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Extracellular respiration

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Summary

Although it has long been known that microbes can generate energy using diverse strategies, only recently has it become clear that a growing number involve electron transfer to or from extracellular substrates. The best-known example of what we will term ‘extracellular respiration’ is electron transfer between microbes and minerals, such as iron and manganese (hydr)oxides. This makes sense, given that these minerals are sparingly soluble. What is perhaps surprising, however, is that a number of substrates that might typically be classified as ‘soluble’ are also respired at the cell surface. There are several reasons why this might be the case: the substrate, in its ecological context, might be associated with a solid surface and thus effectively insoluble; the substrate, while soluble, might simply be too large to transport inside the cell; or the substrate, while benign in one redox state, might become toxic after it is metabolized. In this review, we discuss various examples of extracellular respiration, paying particular attention to what is known about the molecular mechanisms underlying these processes. As will become clear, much remains to be learned about the biochemistry, cell biology and regulation of extracellular respiration, making it a rich field of study for molecular microbiologists.

Introduction

Beginning in the 1880s when Martinus Beijerinck and Serge Winogradsky began studying nitrogen fixation and chemolithotrophy based on the oxidation of inorganic compounds such as sulphur and iron (Beijerinck, 1888; Winogradsky, 1889), an astounding number of metabolic pathways have been described for microorganisms. Today, it is well recognized that the capacity to derive energy for growth by catalysing diverse chemical reactions is one of the hallmarks of the microbial world. Many of the most important discoveries in biochemistry during the past century have stemmed from mechanistic studies of the enzymes and protein complexes involved in these metabolisms. For example, the first crystal structure of a membrane-bound protein came from the work of Johann Deisenhofer, Robert Huber, Hartmut Michel, who studied the photosynthetic reaction centre from *Rhodospseudomonas viridis* (Deisenhofer and Michel, 1989), and much of what we know about proton movements coupled to ATP synthesis stems from early work by Peter Mitchell and colleagues on chromatophores from *Rhodospirillum rubrum* (Scholes *et al.*, 1969).

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In recent years, more and more attention has been paid to microbial metabolisms that involve electron transfer between cells and extracellular substrates, such as minerals. In part, this subject has become popular thanks to the interest and support of the earth sciences community, in recognition of the key roles such metabolisms play in weathering reactions today (Druschel *et al.*, 2004) and might have played in ore deposit formation in the past (Johnson *et al.*, 2003). Environmental microbiologists have contributed greatly to the identification of both bacterial and archaeal strains that respire iron and manganese (hydro)oxides (Lovley and Phillips, 1988; Myers and Nealson, 1988), and microbial ecologists have documented that they are widespread in nature (Ghiorse, 1984; Lovley, 1991; Nealson and Saffarini, 1994; Ehrlich, 2002; Lovley *et al.*, 2004). Only relatively recently, however, have molecular microbiologists turned their attention to them, and our understanding of how organisms catalyse mineral transformations at the molecular level is still incomplete and, thus, a promising area for future biochemical, genetic and cell biological research.

In this MicroReview, we will describe several examples of what we term 'extracellular respiration'. By this we mean the use of a substrate (be it a solid or a solute) that interacts with the cellular electron transport chain through a protein that resides on either the cell surface, a cellular appendage that protrudes into the extracellular space, or a small molecule that traverses the space between the cell and the extracellular substrate (Fig. 1). The substrate is either used as an electron donor or electron acceptor to power oxidative phosphorylation. As will be detailed in the sections that follow, these substrates run the gamut from poorly soluble minerals to electrodes to solvents such as dimethyl sulphoxide (DMSO). Our objective is to highlight recent work that has advanced our understanding of extracellular respiration at the molecular genetic and/or biochemical level. Beyond the intriguing molecular and cell biological challenges extracellular respiration poses, this processes is also of applied interest, particularly in the context of microbial fuel cells (MFCs) (Rabaey and Verstraete, 2005; Gorby *et al.*, 2006).

Extracellular respiration of 'insoluble' substrates

Minerals

To date, the subject of extracellular respiration has been most thoroughly explored through studies concerning the reduction or oxidation of iron and manganese minerals. As several reviews have been published on this topic recently (Croal *et al.*, 2004; Tebo *et al.*, 2005; Weber *et al.*, 2006), here, we restrict our discussion to a few general comments.

