed by the rate of biological iron oxidation. The second challenge facing neutrophilic iron oxidising bacteria is that Fe(III) is rapidly precipitated by oxygen

into insoluble Fe(III)oxides at neutral pH, meaning that these bacteria must also deal with insoluble iron oxides that are generated by the cell during respiration.

Neutrophilic iron oxidising bacteria that have been studied in isolation include bacteria of the genera *Sideroxydans* and *Gallionellacea.* These organisms grow very slowly (typical doubling times of ~ 8 hours) and to low cell culture densities, limiting studies to genome analysis, growth conditions and electron microscopy (Emerson et al., 2013; Emerson & Moyer, 1997; Neubauer et al., 2002). Like *A. ferrooxidans* these organisms couple the extracellular oxidation of Fe(II) to the reduction of O_2 on the cytoplasmic membrane but the oxidised Fe(III) product rapidly precipitates into insoluble Fe(III)oxides that form on the surface of the cell. The *Sideroxydans* genus only contains two known species currently: *Sideroxydans lithotrophicus* ES-1 (Emerson & Moyer, 1997) and *Sideroxydans paludicola* (Weiss et al., 2007). During aerobic respiration by *S. lithotrophicus* ES-1 the Fe(III) has been shown to precipitate as nanoparticles on the surface of the cell, these nanoparticles are proposed to separate from the cell during the bacterial life-cycle of the cell (Emerson et al., 2013).

The *Gallionella* genus is composed of several known species and subspecies: *Gallionella ferruginea* is one of the earliest recognised iron oxidising bacteria, having been identified in the 19th century together with associated iron precipitates that, under a microscope appeared as twisted stalks (Ehrenberg, 1836). Like *S. lithotrophicus, G. ferruginea* oxidises iron at the surface of the cell, but the precipitation of the iron is tightly controlled. Rather than generating iron oxide particles these bacteria process the iron into twisted ‘stalks’ that are made from a mixture of microbial expolymer and Fe(III)hydroxide that extend from one side of the bacteria (Chan et al., 2011; Ghiorse, 1984). These stalks have been proposed to have a number of roles, including tethering the microorganism to sediment in flowing water, preventing the bacteria from becoming

encrusted in iron oxide, and protection against reactive oxygen species produced through the oxidation iron (Chan et al., 2009; Hallbeck & Pedersen, 1995).

Iron oxidation is often used as a supplemental source of electrons by metabolically diverse bacteria. For example photrophic purple bacteria can use iron oxidation to obtain electrons for carbon fixation (Ehrenreich & Widdel, 1994). The oxidised iron is thought to precipitate away form the cell surface as Fe(III)(hydr)oxides, which then slowly convert to lepidocrocite and goethite, although the mechanism is unclear (Kappler & Newman, 2004). There have been reports of anaerobic iron oxidising bacteria that use ferrous iron as a supplement rather than being lithoautotrophic anaerobes (Roden, 2012). Most require supplements of acetate or other organics in order to stimulate iron oxidation (Chakraborty & Picardal, 2013), and it has been proposed that the oxidation of nitrate (and in some instances chlorate) is part of a detoxification strategy as well as having a metabolic role (Carlson et al., 2012).

1.1.2 Mineral reducing bacteria

Mineral reducing bacteria are a globally disperse group of bacteria that are typically found in the anoxic subsurface and can range in depth from a few centimetres, to several kilometres (Nealson, 1997). These microbes can utilise a broad range of substrates as electron donors, including organics such as lactate, acetate or glycerol, or inorganic such hydrogen, with most organisms able to utilise a broad range of substrates. The number of electron acceptors is also broad, ranging from soluble radionuclides, through insoluble metal oxides to synthetic anodes (Guo et al., 2015; Lovley, 1993; Nealson et al., 2002; Wei et al., 2011). Many bacteria have the capability of extracellular reduction, although many of these require the use of exogenous mediators, such as neutral red, to facilitate electron transfer (Taskan et al., 2015). However, many microorganisms already present in the environment, both Gram

negative and Gram positive, are capable of reducing metal oxides or synthetic electrodes without the addition of mediators (Chabert et al., 2015). The most studied mineral reducing organisms are the genera of *Shewanella* and *Geobacter*. Bacteria of the *Shewanella* genus are typically facultative anaerobes that can often found in the shallow sediments of lakes rivers and oceans (Fredrickson et al., 2008). The strict anaerobes of *Geobacter* have also been isolated from a range of different geographical locations, and are often identified in the deeper subsurface (Nevin & Lovley, 2002).

The environmental impact of *Shewanella* spp. was first identified in the sediments of lake Oneida, where the concentrations of manganese in the lake waters were observed to increase in the summer, and decrease in the winter as manganese nodules were deposited within the sediment layer. Enrichment of the sediments identified an organism initially known as *Alteromonas putrefaciens* MR-1 (Later known as *Shewanella oneidensis* MR-1) as the biotic component in the sediment responsible for manganese reduction (Myers & Nealson, 1988). This organism explained the observed manganese cycle within the lake. During the summer months microbial activity increased, leading to an increase in the concentration of soluble, reduced manganese Mn(II) in the lakewater through the process of mineral respiration. During the winter months microbial activity decreases, and the soluble Mn(II) is chemically oxidised back into insoluble manganese Mn(IV) nodules on the surface of the lake sediment, as the lake warms during summer the cycle begins again (Aguilar & Nealson, 1998).

Within the same decade, the environmental capability of *Geobacter* to reduce Fe(III) oxide to the semi-reduced mineral magnetite had also been observed. The ability of bacteria to generate and utilise magnetite as a sensor had already been studied but this was the first evidence that bacteria were transforming Fe(III) minerals to Fe(III/II) minerals during respiration (Lovley et al., 1993). Both *Geobacter* and *Shewanella* are genetically tractable, can be grown as single colonies on plates and can utilise a range

of different organic substrates. They have been the source of careful study for almost 30 years, and as a consequence the mechanisms by which each bacteria performs mineral respiration are better understood than for many other mineral respiring organism.

1.2 Model iron oxides used for measurement of microbial biochemistry.

One of the principle challenges in the study of microbe-mineral respiration is defining the metabolic substrates used by the bacteria. The subsurface environment is comprised of an almost infinite variety of different substances, including minerals, metal chelates, complex organics such as humic acids, as well as mineral clay substrates. In order to survive in the environment microorganisms must be able to interact with these. The morphology of minerals, and complex organics such as humic acids tend to vary according to environmental conditions and the process whereby they were made. In light of this attempts to understand the mechanisms of microbial mineral reduction have relied on a relatively small range of soluble metal chelates and insoluble metal oxides and hydroxides.

Unlike conventional substrates used in biochemical and enzymatic analysis, insoluble metal oxides and hydroxides are heteregenous and cannot be defined by conventional enzyme-substrate kinetic models. There is also competition between microbial catalysis and conventional chemical redox processes. For example the neutrophilic iron oxidising *Sideroxydans lithotrophicus* survives at the oxic / anoxic interface by outcompeting oxygen in ferrous iron oxidation. At atmospheric oxygen concentrations iron oxidation is predominantly a spontaneous chemical process, however at micromolar oxygen concentrations chemical iron oxidation becomes slow enough for *S. lithotrophicus* to oxidise ferrous iron at the cell surface through a biologically driven reaction. Likewise the *Shewanella* outer membrane cytochrome MtrC was shown to become saturated by increasing Fe(III) citrate on the surface of the cell.

There are two approaches used to study biological iron reduction. The first, and simplest, is the reduction of soluble chelates of iron; the second is reduction of iron minerals, which is potentially more physiologically relevant but requires more careful definition. Soluble iron chelates commonly used include Fe(III)citrate, Fe(III)NTA and Fe(III)EDTA. Fe(III)EDTA is the simplest, comprising a single ferric iron atom chelated by an single ethylenediaminetetracetate (EDTA), however this is an artificial substrate that is unlikely to exist in the environment. Both Fe(III)NTA and Fe(III)citrate will form chelates with different structures depending on the Fe(III):chelate ratio. At stoichiometric ratios complexes containing $Fe(III)_2NTA_2$ and $Fe(III)_2citrate_2$ were observed to bind to defined sites on the surface of the outer membrane cytochrome UndA. Studies using these soluble cytochromes have indicated that the redox potential of the chelate largely dictates the observed rate of reduction.

For insoluble minerals, both physical and chemical properties must be considered when determining the availability of Fe(III) and how readily it will reduce. The chemical formula and arrangement of iron and oxygen in the crystal lattice define the mineral and its associated chemical and physical properties. There are 15 different types of iron oxides and hydroxides found in soils and sediments (Schwertmann, 2000). The most commonly studied in microbial reduction are the iron hydroxides ferrihydrite, goethite and lepidocrocite and the iron oxide hematite. These range in chemical formula, crystallinity, shape, size and particle surface area (Table 1). The co-ordination of Fe(III) in the mineral crystals is octahedral. In goethite and hematite the O and OH ions form layers that are hexagonally close packed (α-phase) and in lepidocrocite they are cubic close packed (γ-phase). This affects the crystallinity, surface texture and general reactivity of the mineral. Goethite is found in most soils, hematite occurs in more tropical regions where there are higher temperatures and lower water activities and lepidocrocite occurs in water-logged environments that are deficient in oxygen. Ferrihydrite is a

commonly-found, less ordered substance that varies in composition. It has a higher redox potential and consists of smaller particles making it generally more reactive than the other minerals. Ferrihydrite is unstable and over time transforms into the mineral oxides goethite and lepidocrocite. Lepidocrocite, while stable, has a more positive redox potential than goethite or hematite, making it the next most reactive in the series.

For particulate substrates, only surface-exposed metal ions will be accessible to electron transfer. Transmission electron microscopy can be used to measure the average dimensions of the mineral nanoparticles and, from this, the surface area per gram of mineral estimated. However, this does not take account of the surface texture. To account for the surface the Brunauer-Emmet-Teller (B.E.T.) method determines the 'specific surface area' by measuring the number of gas molecules, near their condensation temperature, that form a monolayer on a specific mass of particles (Schwertmann, 2000). Rates of iron reduction can then be defined in terms of moles Fe reduced m^{-2} s^{-1} that normalises the rates in terms of the physical properties of the minerals.

It has been shown that for abiotic mineral dissolution, the rate of Fe(III) reduction depends not only on the physical properties of the mineral particles but also on the thermodynamic properties of the ferric oxide phase (Roden, 2003). For reduction by a soluble electron donor, such as ascorbate, electron transfer is rapid and detachment of Fe(II) ions from the mineral surface is the rate limiting step as Fe(II) produced by chemical reduction can re-associate with the mineral surface. However, for biological iron oxide reduction the process requires recognition of the mineral surface by catalytic sites on the surface of the bacterium before microbe to mineral electron transfer can take place. In this case, electron transfer is the rate limiting step rather than detachment of Fe(II). From the examples shown in Table 1, it can be observed that, for equivalent surface areas, ascorbate dissolution rates are typically 10 fold faster for lepidocrocite

than goethite; whereas for microbial reduction with *Shewanella* there is less dependence on mineral type and a more direct correlation with specific surface area. Reviewing literature reports of bacterial reduction rates, Roden *et al.* (2003) commented that similar rates of mineral reduction and the same trend with respect to mineral type has been observed for both *Shewanella* and *Geobacter* (*S. Putrefaciens CN32, S. Alga BrY, G. Sulferreducens, G. Metallireducens*). It must be noted that the results for ferrihydrite do not fit the expected rates of abiotic and biotic reduction. Although the chemical and physical properties predict that it should be the most reactive mineral studied, literature values reported for abiotic reduction of ferrihydrite vary greatly and the production of Fe(II) from ferrihydrite by *Shewanella* is significantly slower than that found with the other minerals. This can be explained by the varied composition of ferrihydrite, its instability and the fact that the small ferrihydrite particles have a tendency to aggregate.

Table of soluble and insoluble iron oxides.

2 Biological Electron Transport across the Cell Envelope.

The majority of organisms that perform the metabolic oxidation or reduction of metal species transport electrons to the cell surface where the catalytic redox reaction takes place. For bacteria that utilise minerals as terminal electron acceptors, this requires the transport of electrons generated in the cytoplasm to be transported to the surface of the cell. For bacteria that use minerals as an electron source, the electrons must be brought into the cell either to the cytoplasmic membrane in order to reduce terminal electron accepters, or to cytoplasmic NADH. The outer membrane of Gram-negative bacteria is an insulating barrier that requires a conduit for electron passage, while in Gram-positive bacteria the cell outer wall acts as a barrier between cell and mineral surface.

Consequently in order to transfer electrons from inside the cell to the cell surface bacteria use a diverse range of cofactor rich proteins. Figure 2 shows examples of currently proposed electron transport systems for both mineral oxidising and reducing bacteria. All organisms use the quinol pool, either in the form of menaquinol or ubiquinol, as mediators of electrons across the cytoplasmic membrane, and the current understanding of the structural mechanisms that these bacteria use to transport electrons across the cell envelope and exchange electrons exchange with iron (hydr)oxides will be discussed in the remainder of this review.

[Figure 2. Iron reduction and oxidation pathways in microorganisms]

2.1 Extracellular electron transfer in gram-positive bacteria and archae.

A number of gram-positive bacteria have been shown to be capable of reducing insoluble metal oxides, either as isolated strains or as part of a bacterial consortium. As gram positive bacteria do not have an outer membrane, there is no requirement for electron transfer through porin-cytochrome systems, however the thicker cell walls limits the ability of the cell to transfer electrons directly from the cytoplasmic membrane and into insoluble extracellular mineral. The gram positive *Sulfobacillus sibiricus* is an moderately thermophilic acidophile that oxidises soluble Fe(II) to soluble Fe(III) (Dinarieva et al., 2010; Ilbert & Bonnefoy, 2013). Fe(II) is oxidised at the cytoplasmic membrane and energy is obtained through the reduction of oxygen on the same membrane. It is not clear which enzyme is responsible for iron oxidation, whether Fe(II) is oxidised directly by the terminal oxidase or if a cytochrome b electron shuttle mediates electron transfer between Fe(II) and the terminal oxidase (Figure 2), but for the gram positive acidophilic iron oxidisers, it is not necessary to have an electron conduit

between the cytoplasmic membrane and cell surface as both substrate and product can diffuse through the cell wall.

