

## Review

# Extracellular electron transfer

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**Abstract.** Results from several laboratories indicate that extracellular electron transfer may be a general mechanism whereby microorganisms generate energy for cell growth and/or maintenance. Specifically, bacteria can use redox-active organic small molecules, generated outside or inside the cells, to shuttle electrons between reduced and oxidized compounds. Electron shuttling has now been reported for several different bacterial species, and

exchanges of shuttling compounds may even syntrophically link diverse organisms in nature. Biofilm systems in both geological and clinical settings are likely to be important environments for metabolisms that employ extracellular electron transfer. Both structural and functional analyses suggest that electron shuttles and some virulence factors may be related to one another.

**Key words.** Iron respiration; biofilm; extracellular electron transfer; electron shuttle.

## Introduction

Electron transfer reactions are fundamental to metabolism. Whether an organism is autotrophic or heterotrophic, free living or an obligate parasite, every cell must solve the problem of energy generation in order to survive. At the cellular level, most of our knowledge of electron transfer springs from mechanistic studies of photosynthesis and respiration, both in prokaryotic and eukaryotic systems. While today we know in exquisite detail the structure and function of various membrane-bound proteins that are involved in these electron transfer processes (such as the photosynthetic reaction center, cytochrome oxidase, and NADH dehydrogenase), we know much less about small molecules that participate in extracellular electron transfer [1, 2].

Given that the most productive routes for energy generation typically involve the establishment of a proton-mo-

tive force across a membrane [3], not surprisingly most studies of electron transport metabolisms have focused on molecules that are embedded in or associated with membranes. Recent work on electron transport pathways in bacteria that respire by using insoluble minerals as terminal electron acceptors, however, has drawn attention to the possible importance of small molecules in metabolisms that involve extracellular electron transfer [4, 5]. In this review, we summarize the evidence that supports this type of electron transport pathway, discuss a limited set of small molecules that might participate in this process, and propose a model for how these molecules function in a microbial biofilm where extracellular electron transfer may be essential for survival. Although much of our discussion centers on metal reduction, this is only one specific context in which extracellular electron transfer may be important. Indeed, bacteria in diverse settings (both natural and clinical) are likely to benefit from this type of metabolism.

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## How do microbes breathe minerals?

At a fundamental molecular level this question remains unanswered, despite the fact that more than a decade has passed since environmental microbiologists first came to appreciate the importance of mineral respiration in a variety of biogeochemical cycles and its potential use for the bioremediation of organic and inorganic contaminants [6]. The reasons for this delay are twofold. First, the problem of mineral respiration is difficult to solve, involving as it does a complex set of parameters ranging from environmental chemistry to species-specific electron transfer strategies. Second, only recently have the genomes of two organisms that engage in mineral respiration (*Shewanella oneidensis* strain MR1, formerly *Shewanella putrefaciens* strain MR1, and *Geobacter sulfurreducens*) become available, making practical a genetic approach to studying the mechanisms of their electron transfer pathways. Here, we will restrict our discussion to iron minerals (specifically, iron oxides and/or hydroxides), although microbially mediated redox transformations of other metals (such as manganese oxides) are also significant [7–9].

Unlike other metabolisms where the final electron acceptor is a freely diffusible gas or a readily soluble species that the cell can easily access, metal reducers face the task of transferring electrons to a solid form. Although Fe(III) is almost insoluble at circumneutral pH (table 1), it is one of the most important electron acceptors for microorganisms in anaerobic environments [6]. One way to respire iron

minerals is to use an outer-membrane reductase to receive and transfer the reducing power generated by internal metabolism to the insoluble oxidant outside. For example, *G. sulfurreducens* uses a membrane-bound NADH dehydrogenase coupled to a *c*-type cytochrome to transport electrons to iron [10]. In *Shewanella* species, a series of electron carriers such as cytochromes and menaquinones are required to transport the electrons to iron down a long respiratory chain [11]. Although the specifics of energy generation have not yet been determined, a proton-motive force is presumably established by proton pumping by electron carriers such as menaquinone and/or the NADH dehydrogenase [12, 13]. Whether in *Geobacter* or *Shewanella* species, all of these pathways assume direct contact between microbes and minerals.