Thanks to numerous biochemical and genetic studies over the past two decades in model organisms such as *Shewanella oneidensis*, *Geobacter sulfurreducens*, *Acidithiobacillus ferroxidans*, *Bacillus* sp. strain SG-1 and *Pseudomonas putida*, the general scheme for electron transfer in iron/manganese-oxidizing/reducing bacteria is now known. Diverse membrane-bound and -soluble (i.e. periplasmic) electron carriers, such as *c*-type cytochromes, quinones and multicopper oxidases, can play a role in these systems. Commonly, proteins involved in electron transfer to or from iron/manganese (hydr)oxides are localized to the outer membrane of Gram-negative bacteria or the exosporium of spores from Gram-positive bacteria (Myers and Myers, 1992; Francis *et al.*, 2002; Yarzabal *et al.*, 2002; Mehta *et al.*, 2005; 2006). It is generally assumed that this subcellular location facilitates direct interaction with the metal substrate. In addition to redox-active proteins, extracellular polymeric substances may also affect adherence to mineral surfaces and mediate metal reactivity, as seen in acidophilic iron-oxidizing bacteria (Sand and Gherke, 2006). Biophysical experiments with *S. oneidensis* have shown that specific attractive forces are induced between the cell and a mineral surface during conditions in which electron transfer from the cell to the mineral is expected (Lower *et al.*, 2001). Although these forces have not yet been shown to be due to any particular protein, recent biochemical experiments with OmcA, an outer membrane *c*-type cytochrome from *S.*

oneidensis (Myers and Myers, 1998), have indicated that it can bind hematite with high affinity and transfer electrons to it directly (Xiong *et al.*, 2006). Interestingly, however, OmcA did not bind goethite, the mineral used in the Lower *et al.* study. How different minerals are recognized by the cell is unknown, but may involve a number of subtle effects, as *S. oneidensis* appears capable of discriminating even between different single crystal faces (Neal *et al.*, 2005). In a separate study, scanning tunnelling microscopy and tunnelling spectroscopy were used to characterize the electron tunnelling properties of OmcA and MtrC (also known as OmcB, another outermembrane *c*-type cytochrome) immobilized as molecular monolayers on Au(III) surfaces (Wigginton *et al.*, 2007). In this case, modified versions of the native proteins containing a tetracysteine sequence were used, and it remains to be seen whether the conclusions from these *in vitro* biophysical experiments apply *in vivo* in the presence of iron oxides. For more details on this topic, see the review by Shi *et al.* (2007).

In addition to direct electron transfer to minerals, it has also been established that indirect electron transfer can occur via small molecules that act either to chelate metals and deliver them to an intracellular metal oxidoreductase, or by themselves serving as electron shuttles. A large number of research groups have made important contributions to this area; for a thorough review of this topic, we refer the reader to Kappler and Straub (2005). In the case of metal chelators, ligands such as citrate and nitro-triacetic acid (NTA) are able to deliver soluble iron to *c*-type cytochromes inside the cell, although it is clear that some metal chelates are also reduced extracellularly (Beliaev and Saffarini, 1998; Beliaev *et al.*, 2001; Myers and Myers, 2002; Lies *et al.*, 2005). In the latter case, a host of molecules – organic and inorganic, endogenously or exogenously produced – can shuttle electrons back and forth between cells and mineral surfaces. This might be particularly relevant in the context of biofilms, where the majority of cells are not in direct contact with the mineral surface (Hernandez and Newman, 2001). Although the oxidoreductases that act on these molecules and their respective subcellular locations have not been determined for the majority of the known electron shuttles, it was recently shown that at least some of these molecules require the same outer membrane proteins for their reduction that are required for mineral reduction, which blurs the distinction between the ‘direct’ and ‘indirect’ pathways (Lies *et al.*, 2005).

Irrespective of the potential substrate promiscuity of the oxidoreductases involved in these processes (see below), a novel mechanism of extracellular electron transfer has been described recently in studies with *G. sulfurreducens* (Reguera *et al.*, 2005) and *S. oneidensis* (Gorby *et al.*, 2006) that invokes electron transfer through microbial ‘nanowires’ (Fig. 2). In the case of *G. sulfurreducens*, these nanowires appear to comprise a new class of pili (the so-called ‘geopilins’) that are required for Fe(III) oxide reduction. Interestingly, these pili appear to localize to one side of the cell and are induced only under certain conditions. Twitching motility does not appear to occur under these conditions; thus, Reguera *et al.* (2005) concluded that the geopili do not play an indirect role in Fe(III) oxide reduction by mediating surface motility. Moreover, the geopili are not required for Fe(III) oxides to attach to the cell (i.e. mutants lacking geopili can still bind mineral particles on the cell surface), yet when they are expressed they have a high affinity for Fe(III) oxides. Based on measurements of their electrical conductivity using atomic force microscopy, the geopili were shown to be electrically conductive. From this, it was suggested that geopili serve as direct electron conduits between the cell and the mineral surface. How the amino acids that comprise the pili conduct electrons is not yet understood, nor is it known with which electron-donating proteins they interact inside the cell.