The acidophilic archaea *Ferroplasma acidarmanus* and *Sulfolobus* spp. have also been show to be capable of iron oxidation through direct reduction of iron at the cytoplasmic membrane. *F. acidarmanus* oxidises iron using a copper protein, sulfocyanin, that directly transfers electrons to a terminal aa_3 oxidase in order to supplement the proton motive force (Figure 2) (Castelle et al., 2015; Dopson et al., 2007; Dopson et al., 2005). In *Sulfolobus* spp. a more complex pathway appears to have evolved. A cytochrome b complex (FoxCD) oxidises ferrous iron to ferric iron and sends the electrons to an iron-sulfur protein with [Fe-S] domain (FoxG) and then the electrons are transported to a multicopper oxidase (Mco) before they arrive to the terminal oxidase and the NADH dehydrogenase (Figure 2)(Bathe & Norris, 2007).

In contrast the gram-positive thermophilic iron reducing *Carboxydothermus ferrireducens* (originally called *Thermoterrabacterium ferrireducens*) has been shown to reduce ferrrihydrite through direct contact between microbe and mineral (Gavrilov et al., 2012; Gavrilov et al., 2007). This pathway requires an electron transfer pathway to transport electrons directly from the cytoplasmic membrane to the extracellular minerals on the cell surface. The pathway has yet to be fully defined but it is reported to involve the reduction of Fe(III)oxides using cytochromes embedded within the S-layer of the cell wall, and pili that are expressed during respiration on insoluble minerals. At least one cytochrome Fe-(EDTA) reductase has been identified that attached to the cytoplasmic membrane, although it is not clear whether this is the only protein that is reduced by the cytochrome bc_1 complex, or how electrons from this complex are transferred either to pili or through the cell wall into the cytochromes embedded in the S-layer (Gavrilov et al., 2012) (Figure 3).

*[*Figure 3: Mechanism of extracellular electron transport in the Gram-positive *Carboxydothermus ferrireducens*.]

2.2 The porin-cytochrome complex as a transmembrane electron conduit.

The most commonly studied, and best understood, of the complexes that allow electron transfer across the outer membrane of Gram negative bacteria is the MtrCAB porin-cytochrome complex of *Shewanella oneidensis* MR-1, which is composed of three proteins encoded by the *mtrCAB* operon (Hartshorne et al., 2009; Ross et al., 2007). *S. oneidensis* also produces a second outer membrane cytochrome OmcA and has an *mtrDEF* gene cluster paralogous to *mtrCAB*. OmcA is co-expressed with MtrCAB, while the expression of MtrDEF is under a separate promotor and appears to be preferentially expressed in biofilms or aggregated cells (McLean et al., 2008).

Both *mtrA* and *mtrC* encode for two decahaem cytochromes, with molecular weights of 37 kDa and 75 kDa for MtrA and MtrC respectively. *mtrB* encodes MtrB, a transmembrane β-barrel with a molecular weight of 70 kDa. MtrA is periplasmic, while MtrC is transported by the type-II secretion system to the surface of the cell where it associates with the outer membrane through an N-terminal lipid anchor (Ross et al., 2007). The three proteins come together as a transmembrane complex that allows electrons from MtrA to be transferred to MtrC through MtrB (Figure 2). It is proposed that MtrB functions as a porin into which both MtrA and MtrC insert far enough to allow direct haem-to-haem electron exchange (Hartshorne et al., 2009; Richardson et al., 2012). In support of the proposed 'porin cytochrome mechanism' there is no evidence for cofactors in MtrB that would mediate electron transfer across the ~ 50 nm of the hydrophobic lipid bilayer. Recombinant MtrB cannot be expressed in the folded state without co-expression of MtrA, however, if the genes for expression of outer membrane

proteases are deleted, isolated MtrB can be expressed but the protein is produced in an unfolded state (Hartshorne *et al., 2009;* Schicklberger *et al.,* 2011*)*.

It is proposed MtrA extends into the periplasm where it contacts other periplasmic electron transfer proteins. Once electrons enter MtrA, they are transported through MtrA and into the adjoining MtrC with MtrB acting as an insulating sheath. Because electrons move through the haem chain by tunnelling between adjacent haems the porin-cytochrome complex is more an 'electron hopping conduit' than a true wire, with electrons being able to move in both directions across the membrane depending on the potential difference across the membrane (Hartshorne et al., 2009; White et al., 2012; White et al., 2013). Several groups have exploited this by growing *S. oneidensis* on an electrode and lowering the potential to drive electrons back into the bacterium, thereby altering the metabolic properties of the organism and causing the formation of various intermediates, most notably succinate (Grobbler et al., 2015; Ross et al., 2011).

Structurally much of the MtrCAB porin-cytochrome complex is still unresolved. The best structurally resolved component is MtrC, where a structure of the soluble MtrC protein from *Shewanella oneidensis* MR-1 has been solved to a resolution of 1.8 Å. The dimensions of the protein are approximately 80 x 60 x 30 Å, giving the protein a disc-like appearance (Edwards et al., 2015). Much less is known about MtrB beyond theoretical modelling; MtrB contains up to 28 β-strands as predicted from topology modelling software, and a short N-terminal domain that contains a CXXC amino acid motif. The role of the two cysteine at the N-terminus are unclear, but they are known to be on the surface of the cell, are redox active and at least one is required for successful assembly of the MtrCAB complex. In contrast to the orientation of most known β-barrel porins the MtrB porin is inverted, with the long soluble loops facing the periplasmic side of the outer membrane, and short loops facing the surface of the protein (White et al., 2013)

(Huysmans et al., 2010; White et al., 2013). This inverted state is suggestive of an alternative assembly mechanism and is consistent with the need for MtrA to stablise MtrB in the barrel – it is possible that MtrB assembles around MtrA in or on the outer membrane before formation of the stable complex.

The MtrA protein has been spectroscopically studied by several groups and the haems within the protein are known to cluster around two groups of standard electrode potentials, a high potential group around -200 and a low potential group around -100 mV; the split of haems between each group is approximately equal, with five haems in each group (Bewley et al., 2012; Pitts et al., 2003). There is no high resolution structural information available, so nothing is known about the way that the haems arrange within the MtrA structure, but a low resolution SAXS envelope of MtrA has been proposed by Firer-Sherwood and colleagues, which has an approximate length of 100 Å, a diameter of 40 Å and tapers to a narrow 30 Å at one end, suggesting that it is capable of inserting into MtrB (Firer-Sherwood et al., 2011). MtrA appears modular, as insertion of a stop codon into the *mtrA* gene after the sequence encoding the first five CXXCH haem motifs results in the expression of a truncated MtrA cytochrome with five bis-his ligated haems (Clarke et al., 2008). The structure of a lipopolysaccharide transporter have been resolved and shown to be a 26-strand β-barrel that contains a second protein as a 'plug' (Dong et al., 2014). This porin-plug complex is the closest structurally resolved protein to MtrB and has approximate dimensions of 60 x 40 nm, suggesting that MtrA would be small enough to insert completely to MtrB while the larger MtrC may be restricted from entering fully (Figure 4). These prediction are supported by proteinase K experiments revealing the complete digestion of MtrC from MtrCAB embedded in liposomes or the cell membrane of *S. oneidensis* (Edwards et al., 2015; White et al., 2013)

[Figure 4: Crystal structures of porins similar in size to Cyc2 and MtrB.]

Taken together, these data suggests that the MtrCAB complex functions by allowing electron transfer from one side of the lipid bilayer to the other through a chain of 20 haems that is formed between two cytochromes stabilised by a large porin. The much smaller diameter of MtrA would mean that this protein inserts further into MtrB than MtrC, such that the majority of MtrC remains exposed on the surface of the cell.

[Figure 5: Theoretical structures of known outer membrane porin cytochromes complexes]

Genes homologous to *mtrCAB* are found in several other bacterial families, including the related genus *Rhodoferax ferrireducens, Aeromonas hydrophilia, Halorhodospira halophilia,* and *Vibrio* spp. These bacteria have the full complement of *mtrCAB* genes, but the proposed role of the genes vary (Shi et al., 2012a). *Rhodoferax* is a known reducer of iron oxides and has been shown to grow using iron oxides as a terminal electron acceptor, it therefore seems likely the role of *mtrCAB* in this organism is the same as for *S. oneidensis:* to facilitate the transfer of electrons to extracellular iron oxides (Finneran et al., 2003). Many have not been shown to respire on iron oxides and it has been suggested that these bacteria actually use the MtrCAB pathway to sequester iron from the environment (Bücking, 2012). Many other bacteria have also been shown to contain homologues of *mtrAB* (Shi et al., 2012a) but relatively few of these have been characterised and the roles of the genes are not known.

Outer-membrane porin-cytochrome complexes with a similar modular configuration to MtrCAB have been identified in *Geobacter* (Figure 5). These complexes (Gpc1 and Gpc2) have been demonstrated to be capable of transferring electrons across a membrane and their deletion has been shown to be detrimental to the reduction of

extracellular electron acceptors by *Geobacter sulfurreducens* (Liu et al., 2014). The Gpc1 gene cluster encodes for OmaB, OmbB and OmcB and the paralagous Gpc2 gene cluster encodes for OmaC, OmbC and OmcC. Both OmaB:OmbB:OmcB and OmaC:OmbC:OmcC have been purified as single complexes from the outer membrane of *G. sulferreducens* and, using proteoliposome models, have been shown to be capable of transferring electrons through a lipid bilayer (Liu et al., 2014). OmaB/OmaC are octahaem periplasmic proteins, OmbB/OmbC are transmembrane porins, OmcB/OmcC are dodecameric extracellular cytochromes. The proposed functions and cellular localizations of OmaB/OmaC, OmbB/OmbC and OmcB/OmcC are analogous to those of MtrA, MtrB and MtrC from *Shewanella oneidensis*. However, although both contain haem-binding motifs typical of c-type cytochromes, there is little sequence homology between Gpc1/Gpc2 proteins and the proteins of the *Shewanella* MtrCAB complex and the distribution of the c-type haems is not conserved.

The porin-cytochrome complexes of mineral respiring *Geobacter* and *Shewanella* are three-component complexes that have been clearly identified as facilitating extracellular electron transfer across a lipid bilayer. In contrast the porin-cytochrome complexes found in iron oxidising bacteria contain only two proteins, which are homologues of MtrA and MtrB (Shi et al., 2012a). Few of these two-component complexes have been characterised so far. The genome of *S. lithotrophicus* ES-1 contains the *mtoA* gene, a decahaem cytochrome with 42–44% % homology to the *mtrA* genes of *Shewnella spp*. The *mtoA* gene is next to *mtoB* in the *S. lithotrophicus* genome, which is predicted to encode a porin. To date only the MtoA protein has been characterised, and shown to be a soluble decahaem cytochrome with a redox potential range slightly higher than MtrA. It has been proposed that together with MtoB it forms a decahaem MtoAB porin-cytochrome complex in the outer membrane that facilitates oxidation of soluble Fe(II) to insoluble Fe(III) (Liu et al., 2012)(Figure 5). There is no

MtrC homologue in the gene cluster, but there is a periplasmic monohaem c-type cytochrome, MtoD, that has been purified and structurally characterised (Beckwith et al., 2015). MtoD is proposed to transfer electrons across the periplasm into the cytoplasmic membrane to either bc_1 oxidase or to a CymA homologue, presumably to insert electrons into the quinol pool where they will ultimately be coupled to NAD^+ reduction (Figure 2). Homologous MtoAB systems have been identified in the genomes of *D. aromatic*a and *G. capsiferriformans,* where extracellular electrons are taken from Fe(II), across the outer membrane through the decahaem porin-cytochrome complex and passed to intracellular cytochromes for reduction pathways on the cytoplasmic membrane (Emerson et al., 2013; Shi et al., 2012a). The PioAB iron oxidising system of *Rhodopseudomonas palustris TIE-1* also has high homology to MtoAB and MtrAB, composed of the decahaem PioA and porin PioB. This phototrophic organism can use soluble Fe(II) as a electron donor for carbon fixation, with the periplasmic PioC, an iron-sulfur containing protein as the periplasmic shuttle between the outer and inner membrane (Jiao & Newman, 2007) (Figure 2).

This family of porin-cytochrome complexes seems to provide the major route of electron transfer across the outer membranes of a broad variety of gram negative bacteria, with known examples found in the α-, β-, γ- and ζ-proteobacteria. The number of haems found within the porin cytochrome complex of iron oxidising organisms is 10, while the porin-cytochrome complexes of iron reducing organisms contain a total of 20 haems. The 10 haems therefore appears to represent the optimum number of haems required to traverse the outer membrane through the porin and allow electron transfer on each side of the membrane. The porins within the complexes are predicted to be composed from between 20 – 28 β-strands which would give pore diameters ranging from 30 – 50 Å, sufficient to allow the insertion of a cytochrome through the centre (Figure 4).