A diversity of microorganisms are now known to engage in electron transfer to iron minerals [14] and, moreover, the environments in which these organisms perform this metabolism differ from one another. For example, *Geobacter* species are strict anaerobes, and have been found to dominate iron-reducing populations in a variety of subsurface environments [15]. While these organisms attach to iron minerals, due to nutrient limitation in these contexts, they do not form thick biofilms. Members of the *Shewanella* genus, on the other hand, are facultative anaerobes, and thrive at redox interfaces [8]. These organisms have been associated with corroding steel pipelines [16] and are capable of forming thick biofilms [17, 18]. Not surprisingly, therefore, the molecular mechanisms of mineral respiration appear to be different for

Table 1. Redox potentials and iron solubility.

Redox potentials	$E_0$ (mV)	Reference
Alcaligin acid ox/red	–588	39
NAD <sup>+</sup> /NADH	–320	26
S <sup>0</sup> /HS <sup>–</sup>	–270	26
2,6-AQDS/2,6-AHDS	–184	33
Humics	–200 to +300	35
$\frac{1}{2} \alpha \cdot \text{Fe}_2\text{O}_3/\text{Fe}^{2+}$	–159	<sup>a</sup>
$\alpha \cdot \text{FeOOH}/\text{Fe}^{2+}$	–88	<sup>a</sup>
Menaquinone ox/red	–75	26
ACNQ ox/red	–71	42
Pyocyanin ox/red	–34	49
am · Fe(OH) <sub>3</sub> /Fe <sup>2+</sup>	+59	<sup>a</sup>
Ubiquinone ox/red	+113	26
O <sub>2</sub> /H <sub>2</sub> O	+820	26

Iron solubility and chelation		log K	Reference
Alcaligin	$\text{Fe}_2\text{L}_3 = 2\text{Fe}^{3+} + 3\text{L}^{2-}$	–64.8	39
Goethite	$\alpha \cdot \text{FeOOH}_{(\text{s})} + \text{H}_2\text{O} = \text{Fe}^{3+} + 3\text{OH}^-$	–41.5	37
Ferric hydroxide	$\text{am} \cdot \text{Fe}(\text{OH})_{3(\text{s})} = \text{Fe}^{3+} + 3\text{OH}^-$	–38.8	37
Hematite	$\frac{1}{2} \alpha \cdot \text{Fe}_2\text{O}_{3(\text{s})} + \frac{3}{2} \text{H}_2\text{O} = \text{Fe}^{3+} + 3\text{OH}^-$	–42.7	37
Siderite	$\text{FeCO}_{3(\text{s})} = \text{Fe}^{2+} + \text{CO}_3^{2-}$	–10.7	37
Pyrrhotite	$\text{FeS}_{(\text{s})} = \text{Fe}^{2+} + \text{S}^{2-}$	–18.1	37
Vivianite	$\text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}_{(\text{s})} = 3\text{Fe}^{2+} + 2\text{PO}_4^{3-} + 8\text{H}_2\text{O}$	–36.0	37

<sup>a</sup> Redox potentials were calculated from Morel and Hering [37] assuming  $[\text{Fe}^{2+}] = 1 \mu\text{M}$ .

these groups. While direct contact between microbe and mineral appears to be required for *Geobacter* species to reduce iron in the absence of exogenous electron shuttles (see discussion below) [19], direct contact is not required for *Shewanella* species [5], contrary to previous reports [17]. Although a number of studies point to the role of outer-membrane proteins and cytochromes in catalyzing electron transfer to minerals in both *Geobacter* and *Shewanella* species [10, 20–25], at least in the case of *Shewanella*, more than one pathway for mineral respiration may exist: a pathway that requires direct contact and one that does not. It is the latter, indirect pathway, that is the focus of this review.