Geobacter sulfurreducens is not alone in its ability to produce nanowires. Gorby *et al.* (2006) showed that *S. oneidensis* produces electrically conductive nanowires in response to electron acceptor limitation. In comparing the electrical conductivity of the nanowires produced by the wild type with that of mutants defective in the production of the outer membrane *c*-type cytochromes OmcB (also known as MtrC) and OmcA by scanning tunnelling

microscopy, evidence was found that suggested the nanowires were poor electrical conductors in the absence of these cytochromes. Whether these cytochromes are physically incorporated into the nanowires themselves, or whether they facilitate electron transfer through the nanowires indirectly, is not known. An important additional finding of the Gorby *et al.* study was that nanowires can also be produced by other bacterial genera in addition to mineral reducers. They identified electrically conductive nanowires between cells of the photosynthetic cyanobacterium *Synechocystis* strain PCC 6803 when they were cultivated under conditions of CO₂ limitation. In addition, electrically conductive nanowires were also produced by the thermophilic fermentative bacterium *Pelotomaculum thermopropionicum* when grown in monocultures on fumarate or in co-cultures with *Methanothermobacter thermoautotrophicus* on propionate. It seems possible therefore that nanowire production might serve as a general mechanism for electron transfer both within and between species under certain conditions. Elucidation of what exactly these conditions are, how nanowire production serves the physiological needs of the cell under these conditions, and the mechanisms of their biosynthesis and cellular localization, are priorities for future work.

Electrodes

Electrodes are an important substrate for extracellular respiration in a more applied context. Since the 1970s, understanding how microbes catalyse the conversion of substrates directly into electricity has been relevant to optimizing the performance of MFCs (Rabaey and Verstraete, 2005). Electrodes can serve either as electron donors or as electron acceptors for microorganisms, depending on whether the electrode is functioning as a cathode or anode respectively. Many different types of microorganisms with different physiological capabilities (some fermentative, others respiratory) have been shown to catalyse electricity generation in fuel cells. These organisms have been enriched from diverse environments, ranging from organic wastewaters to aquatic sediments (Bond *et al.*, 2002; Tender *et al.*, 2002; Holmes *et al.*, 2004; Kim *et al.*, 2004; Rabaey *et al.*, 2004; Reimers *et al.*, 2006). The manner in which they generate electricity can vary from catalysing electron transfer directly via outer membrane proteins, to oxidizing/reducing soluble redox mediators that shuttle electrons to/from the electrode, in direct analogy to what occurs for minerals. The potential for artificial mediators to facilitate electron transfer in MFCs has been known for a long time (Roller *et al.*, 1984; Park *et al.*, 2000), but more recently, some MFC bacteria have been shown to produce mediators themselves (Bond and Lovley, 2005; Rabaey *et al.*, 2005). A recent study of electricity production by *G. sulfurreducens* growing on graphite electrodes demonstrated that the same 'geopilins' that play a role in Fe(III) oxide reduction by this organism also contribute to maximum power output when *G. sulfurreducens* is growing as a multilayered biofilm (Reguera *et al.*, 2006). Interestingly, if *G. sulfurreducens* grows on electrodes under conditions where cells do not stack on top of each other, the geopili do not appear to affect current production (Holmes *et al.*, 2006). Although there are obvious parallels between electron transfer to/from electrodes and to/from minerals, it appears that the particular proteins needed to catalyse electron transfer reactions in these two contexts differ (Holmes *et al.*, 2006). Moreover, in natural environments, with mixed communities colonizing deployed electrodes, a variety of mechanisms might operate simultaneously, with both organic and inorganic substrates (e.g. sulphur species in the marine environment) potentially participating in electron transfer reactions at both the anode and the cathode (Reimers *et al.*, 2006).

Extracellular respiration of 'soluble' substrates

As described above, extracellular respiration pathways exist for utilization of insoluble substrates as electron acceptors for anaerobic respiration. Mounting evidence suggests that soluble substrates can also be utilized extracellularly. In this section we will discuss this less intuitive class of extracellular respiration.

Dimethyl sulphoxide

Laboratory perceptions of what is 'soluble' might not reflect the ecological perspective of microorganisms. To illustrate this point, we will discuss DMSO, which can be found in significant concentrations (μM to nM) in aquatic environments (Lee and de Mora, 1999). There are several natural sources for DMSO: photochemical (Brimblecombe and Shooter, 1986) and biological (Hanlon *et al.*, 1994) oxidation of dimethyl sulphide (DMS) and production, either directly or indirectly, from algae (Andreae, 1980; Lee and de Mora, 1999). Although DMSO is considered to be a robust solvent (David, 1972), because it is a highly polar molecule it readily adsorbs to active surfaces such as glass (Simo *et al.*, 1998). In aquatic environments, these surfaces might be the silica frustules of diatoms or the gel-like matrices of marine snow. Such active surfaces might provide a reservoir of 'insoluble' DMSO in the environment that an extracellular respiratory mechanism would allow a bacterium to exploit. What evidence is there that this is the case?