2.3 The Cyc2 outer membrane fused porin-cytochrome.

While the porin-cytochrome complex system is the most commonly identified system in iron respiring bacteria, there is at least one other system identified that regulates electron transfer across the outer membrane. The outer membrane cytochrome Cyc2 from *A. ferrooxidans* was the first outer membrane electron transfer protein to be identified (Appia-Ayme et al., 1998), and was shown to be a monohaem c-type cytochrome localised to the outer membrane. Its proposed role is to obtain electrons from Fe(II) on the cell surface and transfer them to rusticyanin (RusA), a high-poteintial periplasmic copper protein (Figure 2) (Yarzabal et al., 2002). The electron transfer network then diverges as RusA transfers these electrons to either Cyc1 or Cyc42, which are two di-haem periplasmic cytochromes c. Electrons on the haems of Cyc1 are transferred to terminal oxidases, while Cyc42 will transfer electrons through the cytochrome bc_1 complex and the quinol pool to NADH dehydrogenases on the inner membrane (Roger et al., 2012) (Figure 2). Supercomplexes of Cyc2, RusA, Cyc1 and cytochrome oxidase have been isolated, suggesting that there are stable networks of electron transfer proteins from the cell surface to the inner membrane in these bacteria, although it is unclear how Cyc42 would accept electrons in these systems (Castelle et al., 2008). The structure of Cyc2 is poorly understood; it contains a single c-type cytochrome with a measured redox potential of + 560 mV vs. SHE at pH 4.8, making it possible to accept electrons from Fe(II) (Ilbert & Bonnefoy, 2013). Secondary structure predictions suggest that the N-terminal of Cyc2 contains a single CXXCH c-haem motif in an N-terminal region of approximately 30 amino acids, followed by a porin domain of 18 β-strands (Yarzabal et al., 2002). Similar sized porins have been structurally resolved using X-ray crystallography, such as the trimeric ScrY. The monomers of this sucrose transporter have 18 β-strands and form a channel with approximate dimensions of 25 x

40 Å (Figure 3). The most obvious way that the 30 amino acid haem domain could fold is with the 18 β-strands of the porin domain wrapping around an N-terminal haem domain (as is the typical folding pattern for most outer membrane β-barrel proteins). Cyc2 could therefore be representative of a family of fused porin-cytochromes where a monohaem cytochrome domain forms the 'plug' within the β-barrel. It is currently unclear whether the N-terminal haem domain faces the periplasm or the surface, a periplasmic facing haem domain would require soluble Fe(II) to diffuse and be oxidised within the barrel. If the cytochrome is exposed on the surface then it might be expected that Rustacyanin would have to insert into the porin from the periplasmic side in order to form an electron conduit (Figure 6). However, rustacyanin only contains a single copper atom on the surface of the protein and it unclear how this could orient in a stable complex to exchange electrons between the haems Cyc2 and Cyc1A/Cyc42.

[Figure 6: Possible configurations of a Cyc2 fused porin-cytochrome.]

The *Leptospirillum* spp. are a second group of acidophilic aerobes that utilise a system with homology to Cyc2. The outer membrane contains a large amount of an outer membrane protein called cytochrome 572 (Cyt_{572}) that is also predicted to contain a small monohaem N-terminal domain followed by a 15 β-strand C-terminal domain. A periplasmic monohaem cytochrome called cytochrome 579 is predicted to mediate electron transfer from the outer membrane to the inner membrane (Figure 2). Despite the low sequence homology between Cyt_{572} the secondary structure analysis is consistent formation of a fused porin-cytochrome similar to the *A. ferrooxidans* porin-cytochrome, allowing *Leptospirillum* spp. to obtain electrons from Fe(II) in the acidic extracellular environment. Both Cyc2 and Cytochrome 572 have been shown to oxidise

reduced iron under acidic conditions (Castelle et al., 2008; Jeans et al., 2008) and have periplasmic electron transfer partners that mediate electron transfer across the membrane. Unlike the porin-cytochrome complexes of *Shewanella* and *Geobacter*, Cyc2 has been isolated from the cellular membranes of both *A. ferrooxidans* and *Leptospirillum* and shown to be a single subunit protein. This suggests that neither rustacyanin or cytochrome 579 bind tightly to Cyc2, but suggests that they interact transiently with the base of the porin and that the haem domain lies at the periplasmic side of the barrel as shown in figure 6.

The first marine iron oxidiser to be characterized was *Mariprofundus ferrooxydans*. Found in iron-rich maths in hydrothermal fields it has recently been studied and its genome and proteomes sequenced. Two of the most common cytochromes expressed by *M. ferrooxydans* are homologues of Cyc2 and Cyc1 of *A. ferrooxidans*, suggesting that this neutrophilic microorganism utilises the same pathway as the acidophilic iron oxidising organisms (Barco et al., 2015). Perhaps surprisingly the genomes of both *Sideroxydans lithotrophicus* and *Gallionella capsiferriformans* contain genes homologus to *cyc2* of *M. ferrooxydans*, suggesting that these organisms contain both porin-cytochrome complex, and the porin-cytochome fusion systems. It is currently unclear which of these two systems would be responsible for neutrophilic iron oxidation for these organisms.

2.5. Electron transfer through the outer membrane of gram negative bacteria.

Current research has identified two separate systems by which electrons might pass through the outer membrane of Gram negative bacteria. The first, the MtrAB porin-cytochrome complex, represents the best characterised of the electron transfer conduits, with representative complexes isolated from the iron reducing *Shewanella, Geobacter* and lithotrophic iron oxidising *Sideroxydans* and *Gallionella*. It appears a key feature of

respiration using the transfer of electrons to and from iron under conditions where iron oxides develop and is important when dealing with insoluble metal oxides. The second system is the Cyc2 fused porin-cytochrome system that is common amongst microorganisms that utilise extracellular soluble Fe(II), such as *A. ferrooxidans,* this much simpler system is used under acidic conditions when insoluble iron oxides are unlikely to develop. There is also overlap within these systems, *S. lithotrophicus* contains homologues of both the MtrAB porin-cytochrome complex and the Cyc2 fused porin-cytochrome while certain strains of *Geobacter* contain homologues of MtrAB, Cyc2 and also generate conductive pili. It is not known whether these systems have overlapping functionality or perhaps work together to create an efficient iron metabolic pathway. Despite the observation of these conserved pathways for outer membrane electron transfer there are also several organisms that have as, yet unknown mechanisms for electron transfer through their outer membranes. For example *Rhodobacter ferrooxidans* does not contain the genes for porin-cytochromes in their outer membranes, and yet can oxidise metal on the cell surface under neutrophilic conditions (Hegler et al., 2008; Saraiva et al., 2012)(Figure 2).

3. Structures at the Interface of Microbe Mineral Interaction.

In order to utilise extracellular electron acceptors and donors as substrates during respiration bacteria have generated molecular conduits, detailed in the previous section, to transport electrons to the cell surface. From here it is necessary to then transfer the accumulating electrons to terminal electron donors and acceptors. This step is perhaps the most complicated and variable step of all, as it results in the transformation of a wide range of different substrates into different products and intermediates. For acidophilic iron oxidisers this is not a substantial challenge as both Fe(II) and Fe(III) are soluble and

readily diffuse around the cell. However for neutrophilic iron oxidising organisms the challenge is to prevent the accumulation of insoluble iron oxides on the surface of the cell, effectively entombing the bacteria, while for iron reducing organisms the challenge is to move the electrons from the surface to an insoluble, non-diffusive iron oxide particle.

In addition to the challenges posed by the transformation of iron between different mineral states, there is the challenge of access for bacteria attempting to utilise insoluble substrates as electron acceptors. Bacteria typically live either in planktonic suspension or as an aggregate biofilm. For bacteria living planktonically there is a challenge of obtaining regular access to the surface of insoluble substrates when such interactions might only occur transiently. For mineral respiring bacteria in a biofilm the challenge of nutrient access is on two fronts. Aggregated bacteria directly on the surface of the mineral have the greatest access to terminal electron acceptors but are furthest away for nutrients accessible from the media while bacteria on the surface of the biofilm have access to nutrients, but not to an electron donor (Bond et al., 2012). It is essential that all bacteria have access to both nutrients and the mineral oxide buried at the base of the biofilm to survive.

In order for bacteria to transfer electrons to minerals surface there have been three possible pathways postulated for iron (hydr)oxide reducing bacteria. The possible mechanisms by which electrons can transfer from cell to terminal acceptor have been loosely defined into three separate categories; direct, indirect and mediated. Direct mechanisms involve contact between the cell and the acceptor such that cytochrome and other electron transfer proteins on the outer membrane surface could make contact and transfer electrons directly. Indirect electron transfer involves the production of extracellular wire-like appendages that allow electron flux from the cell surface and into an electron acceptor several cell lengths distant. Mediated electron transfer is a broad

category where soluble iron chelators and siderophores, secreted organics and environmental mediators might be utilised as soluble electron shuttles from electron transfer proteins on the cell surface and into the terminal acceptors. There is evidence for all in the literature and it is likely that all three mechanisms can contribute under different environmental stresses (Brutinel & Gralnick, 2012; Lovley, 2008; Nealson et al., 2002; Richardson et al., 2012).

3.1 Extracellular electron transfer through conductive filaments.

In a biofilm actively respiring on a mineral or electrode surface only the first layer of cells will directly contact the mineral or electrode, meaning that the remaining cells require an alternative method of transferring electrons into the acceptor surface (Bond et al., 2012; Lovley, 2008). Many bacteria generate conductive filaments that can be seen under electron microscopy to extend from the surface of bacteria and are long enough to make contact either with a terminal electron acceptor such as a mineral or electrode, or another bacteria (Figure 7). Studies on these filaments has been greatly helped by the application of conductive atomic force microscopy, which allows the conductivity of individual filaments to be measured (Gorby et al., 2006; Reguera et al., 2005).

[Figure 7: Scanning electron microscopy image of *S. oneidensis* grown on a vitreous carbon anode.]

For *S. oneidensis MR-1* two types of filament have been described, a type IV pilin that has been structurally determined (Gorgel et al., 2015) and a second type of filament that is an extension of the outer membrane. These membrane extensions are known to be conductive with reported values of 1 S cm^{-1} and lengths measured of up to 9 μm (Pirbadian et al., 2014). The conductive properties of the *S. oneidensis* MR-1 nanowires

have also been shown to be non-metallic, meaning that electrons are likely to travel through redox centres along the filament, a process known as super-exchange conductivity (Bond et al., 2012; Pirbadian & El-Naggar, 2012).

Type IV pili in Gram negative bacteria bacteria are associated with a range of different properties, including adhesion, motility, DNA transfer and electrical conductivity (Giltner et al., 2012). The filaments that extend from the surface of *Geobacter sulfurreducens* were originally shown to be a member of the type IVa family of pili, have a conductivity of 5 mS cm^{-1} and be essential for Fe(III)oxide reduction (Malvankar et al., 2011; Reguera et al., 2005). The structure and mechanism by which the pili transfer electrons from *Geobacter* to the surface of the cell remains the subject of intense research by several groups. Wild type pili that are expressed by *G. sufurreducens* are up to 20 μm long and are coated in an extracellular cytochrome called OmcS. OmcS is a hexahaem outer membrane cytochrome that is essential for reduction of Fe(III)oxides or on electrodes (Holmes et al., 2006; Mehta et al., 2005). However the distance between adjacent OmcS cytochromes on the pili surface is not sufficiently close to allow for electron transfer between cytochromes, suggesting that electron transfer occurs through the pili, and not through the associated cytochromes (Aklujkar et al., 2013).

[Figure 8: The structures and configuration of pilin associated with mineral reducing bacteria.]

The intrinsic conductivity of the Pilin is proposed to be metallic-like, with electrons travelling through of aromatic residues with closely packed pi orbitals, a process known as metal-like conductivity (Boesen & Nielsen, 2013). A substantial challenge has been to prove the packing of aromatic amino-acids at the centre of the pilin. The PilA subunit of the pilin from *G. sulfurreducens* contains 5 conserved aromatic amino acids at the

carboxy terminus that, when substituted for alanines substantially decreases the conductivity of the pilin without noticeably interfering with the structure (Vargas et al., 2013). This is promising work implicating aromatic amino acid residues in the conductivity of pili, but it is still difficult to rationalise how the PilA subunits, which contain 5 aromatic residues in a total of 66 amino acids would be able to come together to form a continuous chain of pi-stacked orbitals over a distance of several cell lengths. In helping to understand how these five aromatic residues the NMR structure of PilA subunit has been determined and shown to have an α-helical content of approximately 85 % with the five aromatic residues clustered at the carboxy terminus of PilA (Reardon & Mueller, 2013) (Figure 8). Arrangement of this NMR structure into a pilin superstructure based on the pilin arrangement of *Neisseria gonorrhoeae* did not generate a conformation that would allow a continuous chain of stacked aromatic amino acids, but clustered the aromatic amino acids within a 15 Å sphere that was separated from adjacent spheres by an zone devoid of aromatic amino acids. The alternative model, based on the pilus assembly of *Pseudomonas aeruginosa* suggested a continuous chain of aromatic residues within the centre of the pilin with the aromatic amino acids arranged in a helical pattern on the within 3.6 Å of each other that could allow for metallic-like electron transfer (Malvankar et al., 2015) (Figure 8C, 8D). This helical arrangement required three aromatic amino acids to form a continuous chain, and in principle disruption of any of these three should break the chain. It is surprising that five aromatic residues required substitution in order to create the observed decrease in conductivity when deletion of just one of the three aromatic residues should have an effect.

While the pili of *G. sulfurreducens* are the most studied of all the conductive nanowires, there are a host of other microorganisms that have been shown to produce conductive filaments, including *Synechocystis sp PCCC6803, Pelotomaculum thermopropionicum*

(Gorby et al., 2006) and *C. ferrireducens* (Gavrilov et al., 2012). For most of these bacteria the composition of the filaments is not yet known and so it is unclear whether superexchange or metal-like conductive mechanism is favoured by the majority of other bacterial species.

Although the pili of *Geobacter* and several other microorganisms have been shown to be conductive, it has been less clear as to whether the *S. oneidensis* pili are also conductive. Genetic studies showed that deletion of the genes that encode the biosynthetic expression system *mshH-Q* decreased the current generated by a *S. oneidensis* fuel cell, however deletion of the genes *mshA-D* that actually form the pili only caused a 20 % decrease in current, suggesting that the assembly system, rather than the pili were required for extracellular electron transfer (Fitzgerald et al., 2012). The structure of the *S. oneidensis* type IV pilin has also been solved and, like *G. sulfurreducens* contains a number of aromatic amino acids that could be modelled to generate two clusters of parallel aromatic residues approximately 4 -7 Å apart, however there was a maximal distance of 11 Å between the two clusters, which would make electron transfer across the cluster gap unlikely without the presence of a mediator (Gorgel et al., 2015). As a consequence it is still unclear as to whether the type IV pili of *S. oneidensis* are conductive.