### Indirect pathways for mineral respiration: extracellular electron transfer

An alternative to direct-contact pathways involves a mobile small molecule capable of undergoing redox cycling (i.e., an electron shuttle). It serves as the terminal electron acceptor and, once reduced, can itself transfer electrons to iron oxides whereupon it becomes reoxidized. Such a shuttle provides a mechanism for an indirect reduction process. In principle, a single shuttle molecule could cycle thousands of times and, thus, have a significant effect on the turnover of the terminal oxidant (e.g., iron) in a given environment. Some examples of organic molecules that could play this role are humic substances, quinones, phenazines, and thiol-containing molecules like cysteine, but anything that is reversibly redox active and has the right redox potential (e.g., poised between the  $E_0'$  of the reductant and the oxidant; see table 1) could serve the purpose [26]. Organic quinone-containing compounds have been shown to stimulate iron mineral reduction most effectively [27]. Although inorganic molecules like sulfide and reduced uranium can also function as shuttles to iron minerals in the laboratory [27], they will not be discussed in this review.

We primarily focus on the identity (table 2) and importance of organic shuttles in metal reduction, but we also provide some examples of electron shuttling in medically related contexts. Overall, we classify the shuttles according to whether they are already present in the environment (exogenous) or whether the cell produces them (endogenous).

### Exogenous shuttles

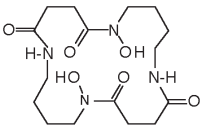
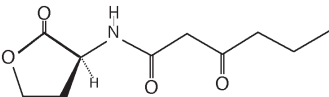
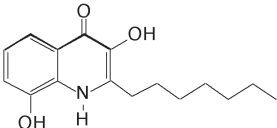
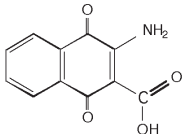
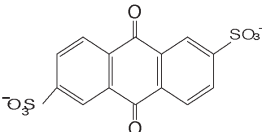
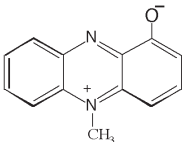
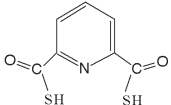
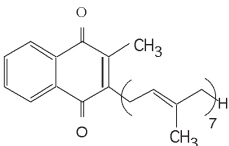
The first evidence for organic shuttle-mediated iron mineral reduction came from studies that attempted to stimulate the oxidation of organic contaminants in an anaerobic aquifer by adding various iron chelators [28]. Chelators, in general, enhance the rate of microbial iron

reduction by making Fe(III) more accessible to the cell. In this experiment, the addition of humic substances (hereafter, 'humics') had the greatest stimulatory effect. Humics are a ubiquitous, significant, and chemically heterogeneous fraction of organic compounds present in aquatic and terrestrial environments; they primarily derive from the degradation of microbial and plant matter [29]. Because humics are only weak chelators of iron, chelation alone could not account for the degree of stimulation observed in these field studies. Further investigations proved that the observed stimulation of Fe(III) reduction involved a redox cycle in which bacterially reduced humics abiotically reduced the iron minerals, themselves being reoxidized in the process [30]. Following these observations, electron spin resonance studies suggested that quinone moieties within humics are the redox active centers. 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 [31]. Furthermore, small quinoid compounds like anthraquinone-2,6-disulfonate (AQDS) were confirmed to function as electron shuttles [table 2] [32]. In experiments with soil microcosms, the addition of extracellular quinones enhanced the rate of iron reduction [27]. All iron-reducing Bacteria and Archaea tested in the laboratory to date, as well as some fermenting bacteria [33], have been found to reduce humics [32, 34, 35]. Accordingly, the indirect reduction of iron via humic shuttles is likely to be a significant extracellular electron transfer process in the environment. Although the energy to be gained from using a redox-active shuttle like AQDS as the terminal electron acceptor is less than that to be gained from using Fe[III] minerals [table 1], the fact that AQDS is highly soluble and bioavailable likely provides the cell with comparatively more energy gained per unit time.