Many organisms are known to utilize DMSO as a terminal electron acceptor for anaerobic growth. In the systems best studied for DMSO respiration, *Escherichia coli* and *Rhodobacter* spp., the terminal reductase is located in the periplasm (McEwan *et al.*, 1985; Stanley *et al.*, 2002). However, our work recently indicated that the DMSO reductase of *S. oneidensis* is localized to the outside of the cell (Gralnick *et al.*, 2006). In support of this, strains defective in type II secretion had a decreased capacity to respire DMSO. Type II secretion was first suggested to be required for the extracellular respiration of iron and manganese by *Shewanella putrefaciens* (DiChristina *et al.*, 2002). Type II secretion is the process of protein translocation across the outer membrane of an organism, and has been extensively studied in systems such as pullulanase from *Klebsiella oxytoca* (d'Enfert *et al.*, 1987) and cholera toxin from *Vibrio cholerae* (Davis *et al.*, 2000). The DMSO reduction defect of type II secretion mutants suggested that respiration required an extra-cellular or surface-attached component. Immunogold labelling of one of the DMSO reductase subunits indicated that it resides on the outer leaflet of the outer membrane under anaerobic conditions (Gralnick *et al.*, 2006). Like other surface-attached proteins such as pullulanase and the outer membrane decahaem *c*-type cytochromes OmcA and OmcB/MtrC from *S. oneidensis* (Myers and Myers, 2003), the catalytic subunit of the *S. oneidensis* DMSO reductase is predicted to be a lipoprotein. Although these different lines of evidence are consistent with the model that DMSO is reduced extracellularly, more work is necessary to conclusively demonstrate this.

Humic substances and other organic compounds

DMSO provides an example of a compound we might typically think of as soluble that might be insoluble in some environments. But for compounds that truly are soluble, what would motivate a cell to localize its enzymatic machinery to the cell surface in order to access them? One reason might simply be that the compounds are too large to be transported into the cell. To illustrate this, let us consider the case of humic substances.

Humic substances (hereafter, 'humics') are high-molecular-weight mixtures of lignin, proteins, carbohydrates and aliphatic polymers that primarily derive from the degradation of microbial and plant matter (McKnight and Aiken, 1998). Humics are among the most widely distributed class of organic compounds on Earth (Stevenson, 1994), accounting for at least half of the organic carbon found in soil (Lal, 2004). These substances can act as electron acceptors for microbial respiration coupled to the oxidation of short-chain fatty acids (Lovley *et al.*, 1996) or pollutants such as toluene (Cervantes *et al.*, 2001) and carbon tetrachloride (Cervantes *et al.*, 2004). In addition, they can also serve as electron donors. Electron spin resonance studies suggested that quinone moieties within humics are the redox-active centres (Scott *et al.*, 1998). A correlation was found between the semiquinone content of a variety of bacterially reduced humics and the amount of reduced iron produced when they were placed in contact

with iron oxides. Furthermore, the small quinoid compound anthraquinone-2,6-disulphonate (AQDS) has been used successfully as a proxy for the reactive moieties of humic substances (Lovley *et al.*, 1996; Coates *et al.*, 1998).

Considerable debate exists regarding the structure of humic substances due to their extraordinarily heterogeneous nature (see reviews by Swift, 1999; Piccolo, 2001; Sutton and Sposito, 2005). A recent view of humic substances describes a micell-like structure, comprised of low-molecular-mass components where hydrophobic constituents are centrally located and hydrophilic substances comprise the surface (Sutton and Sposito, 2005). Though soluble in nature, the size and colloidal nature of these compounds would make it difficult or impossible for bacteria to uptake humics actively, motivating their utilization as electron sources/sinks extracellularly. Although humic substances themselves have yet to be tested, experiments with AQDS are consistent with the hypothesis that humic substances are indeed substrates for extracellular respiration in *Shewanella*.

Mutant strains of *S. oneidensis* lacking various steps in the pathway of metal reduction are defective in reducing AQDS (Shyu *et al.*, 2002; Lies *et al.*, 2005). The degree of the AQDS reduction defect differed from mutant to mutant, with strains lacking menaquinone biosynthetic genes or the *mtrB* gene showing the strongest defects (Lies *et al.*, 2005). MtrB is an outer membrane protein that is required for the proper localization of both OmcA and OmcB (Myers and Myers, 2002). In addition, an OmcB mutant was seriously impaired in AQDS reduction, although not as severely as the MtrB or menaquinone mutants (Lies *et al.*, 2005). These results suggest that most, if not all, of AQDS reduction is catalysed by the same proteins that catalyse metal reduction, which implies that the MtrB/OmcB complex is promiscuous in its choice of substrates (see below). But promiscuity is not necessarily a hallmark of extracellular respiration, as the DMSO pathway in *Shewanella* appears to be specific.