In mixed microbial communities the nanowires can allow the transfer of electron equivalents between different species, for example co-cultures of *G. sulfurreducens* and *G. metallireducens* were able to grown on a mixture of ethanol and fumarate, while isolated cultures were not. The growth was attributed to direct electron transfer (DIET) between the two species through the exchange of electrons via the *Geobacter* pili (Summers et al., 2010). Further studies have shown that *Geobacter* is also capable of growing syntrophically with a range of different bacterial species, including *Methanosarcina barkeri* or *Hydrogenophaga* spp. (Kimura & Okabe, 2013; Rotaru et al.,

2014), surprisingly these bacteria are not known to produce pili that could conduct electrons between species so the mechanism that allows microbes to obtain electrons from *Geobacter* pili is not known.

3.4 Direct contact: The structures of the outer membrane cytochromes of *Shewanella* spp.

The majority of extracelluar electron transfer proteins that have been structurally characterised thus far are the outer membrane cytochromes (OMC) of the *Shewanella* genus (Clarke et al., 2011; Edwards et al., 2014; Edwards et al., 2012b; Edwards et al., 2015; Fredrickson et al., 2008) Phylogenetic analysis revealed that these OMCs could be grouped into four separate clades, called the OmcA, MtrC, MtrF and UndA clades (Edwards et al., 2012a; Edwards et al., 2015). The OmcA and UndA clades are more closely related than to either the MtrC and MtrF clades, consistent with their positions within the gene clusters. OmcA and UndA are interchangeable between different *Shewanella* species while MtrC and MtrF are associated with the porin and periplasmic cytochromes. There is often little sequence homology between the different clade members with pairwise alignments of representative members of each clade giving values for sequence identity of 23-30%. However the arrangement of CXXCH c-type cytochrome binding motifs shows significant conservation (Edwards et al., 2014; Edwards et al., 2012a). The first approximately 200 N-terminal amino acids typically that contain the LXXC lipid binding motif and, in OmcA, UndA , MtrF and half of the MtrC family a $CX_{2-5}C$ motif that forms a disulfide bond. There then follows a CXXCH rich region containing five CXXCH cytochrome binding motifs within 200 amino acids before a gap of around 200 amino acids that contains a completely conserved $CX_{8-15}C$ motif that forms a disulfide bond. The C-terminal ~200 amino acids then contains the final 5 (or 6 in the case of UndA) CXXCH binding motifs. This pattern of interspersed

cytochrome binding regions is specific to this family of OMCs and resolution of the structures of representatives of each of the four clades revealed the significance of this conserved organisation (Clarke et al., 2011; Edwards et al., 2014; Edwards et al., 2012b; Edwards et al., 2015) (Figure 8).

[Figure 9: Cartoon representations of the crystal structures of outer membrane cytochromes.]

Despite the low sequence homology the structures of these cytochrome are markedly conserved. Each cytochrome is comprised of four domains, two alternating β-barrel domains and two multihaem domains. The domains are arranged in a loop so that the distance between the two porphrin rings of adjacent haems in the two multihaem domains are within electron transfer distance of each other, in this way rapid electron exchange between the two domains and across the entire protein is allowed The arrangement of the four domains results in the formation of a cross-like arrangement of the haems. This 'staggered-cross' is so far unique to structures of the outer membrane cytochromes of *Shewanella* with the minimum distance between the porphrin rings of adjacent haems being less than 7 Å, allowing for rapid electron transfer between the four terminal haem groups (Breuer et al., 2012). All the haems in the outer membrane cytochrome crystal structures are *bis*-histidine coordinated, making it difficult to predict which sites are used for entry/egress. Haems 5 and 10 (11 in UndA) are exposed at opposite ends of the structure, and are obvious sites for electron entrance/exit, but haems 2 and 7 are oriented towards the β-barrels that scaffold the two pentahaem domains and may direct electrons into potential binding sites for soluble metal ions or shuttles.

Overlay of the haems of the four OMCs reveals that the highest positional change is observed in haem 5, where the MtrC and MtrF haems are close in spatial arrangement, but the OmcA and UndA haems are widely different. The orientation of haem 5 of UndA is flipped relative to MtrC/MtrF/OmcA and haem 5 of OmcA is displaced. It is tempting to suggest that the high conservation in MtrC and MtrF might be due to their interaction with the MtrDE and MtrAB porin-cytochrome complexes, leaving haems 10 as the site of direct electron exchange. As OmcA and UndA do not form isolatable complexes with either porin-cytochrome complex, the requirement for a conserved terminal haem may not be necessary. In contrast, deletion of MtrC still allows electron exchange through OmcA, so a contrary argument could be made for haem 10, which is conserved. The other obvious area of difference between the homology of the other proteins is the position of the extra haem in UndA, which is located at the interface of domain 2 and the β-barrel. This extra haem could be responsible for the accelerated rates of Fe chelate reduction observed for Fe(III)NTA and Fe(III)EDTA as crystal soaks revealed that both Fe(III)citrate and FE(III) NTA associated within electron transfer of this haem (Edwards et al., 2012b; Shi et al., 2011).

The redox potential windows of the haems of the members of the OMCs from *S. oneidensis* have been measured, (Clarke et al., 2011; Firer-Sherwood et al., 2008; Hartshorne et al., 2007) and range in value from -500 to +100 mV vs. Standard hydrogen electrode. The cytochromes have also been tested for various soluble and insoluble substrates, by measuring the rate at which a fully reduced cytochrome is oxidised in the presence of substrate. The OMCs of *Shewanella* spp. characterised so far are rapidly oxidised by a range of soluble substrates including Fe(III)citrate, Fe(III)NTA, Fe(III)EDTA, FMN and riboflavin, as well as insoluble Fe(III)(hydr)oxides such as Goethite, hematite, lepidocrocite or ferrihydrite .

Initial *in vitro* experiments on the OMCs of *S. oneidensis* revealed that soluble chelated iron species such as Fe(III)citrate and Fe(III)NTA as well as flavins such as FMN and riboflavin, could rapidly oxidise reduced samples of MtrC and OmcA. The oxidation of these OMCs was dependent on the redox potential of the electron acceptor, with Fe(III) chelates fully oxidising OMC, and FMN only partially reducing OMC. Surprisingly the rates of reduction by reduced cytochromes purified from *S. oneidensis* are too low to support physiological respiration However, when soluble reduced OMC were mixed with iron oxides such as Fe(III)hydroxide, the rate of oxidation were less than 0.005 s^{-1}, suggesting that the OMC were not capable of reducing iron oxides fast enough to support respiration (Clarke et al., 2011; Ross et al., 2009). By incorporating the MtrCAB complex into proteoliposomes containing the membrane impermeable methyl viologen, a system was established whereby each MtrC could catalyse the reduction of iron oxides from the reduced methyl viologen inside the liposome. This method had 2 advantages over the use of soluble proteins. (1) Each MtrCAB could transfer <1000 electrons from the liposome interior, thereby functioning as an electron conduit. (2) The MtrCAB complex was correctly oriented, with MtrC on the membrane surface and so would be optimised for correct interaction with the iron oxides, rather than perhaps interacting in an orientation that did not favour electron transfer. These proteoliposome experiments proved for the first time that direct electron transfer from the cell surface to an iron oxide or electrode was possible and that the rate of electron transfer appeared to be proportional to the driving force between electron donor and acceptor. (White et al., 2013).

The remarkable structural conservation, both in domain organisation and haem arrangement, suggest that both the β-barrel domains and the staggered-haem cross are essential features of these mineral reducing cytochromes, however it still not clear what area on the surface of these elliptical proteins would interact with insoluble substrates.

Previously Lower and coworkers used phage-display technology to enrich for peptides that bind to hematite. This work identified a hematite-binding motif, with a conserved sequence of Ser/Thr-hydrophobic/aromatic-Ser/Thr-Pro-Ser/Thr (Lower et al., 2008; Lower et al., 2007). Molecular dynamic simulations with the peptide Ser-Pro-Ser indicated that hydrogen bonding occurs between two serine amino acids and the hydroxylated hematite surface and that the proline induces a structure-binding motif by limiting the peptide flexibility. The location of the residues comprising the proposed hematite binding motif were subsequently identified to be adjacent to haem 10 in the crystal-structures of MtrC and OmcA (Edwards et al. 2014, Edwards et al. 2015).
The current evidence is indicative of an orientation of the MtrC cytochrome with heme 5 interacting with MtrAB and haem 10 capable of interacting with the extracellular environment, however this is still far from certain. The orientation of the cytochromes on the surface of the membrane, along with the interactions between different cytochromes on the membrane surface are still significant questions that need to be addressed before a molecular understanding of the interaction between cytochrome and extracellular environment can be understood.

3.4.2 The role of shuttles in Shewanella extracellular electron transfer.

The use of electron shuttles as mediators during mineral respiration by *Shewanella* spp. has been widely researched, the first experiments separated *Shewanella putrefaciens* spp. 200 from the insoluble mineral goethite by a dialysis membrane and found that the goethite could not be reduced (Arnold et al., 1986). A similar observation was made using *Shewanella putrefaciens* MR-4 and manganite (MnOOH), where dialysis tubing prevented reduction of the insoluble manganese hydroxide (Larsen et al., 1998). Subsequent experiments encased iron oxide particles in microporus alginate beads. The beads had a diameter of 5 mm and pores of 12 kDa with Fe(III)oxide evenly distributed

throughout. While *G. metallireducens* was only able to reduce the Fe(III)oxide exposed on the surface of the beads, *Shewanella alga* BR1Y was capable of reducing much of the internalised Fe(III) as well (Nevin & Lovley, 2002). These later results suggested that certain species of *Shewanella* might be able to reduce insoluble iron oxides indirectly through the use of redox mediators. The first studies on indirect reduction by *S. oneidensis* used Fe(III)oxides encased in porous glass beads, these beads were approximately 50 μm in diameter, with the glass shell around the Fe(III)oxide being 0.3 μm thick. In these experiments *S. oneidensis, Shewanella putrefaciens* CN-32 and *Shewanella* spp. strain ANA-3 were all shown to reduce Fe(III) through the 0.3 μm porous barrier of the glass bead. *S. oneidensis* was further shown to form a biofilm over the surface of the beads, causing them to cluster together (Lies et al., 2005). Together these results using encapsulated Fe(III)oxides suggested that, under certain conditions, *Shewanella* respiration of insoluble metal oxide was at least partly due to the involvement of soluble mediators that could diffuse through the permable glass or alginate and reduce the encapsulated Fe(III).

Initially *S. oneidensis* was reported to secrete quinol-based compounds that were able to restore viability to menaquinone biosynthesis mutants (Newman & Kolter, 2000). These compounds were later identified as being released by lysed cells, rather than intentionally secreted shuttles (Myers & Myers, 2004). Eventually the search for secreted compounds in the extracellular environment in batch culture experiments by *Shewanella oneidensis* revealed the presence of the flavin compounds flavin mononucleotide (FMN) and Riboflavin (Marsili et al., 2008; von Canstein et al., 2008). These two compounds were determined not to be the products of cell lysis and a dedicated transport pathway was identified. FADH is secreted into the periplasm and hydrolysed into FMN by a 5'-nucleotidase called UshA. The FMN is then secreted into the extracellular matrix by a flavin transporter encoded by the *bfe* gene (Covington et al., 2010; Kotloski & Gralnick,

2013). Deletion of the *bfe* gene decreased the ability of *Shewanella* to reduce iron oxides or transfer electrons to carbon anodes but the phenotype could be restored through the addition of riboflavin to the culture mixture. *S. oneidensis* mutants lacking either *mtrC* or *omcA* were limited in their ability to generate current or reduce ferrihydrite, and addition of flavin did not improve this, showing the electron transfer pathway from the *S. oneidensis* cell surface to terminal electron acceptor required both MtrC/OmcA and FMN/Riboflavin (Coursolle et al., 2010) Taken together, these results clearly demonstrated that extracellular flavin in the forms of FMN or riboflavin were essential for Shewanella to respire on either insoluble Fe(III) or electrode surfaces, and that MtrC and OmcA were responsible for reduction (Brutinel & Gralnick, 2012).

Surprisingly, experiments mixing reduced flavins and insoluble iron oxides revealed that the ability of flavins to reduce insoluble Fe(III) was variable depending on the composition of the Fe(III) species. Mixing ferrihydrite or lepidocrocite with fully reduced FMN or riboflavin resulted in flavin oxidation coupled to the release of soluble ferrous iron (Shi et al., 2012b). In contrast experiments involving the reduction of the iron oxides goethite and hematite by FMN reveal almost no electron exchange, indicating that *S. oneidensis* respiration on these minerals was unlikely to occur using soluble flavin shuttles (Wang et al., 2015).

S. oneidensis has been shown to be fully capable of respiring and reducing both goethite and hematite (Learman et al., 2007; Lower et al., 2001; Neal et al., 2003; Ruebush et al., 2006), in these instances an alternative electron transfer system must be applicable if FMN or riboflavin are not capable of reducing them.

4. Summary of electron transport models across the outer membrane.

4.1 The extracellular electron transfer systems of neutrophilic iron oxidising organisms.

S. lithotrophicus, G. *capsiferriformans* and *R. palustris* all appear to utilise a two component porin-cytochrome complex to transfer electrons from Fe(II) at the cell surface to cytochrome electron shuttles in the periplasm. The mechanism by which electrons are abstracted from soluble Fe(II) at the cell surface is not known but the resulting formation of insoluble Fe(III)(hyd)oxides is well documented. In *S. lithotrophicus* the insoluble Fe(III)species precipitate on the surface of the cell as nanoparticles that appear to separate from the cell rather than accumulate (Emerson & Moyer, 1997), while in *Galionella* and related species the iron is oxidised and incorporated into twisted stalks. These stalks have been shown to be predominantly iron and organic carbon and are typically have diameters of 0.4 μm and can be up to 400 μm in length. The stalks are are only produced under growth on iron and are composed of from fibres that have nanometer diameters. The number of both stalks, and fibres within the stalks varies on species and growth conditions.

The mechanism by which electrons are abstracted from Fe(II) in this process is still not understood. It is unclear if the Fe(II) is oxidised to Fe(III) and then precipitated to Fe(III)OOH, or whether the steps of electron abstraction and oxide formation occur simultaneously. By coupling the oxidation of Fe(II) with the formation of an Fe(III)oxide it would be possible to harvest the electron from a Fe-complex at a much lower redox potential. The reduction potential of the haems from MtoA from *S. lithotrophicus* have been measured and shown to vary between +100 mV to – 400 mV (Liu et al., 2012), and the ability of MtoA to oxidise a range of different iron chelates, including Fe(II)citrate and Fe(II)EDTA, was shown to occur at rates between 1 $x10^{-3}$ – 6.3 x 10^{-3} μM^{-1} s^{-1}. It is likely that Fe(II) exists in a chelated form in the environment, attached to humic or organic

acids, and this is the physiologically relevant form of iron utilised by iron oxidising bacteria such as *S. lithotrophicus. S. lithotrophicus* was shown to grow on a range of different Fe(II) species, including FeS, $FeCO_3$ (siderite), $FeCl_2$, and $FeSO_4$ suggesting that the species and consequently the redox potential of the Fe(II) source is not important (Emerson et al., 2013).

4.2 The mechanism of mineral reduction in *Geobacter Sulfurreducens*

While conductive pili are responsible for electron transfer from the surface of *G. sulfurreducens*, the mechanisms by which electrons are transferred through the outer membrane and into the mineral surfaces are likely to involve the expression of cytochromes. The genome of *Geobacter sulfurreducens* contains over 100 c-type cytochromes, some containing up to 64 CXXCH motifs, the canonical c-type cytochrome binding motifs (Methe et al., 2003). These genes appear to have formed through multiple rounds of duplication, suggesting that the expressed cytochromes will form chains of multihaem domains. The structurally resolved cytochromes from *Geobacter* so far include the periplasmic monohaem PccH, dihaem proteins MacA (DCH2), dodecahaem GSU1996, and a trihaem cytochrome *c*7 family of PpcA - F (Dantas et al., 2015; Heitmann & Einsle, 2005; Pokkuluri et al., 2011; Pokkuluri et al., 2010). The only cytochrome associated with the outer membrane that has been structurally characterised is the outer membrane monohaem cytochrome OmcF. Many other key cytochromes of *Geobacter sulfurreducens*, including OmaB, OmcB and OmcS have yet to be crystallographically resolved.

There are excellent reviews describing the role of the periplasmic cytochromes in transferring electrons from the cytoplasmic membrane to the outer membrane (Richter et al., 2012; Santos et al., 2015). The interactions between electron transfer proteins within the periplasm appear to be transient, allowing pathways to alter in response to changes

in the periplasmic redox environment during homeostasis. The electrons are then proposed to leave the cell through the OmaB-OmbB-OmcB porin-cytochrome complex with electrons emerging on the *G. sulfurreducens* surface through OmcB (Liu et al., 2014), rather than electrons being transported through the cell surface via the pili. There is little evidence for electron transfer through pi-orbital stacking within bacterial cells, with the majority of electrons being carefully transported through protein cofactors within 14 Å of each other (Moser et al., 2010). It is interesting to note that the conductivity of *G. sulfurreducens* pili are sensitive to environmental changes such as pH, and as consequence their properties could be expected to change in the hydrophobic environment of the lipid bilayer (Malvankar et al., 2015). if the aromatic stacking is disrupted within the core of the lipid bilayer then pilin electron transfer through the outer membrane will not occur, ensuring the pilin only conduct electrons occurs on the cell exterior, and preventing electrons re-entering the bacterium.

The dodecahaem OmcB is an extracellular c-type cytochrome associated to the outer membrane by a N-terminal lipid anchor. OmcB is expressed during Fe(III) respiration and its repression limits the ability of *G. sulfurreducens* to respire on Fe(III) oxides (Leang et al., 2003). There is currently little experimental biochemical or structural information on OmcB. The amino acid sequence reveals there is little homology to any other characterised cytochrome apart from OmcC, a 71 % homologous protein that is part of a tandem chromosomal repeat on the *G. sulfurreducens* genome (Leang & Lovley, 2005; Liu et al., 2014). The arrangement of CXXCH motifs in the 722 amino acids of the OmcB sequence shows that seven CXXCH motifs are clustered in the first 285 amino acids, while the remaining five are found in the next 284 residues, leaving the final 153 amino acids free. This possibly indicates a tighter clustering of haems around the N-terminal domain of OmcB, and could indicate an electron transfer pathway from the OmbA-OmbB outer membrane complex that involves partial embedding in the OmbB

porin. In support of this hypothesis is previous research that showed that OmcB was partially, but not wholly, exposed on the surface of *G. sulfurreducens* (Qian et al., 2007). The other important outer membrane cytochromes of *G. sulfurreducens* are the hexahaem OmcS and tetrahaem OmcE. These cytochromes are loosely associated to the surface and deletion of either gene causes *G. sulfurreducens* to lose the ability to respire on insoluble Fe(III)oxides, despite being able to reduce extracellular soluble metals (Mehta et al., 2005). Of these OmcS has provoked the most interest as it is localised to the conductive pili, is known to contain six *bis*-histidine coordinated c-type haems that have midpoint redox potentials of – 40 mV to -360 mV vs. SHE. OmcS is also the only cytochrome of *G. sulfurreducens* that has been shown to reduce insoluble iron oxides, although no rates have been measured (Qian et al., 2011). Deletion of *omcS* also prevents respiration on Fe(III)oxides although addition of magnetite nanoparticles to *G. sulfurreducens* cells can restore Fe(III)oxide respiration (Liu et al., 2015), it has been reasonable to deduce from these experiments that OmcS therefore is a catalytically important cytochrome that functions as the final electron donor, from the conductive pili, to insoluble iron oxides.

The number of outer membrane cytochromes that have been shown genetically to be important for Fe(III)oxide respiration suggests that there is a specific electron transfer pathway that runs from the periplasm to the surface of an iron oxide. A recent proposed mechanism for electron transfer across the surface of *G. sulfurreducens* suggested that electrons would pass through the outer membrane via the OmaB-OmbB-OmcB porin-cytochrome, then electrons would pass from OmcB to OmcE across the surface of the cell, electrons would then enter at the base of the pili and pass through the conductive pili to the OmcS that coat the pili (Santos et al., 2015) (Figure 9).

[Figure 10: Mechanism of insoluble mineral reduction by *Geobacter sulfurreducens.]*

This mechanism provides a plausible explanation for the number of different cytochromes required for electron transfer between the outer membrane and Fe(III)oxide surface, but there is still a number of other proteins that are somehow involved in anaerobic respiration for example a multicopper protein OmpB has been shown to be important somehow in mineral reduction, although it is diffusive over the surface of the cell and not localised to the pili (Qian et al., 2007). It is possible that these proteins are not directly involved in Fe(III)oxide respiration but help to maintain the cell under conditions of Fe(III) reduction.

4.3 The mechanism of electron transfer from the cell surface of *Shewanella* spp..

One of the primary advantages in research into mineral respiration of the *Shewanella* spp. has been that the genes *mtrCAB and omcA* responsible for microbial electron transfer were identified early on as being part of a single gene cluster (Beliaev & Saffarini, 1998). Given that deletion of *mtrA* or *mtrB* fully prevented *S. oneidensis* MR-1 from reducing insoluble metal oxides and deletion of *mtrC* or *omcA* caused only partial loss of Fe(III)oxide reduction, it is likely there is overlap between the roles of MtrC and OmcA (Coursolle & Gralnick, 2010; Myers & Myers, 2001). The role of the MtrCAB porin-cytochrome complex as a conduit for electron transport across the outer membrane will result in the accumulation of electrons within the MtrC cytochromes on the cell surface, but the pathway by which electron are then transferred to insoluble Fe(III) and Mn(VI) oxides is less clear.

The mechanism by which *S. oneidensis* transfers electrons from periplasmic electron donors to extracellular substrates has been experimentally shown to be a multistep process that requires the participation of both MtrA, MtrB, MtrC and OmcA as well as extracellular flavins. *S. oneidensis* produces filaments that have been shown to be

conductive, but when examined these conductive filaments were revealed to be extensions of the cell membrane and periplasm that could extend up to 9 μm (Gorby et al., 2006; Pirbadian et al., 2014). These extensions have been shown to be conductive, but have different properties to the metallic like conductivity of the *G. sulfurreducens* pili, instead they show super-exchange conductivity, where the electrons are passing through a number of cofactors. These outer membrane extensions have been shown to contain large numbers of both MtrC and OmcA spread across the surface, allowing for the possibility of electrons being transferred through the filament and into terminal electron acceptors. It is unclear how electrons could travel through these filaments, either through the internal periplasmic space and then out through the porin cytochrome complexes, or directly through MtrC and OmcA on the outside. It is also currently unclear how these filaments are assembled, or how their expression is regulated

The pathway through the outer membrane is well defined, through the MtrCAB porin-cytochrome complex that permits electron transfer to the cell surface. From here electrons can pass from MtrC to OmcA, although it appears possible for electrons to directly move from MtrAB to OmcA as deletion of MtrC still allows for partial Fe(III)reduction and transfer to electrodes (Bretschger et al., 2007; Coursolle & Gralnick, 2010). Both MtrC and OmcA have been shown to interact on the surface of living *S. oneidensis* cells at a ratio of 2 OmcA : 1 MtrC, so each porin-cytochrome outer membrane conduit complex, consisting of MtrCAB-$OmcA_2$, could feasibly hold up to 40 electrons within a haem chain that could transfer electrons to an available electron acceptor (Shi et al., 2006).

It is possible for MtrC to directly reduce mineral oxides at rates that could support physiological respiration, given a sufficient driving force in the form of a lower redox potential on the inner side of the membrane (White et al., 2013). However, the observed increase in Fe(II) when either FMN or riboflavin are added to *S. oneidensis* cells

respiring on insoluble Fe(III) indicates that the flavins have an important role in supporting anaerobic respiration on iron oxides.

The isolated MtrC and OmcA were capable of reducing flavins at physiologically favourable rates showing that flavins and cytochromes could transiently interact to exchange electrons, however no stable complex or measurable association could be determined, indicating the electron transfer interaction was very weak and short lived (Coursolle et al., 2010). Paquete *et al.* measured dissociation constants of 29 and 225 μM between FMN and MtrC or OmcA, suggestive of a very weak interaction that, at the sub-micromolar concentrations of flavin secreted by *S. oneidensis* under batch culture conditions, would favour a transient interaction between cytochrome and soluble shuttle (Paquete et al., 2014). However, an *in vivo* investigation using whole cell EPR spectroscopy coupled with cyclic voltammetry revealed that evidence for tight FMN-MtrC and riboflavin-OmcA complexes on the surface of the cell (Okamoto et al., 2013; Okamoto et al., 2014). A separate study by Wang *et al.* (2015) using the proteoliposome method of White *et al.* (2012) revealed that stoichiometric amounts of flavin binding to MtrCAB was sufficient to accelerate the initial rate of electron transfer to hematite, lepidocrocite and goethite (Wang et al., 2015). A study that tracked the movement of *S. oneidensis* in the presence of Mn(IV)oxide particles showed that many cells remained planktonic, making intermittent contact with the mineral surface in a manner that suggested respiration was occurring through direct contact, rather than through electron shuttles diffused through the media (Harris et al., 2012).

Taken together the *in vivo* and *in vitro* data suggest that these outer membrane flavocytochrome complexes are the dominant form responsible for mineral reduction and electron transfer to electrodes, rather than cytochromes transferring electrons to soluble shuttles. Further studies on MtrC and OmcA revealed that the binding of flavins to either cytochrome was enhanced when the protein was reduced. Rather than the haems, flavin

binding was revealed to be regulated by a conserved disulfide on the surface of the second β-barrel domain observed in the four available outer membrane cytochrome structures (Figure 8). Reduction of this disulfide caused either riboflavin or FMN to associate so tightly to the outer membrane cytochrome that it was possible to isolate the flavin-cytochrome complex through gel filtration. Oxidation through exposure to air caused the disulfide to reform and flavin to dissociate (Edwards et al., 2015). It has been suggested that this control of flavin binding by the redox active disulfide may be a mechanism to protect *S. oneidensis* against the formation of reactive oxygen species, as *S. oneidensis* has a very low tolerance for oxygen species such as peroxide (Ghosal et al., 2005).

MtrC, OmcA, MtrF and UndA were all reported to bind a single flavin, with no observable preference for FMN or riboflavin, in contrast to Okamoto *et al.* (2013) who observed preferential interactions for MtrC with FMN, and OmcA for riboflavin. These data suggest the observed differences between cytochromes and flavins may possibly be due to accessibility on the cell surface, rather than the affinity between cytochrome and flavin. While it is still not possible to rule out the potential role of flavin as a soluble shuttle between *S. oneidensis* and the more reactive minerals such as ferrihydrite and lepidocrocite, it is clear that the mechanism of direct contact between *S. oneidensis* outer membrane cytochrome and mineral oxide will dominate for the more stable minerals such as goethite and hematite. The *S. oneidensis* nanowires first reported by Gorby *et al.* in 2006 utilise the same MtrC/OmcA catalytic mechanism for mineral reduction as the rest of the cell surface (Gorby et al., 2006; Pirbadian et al., 2014), so it is possible to suggest a flexible mechanism by which *S. oneidensis* can interact with insoluble metals under different levels of oxygen (Figure 10). Under anoxic conditions the disulfides on the surface of the outer membrane cytochromes becomes reduced, possibly through proximity with reduced haem groups of the cytochrome or reduced

environmental species. The cytochrome then binds flavin to give a flavocytochrome with enhanced electron transfer activity. This flavocytochrome is capable of reducing minerals, metals and electrodes at much faster rates than the cytochrome form, but when the environment changes from anoxic to micro-oxic the disulfide reforms and the mechanisms switches to a mediated process where flavins may become soluble shuttles to other electron acceptors, such as ferrihydrite.