In addition to humics, we predict that melanins might be an important candidate for extracellular respiration. Melanins are produced both intra- and extracellularly by a wide variety of microorganisms, including several species of pathogenic bacteria, fungi and worms (Nosanchuk and Casadevall, 2003) as well as by metal-reducing bacteria (Turick *et al.*, 2002). The production of melanin by *S. oneidensis* is stimulated by high concentrations of tyrosine (Turick *et al.*, 2002), which is not surprising given that tyrosine is a precursor of melanin. In many respects, melanins are structurally similar to humic substances. Although there is a strong connection between melanization and virulence, very little is understood about the cell biology of this pigment system and even less about whether these pigments are involved in extracellular respiration. We hope that the similarity between melanin and humics will prompt increased attention to this important and enigmatic class of polymers.

Soluble metal ions

Lastly, a final consideration in predicting whether a soluble compound might be a candidate for extracellular respiration is whether the respired product is also soluble. If not, a cell might be wise to localize its respiratory machinery to the cell surface to avoid intracellular precipitation of the product. To illustrate this, we consider the case of certain redox-active metals.

Redox state often has a profound influence on the solubility of metal ions. Many metals used by bacteria for anaerobic respiration exist naturally in a soluble state. Examples of metal and radionuclide species that are soluble in the oxidized state and can be substrates for anaerobic respiration are Uranium, Vanadium, Arsenic, Chromium, Technetium and Selenium (Lovley, 1991; Turick *et al.*, 1996; Lloyd *et al.*, 2000; Carpentier *et al.*, 2003; Stolz *et al.*, 2006). Even though these substrates are soluble and could be respired inside or outside of the cell, some of these metals (Se) and radionuclides (U, Tc) precipitate during microbial reduction due to the

low solubility of their reduced forms. *Acidithiobacillus ferrooxidans*, for example, uses Fe(II) as an electron donor in its metabolism, and precipitates Fe(III) oxides extracellularly. A high-molecular-weight *c*-type cytochrome has been localized to the outer membrane, and is presumed to facilitate Fe(II) oxidation on the cell surface (Yarzabal *et al.*, 2002). Studies of metal reduction in *Shewanella* species support the notion that extracellular respiration/reduction occurs in these cases. For example, mutant strains lacking the extracellular metal reductase complex (OmcA, OmcB/MtrC) have a decreased capacity to reduce U(VI) compared with wild type (Marshall *et al.*, 2006). Purified OmcB/MtrC is able to reduce U(VI) *in vitro* when artificially reduced with dithionite. Consistent with this, another study found an *mtrB* mutant to exhibit a partial defect in U(VI) reduction (Bencheikh-Latmani *et al.*, 2005). Similarly, vanadate {V(V)} reduction proceeds through the extracellular metal reductase pathway, requiring all of the same components as iron and manganese reduction (Myers *et al.*, 2004; Carpentier *et al.*, 2005). While evidence for extracellular V(V) reduction is clear, the evidence that selenite {Se(IV)} reduction in *S. oneidensis* takes place extracellularly is based only on the localization of Se(0) nanoparticles to the outside of the cell (Klonowska *et al.*, 2005). Interestingly, a mutant strain lacking CymA still retains the capacity to reduce SeO₃. If true, this would indicate a process independent of the main metal reductase pathway, as CymA is essential for iron and manganese respiration (Myers and Myers, 1997). However, it is unclear if selenite reduction in *Shewanella* can be coupled to anaerobic growth, as has been seen in other organisms (Stolz *et al.*, 2006). The poor solubility of the reduced forms of V, Se and U is consistent with the strategy of extracellular respiration in preventing precipitation of metals and metalloids in cellular compartments.

Promiscuity of the *mtr/omc* pathway in *S. oneidensis*

Through the efforts of many research groups, it has become clear that the Mtr/Omc electron transfer pathway in *S. oneidensis* is involved in the reduction of a variety of substrates. While mutant strains were initially observed to be defective in the reduction of iron and manganese oxide minerals, other substrates are now recognized including: AQDS, chelated Fe(III), V(V) and U(IV) (Shyu *et al.*, 2002; Croal *et al.*, 2004; Myers *et al.*, 2004; Bencheikh-Latmani *et al.*, 2005; Carpentier *et al.*, 2005; Lies *et al.*, 2005; Marshall *et al.*, 2006; Weber *et al.*, 2006). Neither the extent nor the physiological benefit of this promiscuity is clear, but remains an important area for future research. Moreover, elucidating the mechanism of this promiscuity presents an exciting challenge for biochemists. It is possible that particular proteins (e.g. MtrB) play a generic role in targeting different oxidoreductases (e.g. OmcB, OmcA) to the outer membrane, which in turn are specific for particular substrates; alternatively, it is possible that a single oxidoreductase catalyses the reduction of diverse substrates, either because it itself can recognize and directly reduce them, or because it uses a single mechanism to indirectly reduce them (e.g. an endogenous electron shuttle).