[Figure 11: Proposed mechanisms of extra-cellular electron transfer to insoluble Fe(III) oxide / hydroxide minerals by *Shewanella oneidensis* MR-1.*]*

5. Future perspectives:

The past decades have brought huge insight into the biochemical relationship between microorganisms and the inorganic environment. In particular, our understanding of how the model mineral respiring organisms *S. oneidensis* and *G. sulfurreducens* transfer electrons from the cytoplasmic membrane to extracellular Fe(III)oxides has greatly increased. However, there are still substantial areas in these systems where our understanding in these systems is limited. For *Shewanella* the interactions between mineral, cytochrome and flavin are still unclear, and it appears likely that the microbe-mineral interface is dynamic and adaptive, although how this adaption is driven is unclear. For *Geobacter* the outer membrane cytochromes are poorly characterised, and a mechanistic understanding of their roles, particularly for OmcS, is an important step in understanding how these complexes work. There is evidence for metallic-like conductivity in *Geobacter* pili, but better understanding of the roles of the aromatic residues, as well as improved models for pilin assembly, are still required.

Due to their low growth rates and poor biomass yields, substantially less is known about

the iron oxidising bacteria, and this is the area where more experimental biochemical evidence is needed. Better growth conditions for these bacteria, or expression of iron oxidising systems in suitable bacteria is required before the electron transfer pathways and metabolic pathways of these bacteria can be understood to the same level as the mineral respiring strains.

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Mineral	Goethite	Hematite	Lepidocrocite	Ferrihydrite	Ref
Formula	αFeOOH	αFe_2O_3	γFeOOH	Varied	1
Crystal system	Orthorhombic	Trigonal	Orthorhombic	Amorphous	1
Typical size and shape	20-100 nm x 200-500 nm (needles)	30-50 nm (hexagonal)	10-50 nm x 100-300 nm (lath-like)	<50nm (spheres)	2
Typical B.E.T. surface area ($m^2 g^{-1}$)	38	34	130	230	2
Redox Potential Typical [a](V)	-0.157	-0.121	-0.103	+0.61	2
Dissolution Rate by ascorbate [b](10^{-9} mol m^{-2} s^{-1})	0.4	-	5 – 7	4 - 40	3
Dissolution Rate by ascorbate [b](10^{-9} mol m^{-2} s^{-1})	0.1	0.1	4	0.2	4
Dissolution Rate by *Shewanella* [c](10^{-9} mol m^{-2} s^{-1})	0.06	0.08	0.1	0.01	4

[a] Redox Potential (V vs SHE at pH7) 1 μM total dissolved Fe.
[b] Dissolution Rate: Reduction of iron oxides by 10mM ascorbic acid at pH 3, 25°C, anoxic conditions, determined by concentration of Fe^{2+} released into solution.
[c] Dissolution Rate: Reduction of iron oxides by *Shewanella putrefaciens* strain CN32, pH 7, , 25°C, anoxic conditions, determined by concentration of Fe^{2+} released into solution.

(1) (Schwertmann, 2000) (2) (White et al., 2013) (3) (Larsen & Postma, 2001) (4)(Roden, 2003)

Figure 1: Electron transfer pathways of energy production for mineral-respiring bacteria. (A) Mineral-reducing bacteria transfer electrons into the quinol pool via a quinone reductase in the cytoplasmic membrane. Quinol dehydrogenases on the periplasmic side of the membrane oxidise quinol and electrons are passed through the periplasm, across the outer membrane to the extracellular electron acceptors (M_{ox}). (B) Pathway of iron-oxidising bacteria, electrons obtained on the cell surface from oxidation of iron are transferred through two separate pathways; either into oxidases for production of a proton motive force, or into the quinol pool to power the formation of NADH. Red (grey) lines represent electron transport while dashed lines represent proton transport

Figure 2. Iron reduction and oxidation pathways in microorganisms. (A) Anaerobic iron reducers *G. sulfurreducens* and *S. oneidensis* (Lovley 2006, Shi et al. 2012, Liu et al. 2014); (B) Neutrophilic aerobic iron oxidisers *M. ferroxidans* and *S. lithotrophicus* (Barco et al. 2015, Beckwith et al. 2015); (C) acidophilic aerobic iron oxidisers *A. ferroxidans*, *Leptospirillum* spp., *S. sibiricus*, *F. acidarmonas, Sulfobulbus* spp. (Ilbert & Bonnefoy 2013); (D) Neutrophilic photosynthetic iron oxidizers *R. ferroxidans* and *R. palustris* (Jiao & Newman, 2007, Saravia *et al.* 2012) ; (E) neutrophilic anaerobic iron oxidisers dependent on nitrate, perchlorate or chlorate reduction (Shi et al. 2012). The electron flow is indicated as a dashed line.

Figure 3: Mechanism of extracellular electron transport in the Gram-positive *Carboxydothermus ferrireducens*. Electrons generated through the cytochrome bc_1 complex are transferred to Extracellular iron oxides through direct electron transfer pathways that are proposed to involve cytochromes associated with the cytoplasmic membrane, the microbe-mineral interface involves cytochromes associated with the S-

layer of the cell wall. The electron transfer mechanisms indicated by dashed arrows have not yet been determined.

Figure 4: Crystal structures of porins similar in size to Cyc2 and MtrB. (A) lipopolysaccharide translocon consisting of a 26 β-strand porin LptD and α-helical LptE plug (light grey) (PDB ID: 4N4R). (B)18 β-strand monomer of the trimeric sucrose transporter ScrY (PDB ID: 1A0T).

Figure 5: Theoretical structures of known outer membrane porin cytochromes complexes. (A) MtrCAB complex of *Shewanella oneidensis* consisting of the decaheme MtrA, the 28 β-strand porin MtrB and the decaheme cytochrome MtrC (PDB ID: 4LMB). (B) Gpc1/ Gpc2 complex of *Geobacter sulfurreducens* consisting of the octaheme OmaB, 20 β-strand porin OmbB and dodecaheme OmcB. (C) MtoAB complex from *Sideroxydans lithotrophicus ES-1* consisting of the decahaem MtoA and 28 β-strand porin MtoB. Red diamonds represent c-type haems.

Figure 6: Possible configurations of a Cyc2 fused porin-cytochrome. (A) The haem domain is located on the cell surface, allowing access to Fe^{2+} and insertion of rustacyanin into the barrel. (B) The haem domain located on the periplasmic face of the membrane, allowing reversible association with rustacyanin and Fe^{2+} diffusion through the extracellular-facing barrel entrance. (C) The haem domain is in the centre of the barrel with Fe^{2+} diffusing into the extracellular side and electrons being transferred to a bound rustacyanin at the periplasmic face of the membrane.

Figure 7: Scanning electron microscopy image of *S. oneidensis* grown on a vitreous carbon anode. Filaments similar to the nanowires observed by Pirbadien et al. (2014) are extending from the microorganisms. Image courtesy of Saad Ibrahim (UEA) and Kim Findlay (John Innes Centre).

Figure 8: The structures and configuration of pilin associated with mineral reducing bacteria. (A) The *G. sulfurreducens* PilA structure (Reardon & Mueller 2013, PDB ID: 2M7G) with aromatic residues implicated in electron transfer shown as spheres. (B) PilA structure from *S. oneidensis* (Gorgel et al. 2015, PDB ID: 4D40) with aromatic residues associated with the pilin centre shown as spheres. (C) Side view of proposed assembly of conductive pilin based on homology modeling using *P. aeruginosa* pilin assembly as a template. Aromatic residues shown as spheres with proposed stacking residues highlighted in red. (D) End-on view of *G. pilin* assembly. Structural coordinates for pilin assembly obtained from Malvankar et al. 2014.

Figure 9: Cartoon representations of the crystal structures of outer membrane cytochromes. Structures of MtrC (PDB ID: 4LM8), MtrF (PDB ID: 3PMQ), OmcA (PDB ID: 4LMH) and UndA (PDB ID:3UCP) isolated from *Shewanella* spp. Domains are numbered according to their position in the amino acid sequence (roman numerals). (Centre) Superposition of the haems of each cytochrome shows conservation haem configuration within the cytochrome structure. Haems are numbered according to the position of the CXXCH binding motif in the amino sequence. Numbers refer to MtrC, MtrF and OmcA with numbers in parenthesis referring to UndA. The position of resolved disulphide bonds in each structure are shown as sticks.

Figure 10: Mechanism of insoluble mineral reduction by *Geobacter sulfurreducens.* Electrons from the periplasm are proposed to pass through a porin-cytochrome complex to OmcB on the cell surface, OmcB passes electrons to OmcE with mediates electron transfer to the extended pilin. Electrons transfer from the pilin to pilin-associated OmcS cytochromes that directly reduce the insoluble Fe(III) oxide / hydroxide.

Figure 11: Proposed mechanisms of extra-cellular electron transfer to insoluble Fe(III) oxide / hydroxide minerals by *Shewanella oneidensis* MR-1. (**A**) Periplasmic electrons are passed through the MtrCAB porin-cytochrome complex to the surface exposed MtrC cytochromes. From here they can either be directly transferred to the surface of the mineral or to cell membrane associated OmcA. **(B)** In the absence of oxygen, secreted flavins bind to OmcA and MtrC, which enhances the mineral reductase activity of these cytochromes. Exposure to oxygen causes the flavin to dissociate. **(B)** Reduction of ferrihydrite and lepidocrocite by soluble flavin shuttles can occur but the relative contributions of direct or mediated reduction are not known.

Figure 1:

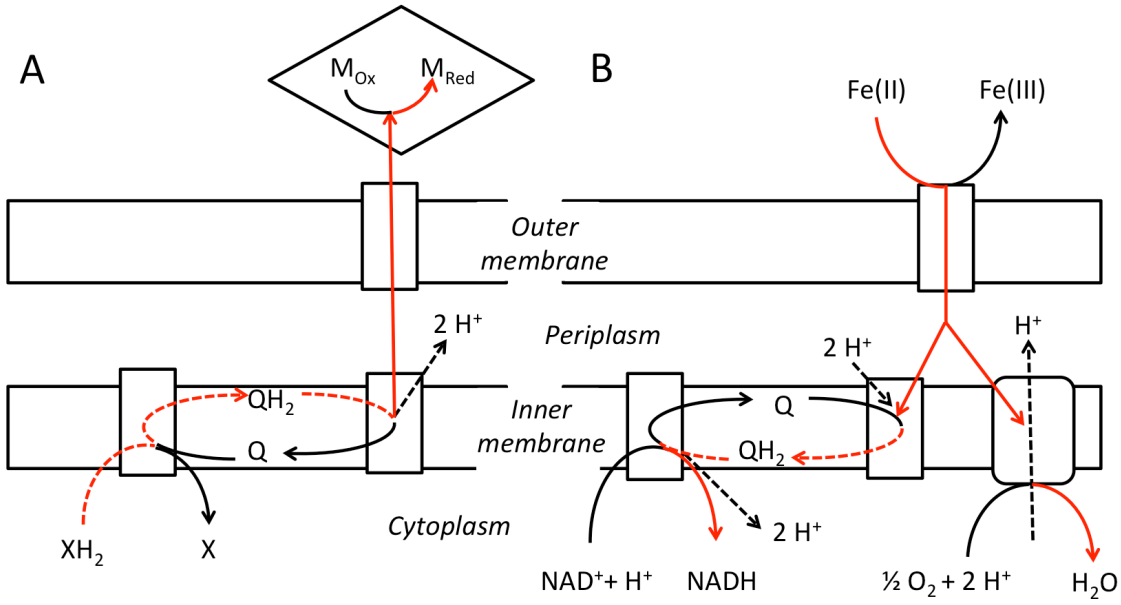

A
M_{Ox}
M_{Red}
B
Fe(II)
Fe(III)
Outer membrane
Periplasm
Inner membrane
Cytoplasm
2 H+
QH_2
Q
XH_2
X
2 H+
Q
QH_2
2 H+
H+
$NAD^+ + H^+$
NADH
½ O_2 + 2 H+
H_2O