Common pathways used in extracellular respiration

In general, studies with *Shewanella* and *Geobacter* species show that electron transfer proceeds through a sequence of soluble and membrane-bound electron carriers from the cytosol to the outside of the cell. Early steps in this process contribute to the generation of a proton motive force (Δp) around the inner membrane, but later steps are uncoupled from proton pumping, and merely serve to deliver electrons to the terminal oxidant. In many of these systems, menaquinone is a key inner membrane-bound electron carrier that presumably contributes to the establishment of a Δp (Lovley *et al.*, 1993; Myers and Myers, 1993; Newman and Kolter, 2000; Saffarini *et al.*, 2002). Many proteins involved in extracellular respiration are *c*-type cytochromes. CymA, a tetrahaem *c*-type cytochrome, interacts with the menaquinone pool directly in *Shewanella* species. This protein is required for almost all forms of anaerobic respiration in *Shewanella*, with the exception of thiosulphate and TMAO reduction (Myers and

Myers, 1997; Saffarini *et al.*, 2002). In each extracellular respiration system described so far, an electron carrier protein is involved in moving electrons across the periplasm. In the metal reduction pathway in *Shewanella* this protein is MtrA, and in DMSO reduction the protein is DmsE. MtrA is a decahaem *c*-type cytochrome (Pitts *et al.*, 2003), while DmsE is also predicted to be one, based on the presence of conserved haem binding motifs (Gralnick *et al.*, 2006). The small periplasmic *c*-type cytochrome PpcA from *G. sulfurreducens* might play a similar electron-carrying role (Lloyd *et al.*, 2003). The terminal reductases used by the extracellular respiratory pathways in *Shewanella* differ, however: in the case of metal reduction, the terminal reductases appear to be decahaem *c*-type cytochromes (Shi *et al.*, 2006), whereas a putative molybdopterin-containing protein is used in DMSO reduction (Gralnick *et al.*, 2006). Outer membrane *c*-type cytochromes have also been identified in *G. sulfurreducens* that are required for metal reduction (Leang *et al.*, 2003; Kim *et al.*, 2005; 2006; Mehta *et al.*, 2005).

Although less is understood regarding the mechanisms involved in localizing the components of the extracellular respiratory systems to the outside of the cell, it appears that type II secretion plays an important role in both *Shewanella* and *Geobacter*. Interestingly, the defect seen in *Shewanella* type II secretion mutants is not as profound as the defect seen in *G. sulfurreducens*. *Shewanella* mutants retain some DMSO and Fe(III) reduction activity (DiChristina *et al.*, 2002; Gralnick *et al.*, 2006), whereas a *G. sulfurreducens* type II secretion mutant is completely defective (Mehta *et al.*, 2006). This could indicate the existence of an additional system of protein translocation independent of type II secretion in *Shewanella*. Another difference is that the defect in *G. sulfurreducens* is specific to insoluble Fe(III), whereas type II secretion in *Shewanella* is required for maximal reduction of both soluble and insoluble metals. Moreover, *G. sulfurreducens* has two gene clusters encoding type II secretion systems, a so-called 'classical' cluster which is similar to the Gsp system of many Gram-negative bacteria (including *Shewanella*) and one similar to the non-classical Xcm secretion system of *P. putida* (de Vrind *et al.*, 2003) that is required for insoluble Fe(III) reduction (Mehta *et al.*, 2006). What role, if any, the classical type II secretion system has in the ability of *Geobacter* to reduce metals is unknown.

Perspectives for future research

Unknown substrates

In this review, we have discussed various substances that are substrates for extracellular respiration, but in all likelihood this set is far from complete. In addition to melanins, it seems reasonable that hydrophobic substrates such as oil, or mineral deposits such as elemental sulphur, or compounds whose processing gives rise to a toxic product, might be metabolized on the cell surface. Even when we restrict our focus to the genomes of *Shewanella* species in search of homologues to proteins known to be required for extracellular respiration, it is clear that there are several gene clusters of unknown function that likely encode novel extracellular respiratory processes. For example, *S. oneidensis* contains two such clusters. One cluster is similar to the metal reductase gene cluster, but is not required for metal reduction (Myers and Myers, 2002; Marshall *et al.*, 2006), while the other cluster is similar to the DMSO reductase cluster, but likely does not play a role in DMSO reduction (Gralnick *et al.*, 2006). As more environments and metabolically diverse bacteria are explored, we expect that additional substrates will be discovered.

Elucidating the mechanisms of extracellular respiration

Many interesting mechanistic questions about extracellular respiration remain to be explored. It is striking that many of the outer membrane proteins in Gram-negative bacteria that are known to be involved in extracellular respiration are lipoproteins. How are they transported to the external face of the outer membrane? After being exported across the inner membrane, do

they insert into the outer membrane on the periplasmic side, and then flip from one face to the other via a Lol-dependent mechanism (Tokuda and Matsuyama, 2004)? Or does the type II secretory system directly target them to the external face of the outer membrane? While genetic evidence suggests the latter pathway is involved (DiChristina *et al.*, 2002; Gralnick *et al.*, 2006), the former pathway cannot yet be excluded because detailed biochemical studies of the transport machinery have not yet been performed. Even in the well-studied pullulanase system, we do not yet know where the lipids of pullulanase are located. In addition, although bioinformatic predictions suggest that many of these outer membrane proteins are decahaem cytochromes, this awaits confirmation from structural biological studies. Once this is known, it will open up the possibility of identifying the sequence of electron transfer events both within these proteins, and between them and their substrate(s) or other proteins in the electron transport chain. Similarly, much remains to be learned regarding the assembly and electron-transfer mechanisms of nanowires. With increased attention from molecular and cellular biologists, as well as bioinorganic chemists, we anticipate exciting advances will be made in the coming decade. Lastly, recent studies of transcription factors, such as ArcA from *S. oneidensis* (Gralnick *et al.*, 2005) and RpoS from *G. sulfurreducens* (Nunez *et al.*, 2006), have shown that their regulons are notably different from those that have been established for *E. coli*, and thus our appreciation of the diversity of biological networks will likely be enhanced by studying how extracellular substrates are sensed. Given the wide distribution of organisms with the capacity for extracellular respiration in the environment, insights gained from detailed mechanistic studies could one day facilitate their use for bioremediation or energy generation in many different contexts.

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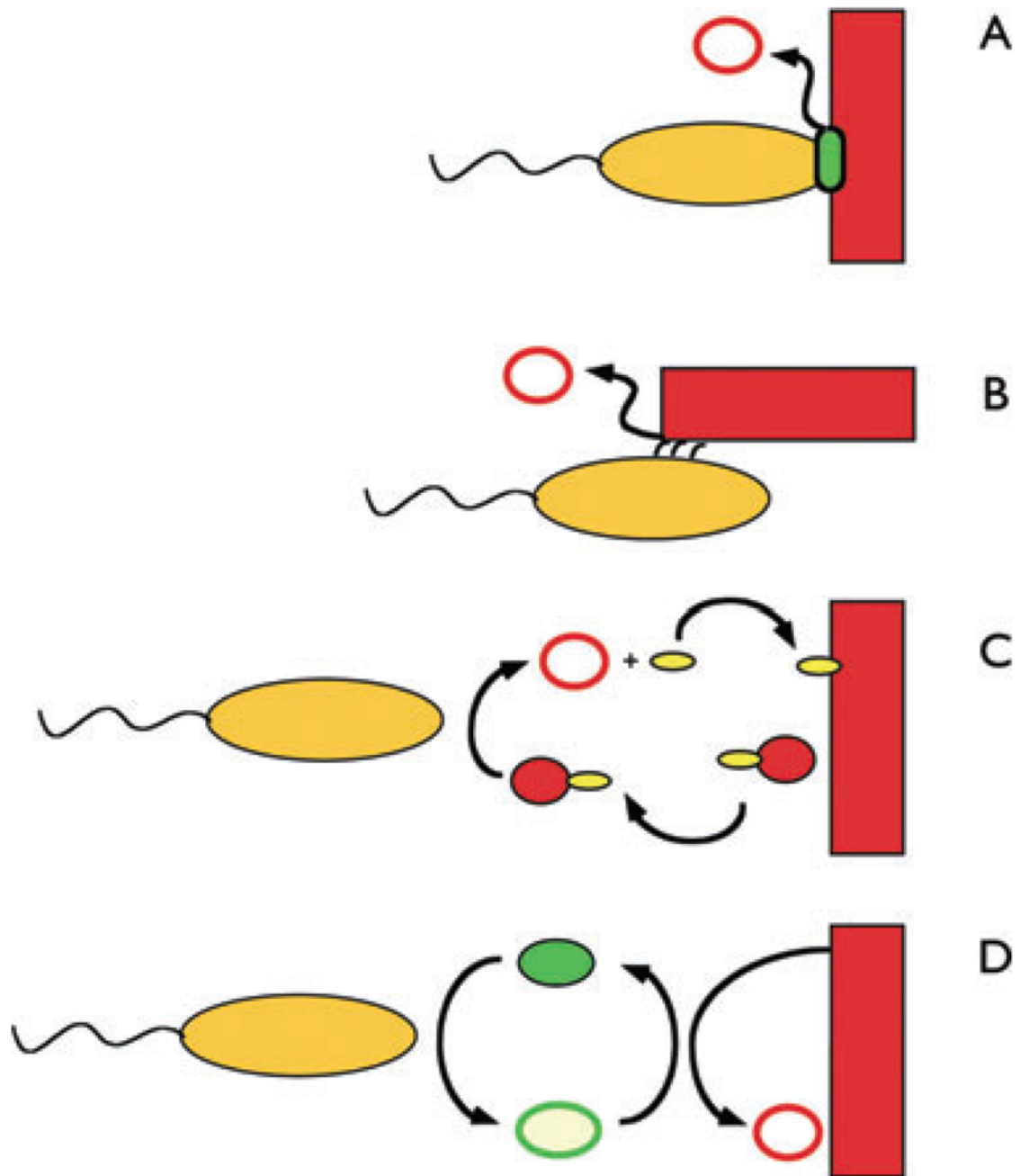
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**Fig. 1.**