Figure 2

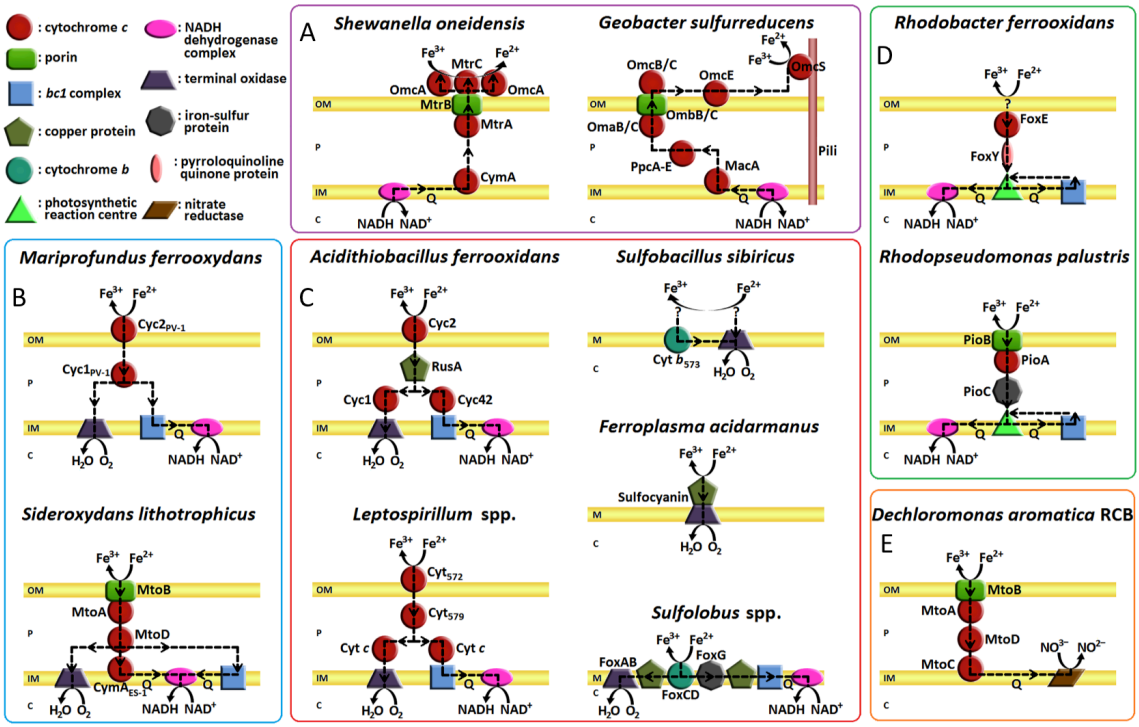

: cytochrome c
: porin
: bc1 complex
: copper protein
: cytochrome b
: photosynthetic reaction centre
: NADH dehydrogenase complex
: terminal oxidase
: iron-sulfur protein
: pyrroloquinoline quinone protein
: nitrate reductase
A
Shewanella oneidensis
Geobacter sulfurreducens
B
Mariprofundus ferrooxydans
Sideroxydans lithotrophicus
C
Acidithiobacillus ferrooxidans
Leptospirillum spp.
Sulfobacillus sibiricus
Ferroplasma acidarmanus
Sulfolobus spp.
D
Rhodobacter ferrooxidans
Rhodopseudomonas palustris
E
Dechloromonas aromatica RCB
OM
P
IM
M
C
NADH NAD+
H2O O2
Pili

Figure 3

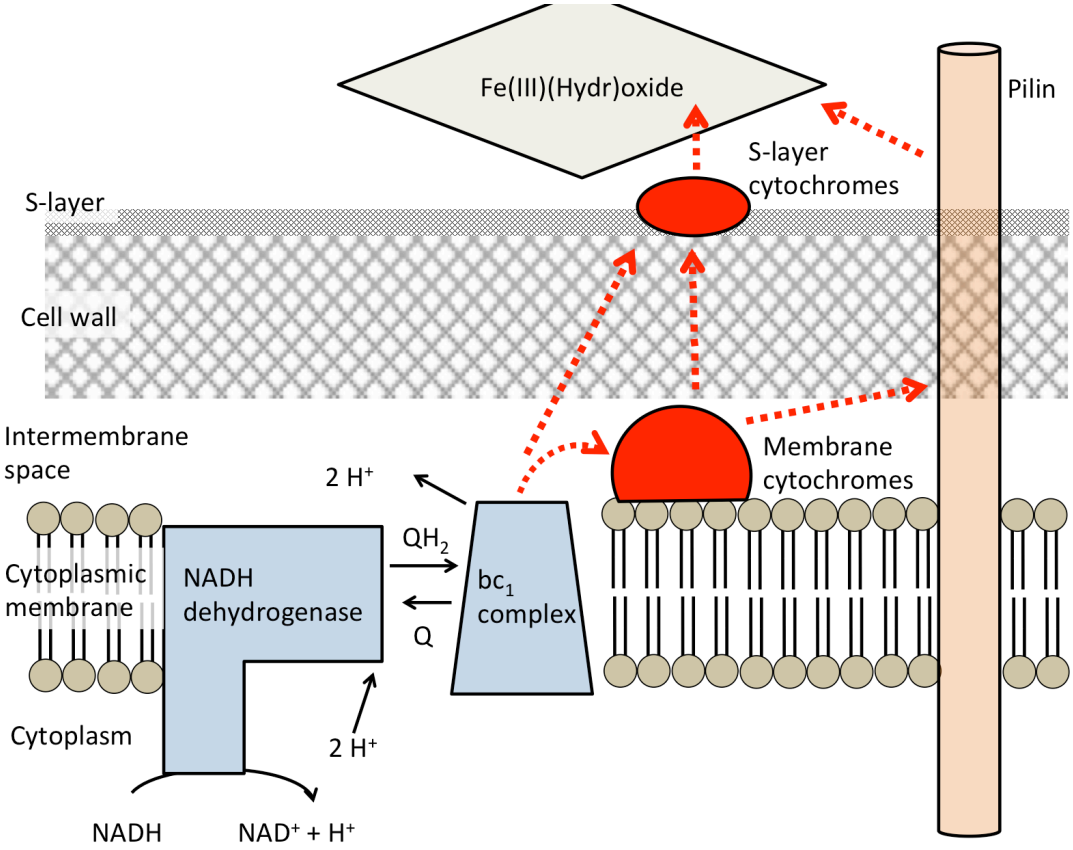

Fe(III)(Hydr)oxide
Pilin
S-layer cytochromes
S-layer
Cell wall
Intermembrane space
2 H+
Membrane cytochromes
QH2
Cytoplasmic membrane
NADH dehydrogenase
bc1 complex
Q
Cytoplasm
2 H+
NADH
NAD+ + H+

Figure 4

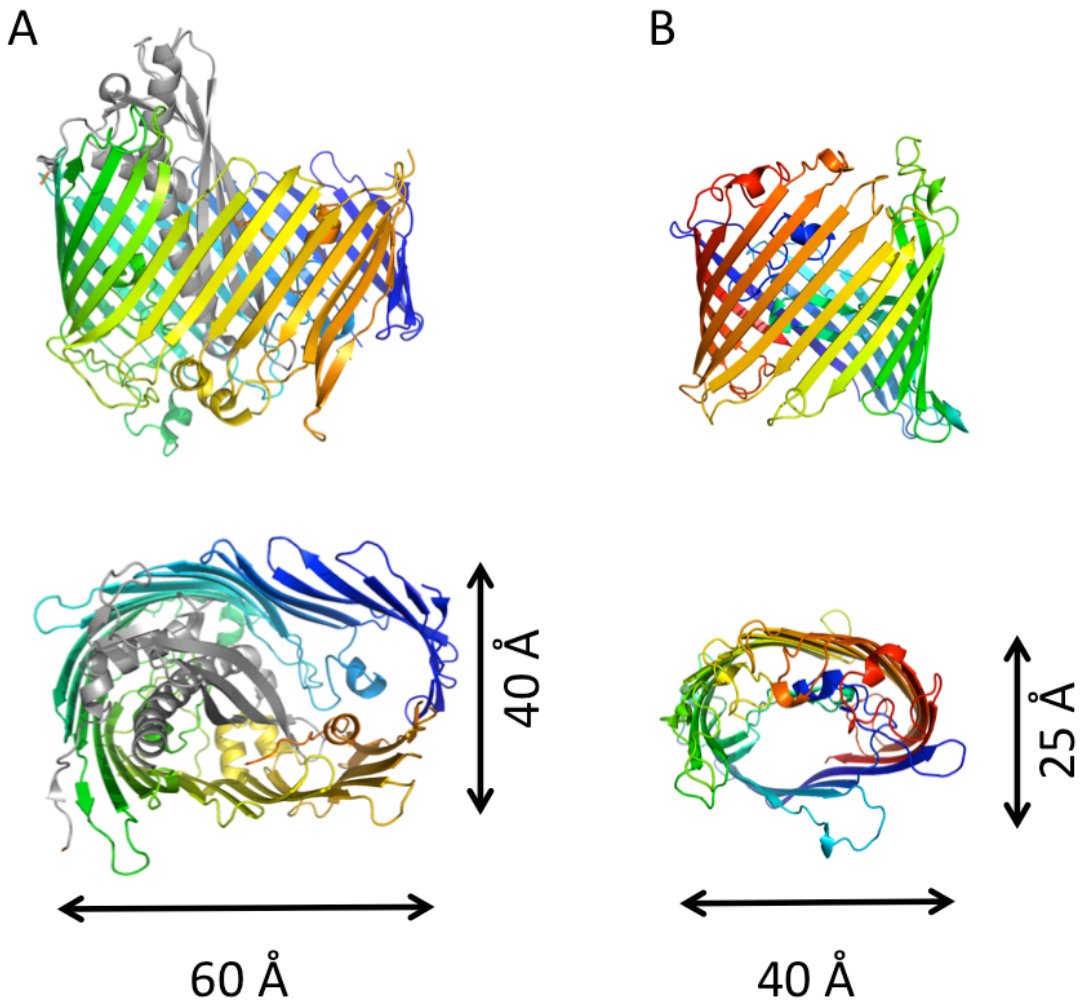


Figure 5

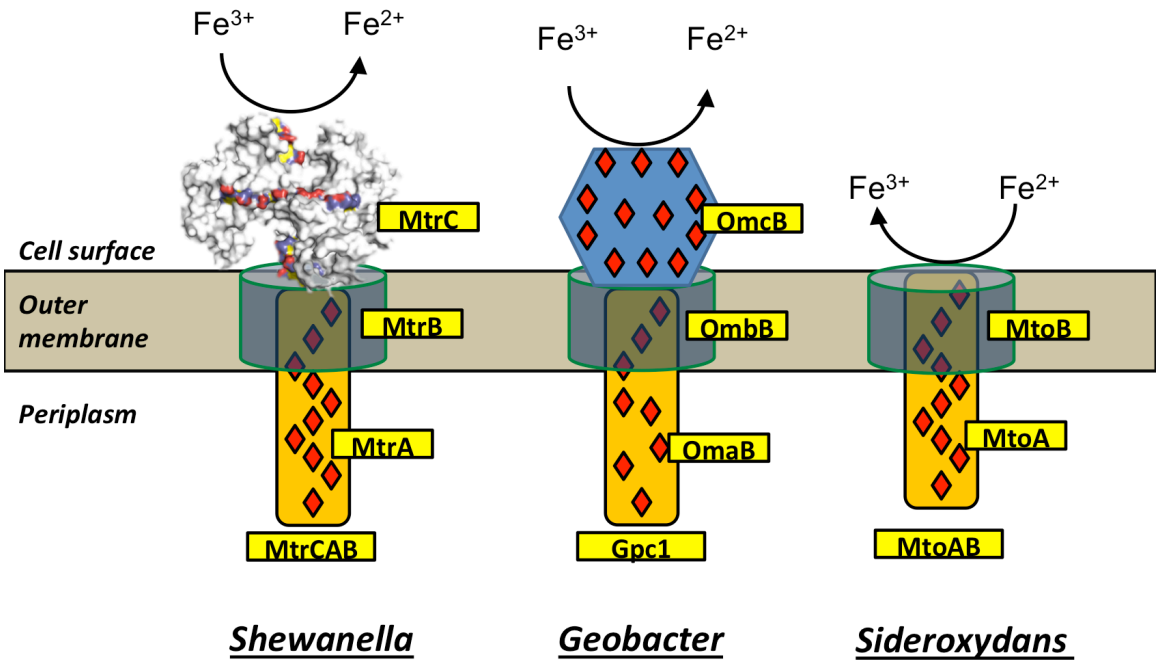

Fe3+
Fe2+
MtrC
MtrB
MtrA
MtrCAB
Cell surface
Outer membrane
Periplasm
Shewanella
Fe3+
Fe2+
OmcB
OmbB
OmaB
Gpc1
Geobacter
Fe3+
Fe2+
MtoB
MtoA
MtoAB
Sideroxydans