Four potential strategies for extracellular respiration. In this cartoon, the substrate is depicted as a solid red rectangle [e.g. an iron (hydr)oxide] and its reduced product (e.g. ferrous iron) as an open red circle. As discussed in the text, this is but one example of extracellular respiration. Note that these strategies can apply to other substrates, and the flow of electrons can also be reversed. A. A protein that resides on the cell surface (green oval) directly interacts with the extracellular substrate to transfer electrons from the cell to the substrate. B. A cellular appendage (such as an electrically conductive pilus) forms a bridge between the cell and the substrate, catalysing electron transfer.

C and D. A small molecule (such as a chelator, C) or an electron shuttle (D) interacts with the substrate outside the cell. In the case of a chelator (small yellow oval), the substrate is brought to the cell for reduction (either on the outside or on the inside); in the case of an electron shuttle (green oval; closed oval is in the oxidized state, open oval is in the reduced state), the substrate remains at a distance from the cell, with the shuttle catalysing the transfer of electrons between the cell and the substrate. The shuttle, in principle, could be reduced on the outside or inside of the cell.

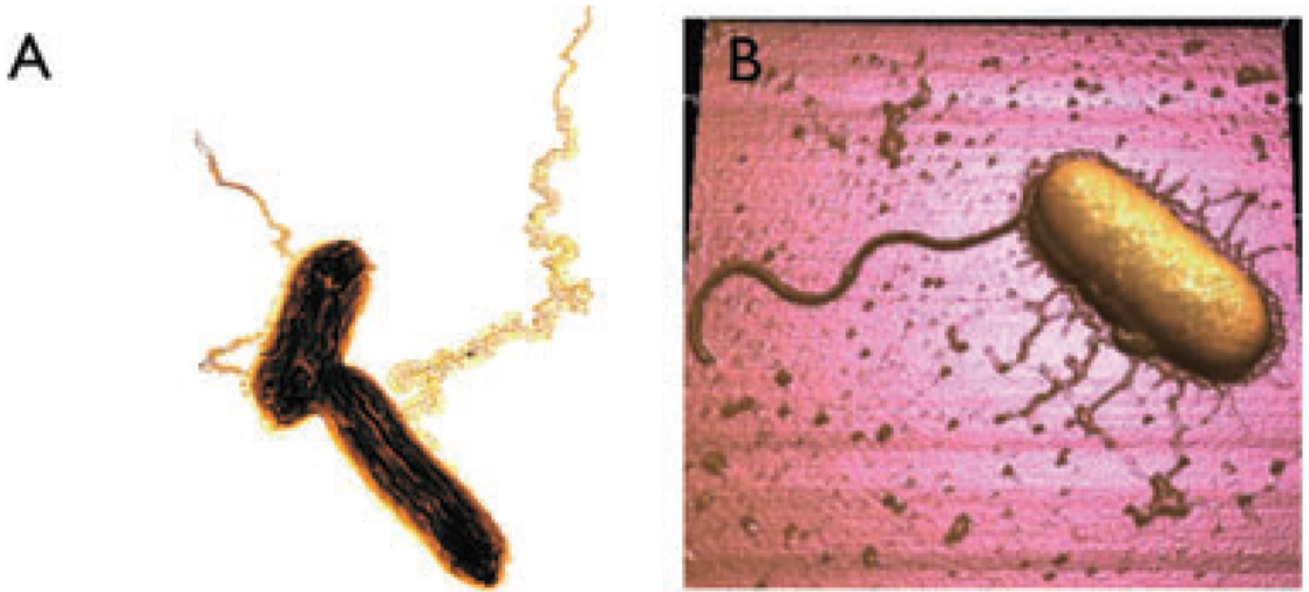


Fig. 2.

'Nanowires' produced by two different Fe(III)-reducing bacteria.

A. Transmission electron micrograph of platinum-shadowed 'geopili' produced by *Geobacter sulfurreducens* (courtesy of Gemma Reguera, Michigan State University). The length of the longer cell is approximately 2 μm . The image has been colorized to enhance contrast.

B. Atomic force micrograph of *Shewanella oneidensis* (courtesy of Pamela Gross, University of Southern California). The large appendage is the flagellum; the shorter pili are electrically conductive nanowires. The length of the cell is approximately 3 μm .