Figure 6

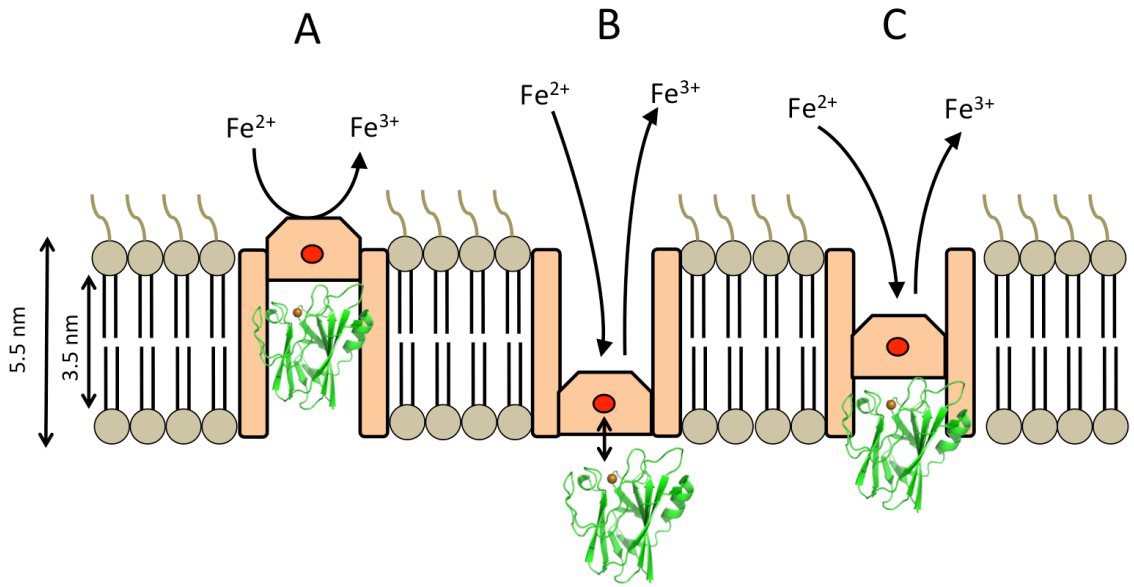


Figure 7

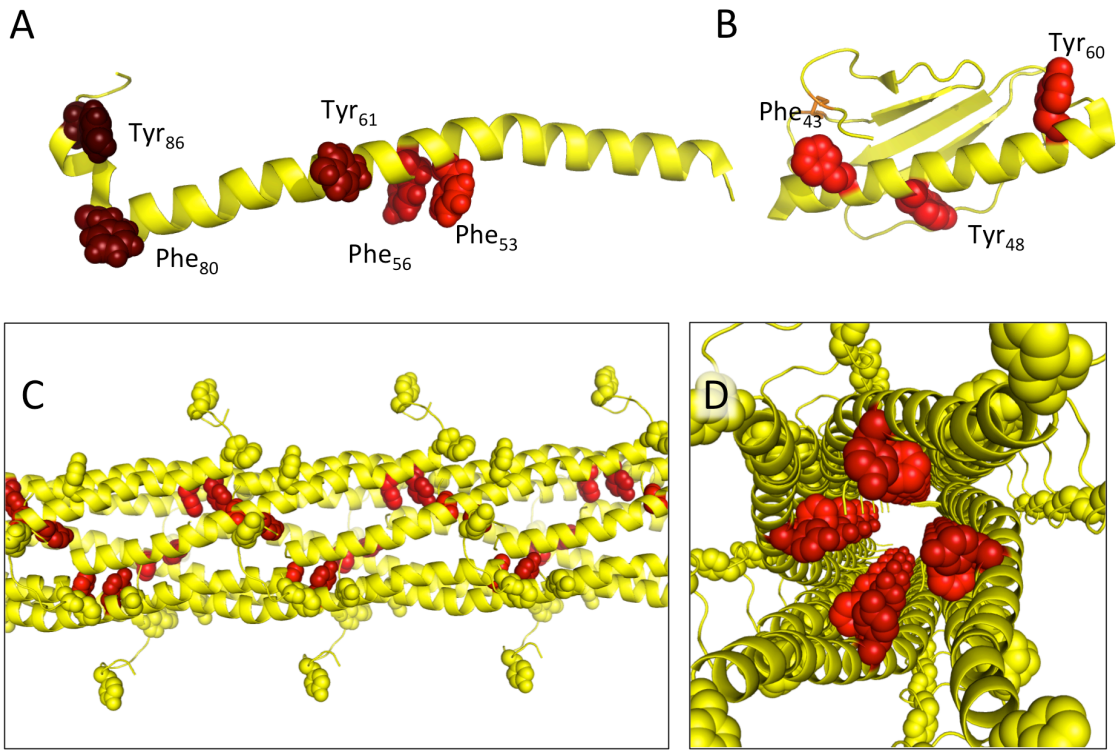


Figure 8:

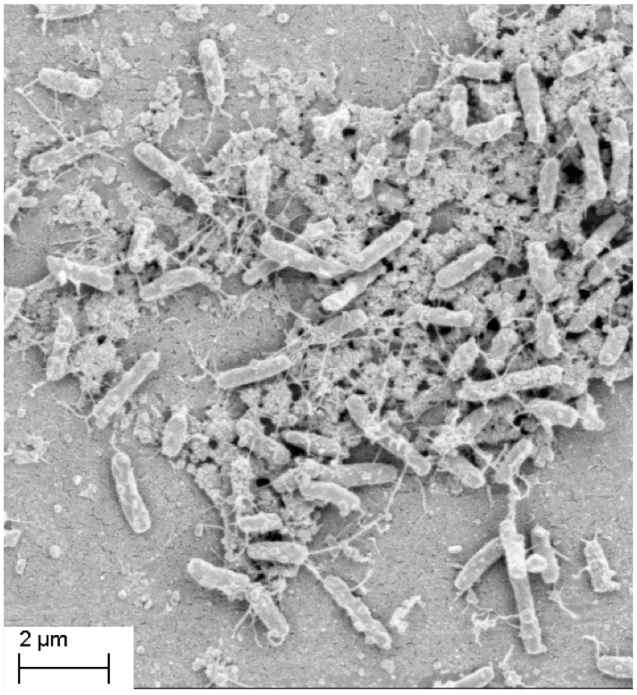


Figure 9

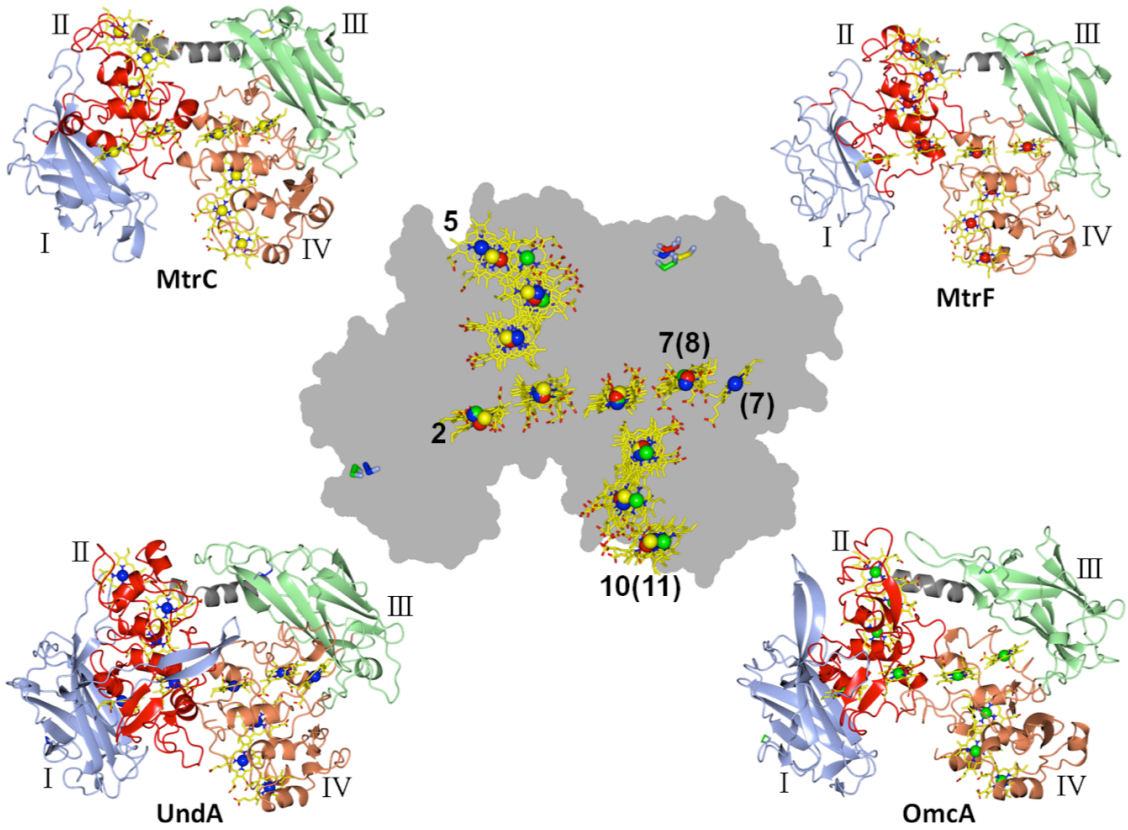

II
III
I
IV
MtrC
II
III
I
IV
MtrF
5
7(8)
(7)
2
10(11)
II
III
I
IV
UndA
II
III
I
IV
OmcA

Figure 10

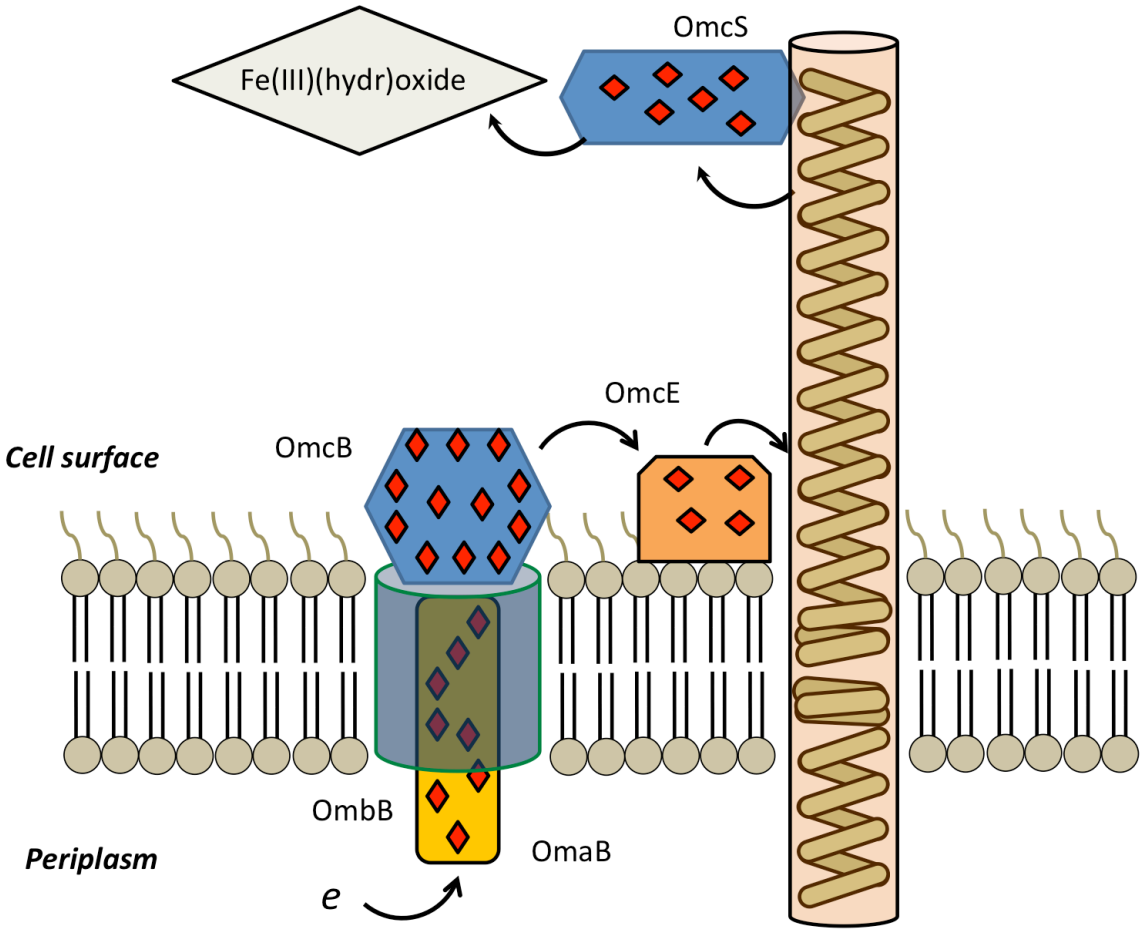
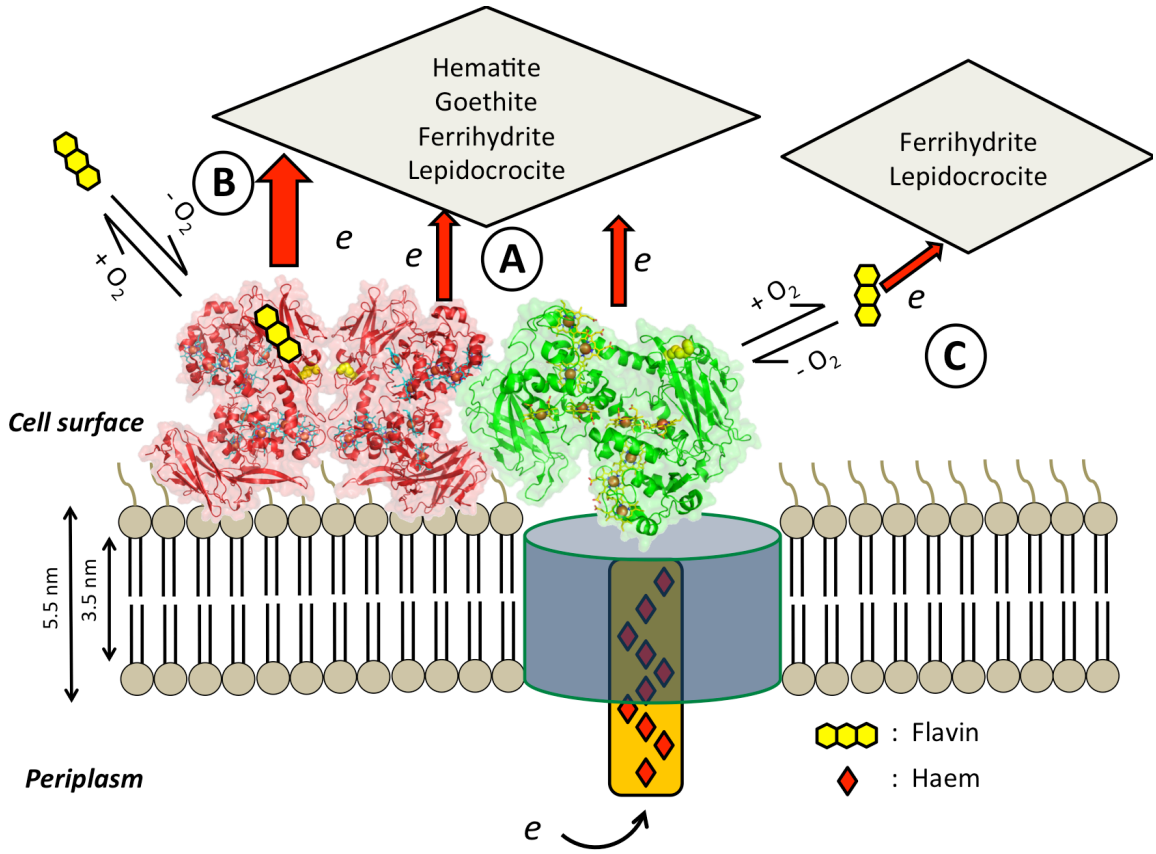

Fe(III)(hydr)oxide
OmcS
OmcE
OmcB
Cell surface
OmbB
OmaB
Periplasm
e

Figure 11


Hematite
Goethite
Ferrihydrite
Lepidocrocite
Ferrihydrite
Lepidocrocite
B
A
C
e
e
e
e
+O2
-O2
+O2
-O2
Cell surface
5.5 nm
3.5 nm
Periplasm
: Flavin
: Haem
e