### Endogenous shuttles

A similar extracellular electron transfer mechanism could employ a microbially produced shuttle. In fact, *S. oneidensis* strain MR-1 was found to excrete a quinone-like molecule that was hypothesized to serve this purpose [4]. This extracellular factor is recovered from cell-free supernatants of an *S. oneidensis* wild-type culture and complements a mutant strain (H2) that is impaired in AQDS and humics reduction. H2 has a disruption in the *menC* gene, which encodes o-succinylbenzoic acid synthase, an enzyme involved in the biosynthesis of menaquinone. Menaquinone is a lipophilic naphthoquinone that can freely diffuse in membranes and shuttles electrons between proteins of low redox potential in the respiratory chain (table 2). The fact that the *menC* mutant is unable to make the extracellular factor shows that the factor and

Table 2. Structures and proposed functions of siderophores, signals, and shuttles.

Name	Structure	Function	Reference
Putrebactin		siderophore	39
3-OC <sub>6</sub> -HSL		signal	53
Quinolone		signal	58
ACNQ		redox shuttle	42
2,6-AQDS		redox shuttle	32
Pyocyanin		antibiotic iron acquisition redox shuttle	45,47,48
P-2,6-bT		siderophore (?) redox shuttle	36
Menaquinone		redox shuttle	26

menaquinone share at least part of a common biosynthetic pathway and suggests that the factor may be a small quinone. Data in support of this are that the extracellular factor has high UV absorption, turns orange upon reduction, and has a molecular mass around 150–300 Da. Ongoing experiments show that the mutant strain can be complemented by supernatant extracts from other bacterial species [M.E. Hernandez and D.K. Newman, unpublished data], suggesting that there might also be interspecies cycling of shuttles. Although direct proof that this molecule is a shuttle is not yet available, other lines of evidence demonstrate that the indirect mechanism of metal reduction is important. Recent studies with another

*Shewanella* species (*S. alga* strain BrY) showed that it can reduce amorphous iron hydroxide [Fe(OH)<sub>3</sub>] trapped inside alginate beads. Because the bacteria cannot directly contact the majority of the Fe(OH)<sub>3</sub> in the beads (the pore size, 12 kDa, is too small to permit their passage), the appearance of large quantities of Fe(II) in the presence of live *S. alga* cells strongly suggests that they produce shuttling compounds that penetrate the beads [5]. An earlier experiment that addressed whether direct contact was required for metal reduction by *S. putrefaciens* strain 200 used dialysis tubing to separate the microbes from goethite (a crystalline iron oxy-hydroxide,  $\alpha$ -FeOOH) [17]. Although no Fe(III) reduction was ob-

served under these conditions, when AQDS was used as a positive control to repeat these experiments, no Fe(III) reduction was observed either [19]. Accordingly, the use of dialysis membranes as an assay for shuttles is suspect, and the iron-bead assay is preferable to determine whether direct contact is required.

Another example of a microbially produced electron shuttle relevant to environmental microbiology is a recently characterized extracellular compound that catalyzes the transformation of carbon tetrachloride ( $\text{CCl}_4$ ) to carbon dioxide [36]. It was isolated from cell-free supernatants of iron-limited cultures of a *Pseudomonas stutzeri* strain. This small molecule, a pyridine-2,6-thiocarboxylate (P-2,6-T), is able to shuttle electrons to  $\text{CCl}_4$ , thereby reductively degrading it. Whether this shuttling mechanism is used to generate energy for growth is not known. P-2,6-T can form complexes with a variety of metals, and was first isolated under conditions of iron limitation. This suggests that it might function as a siderophore, although its metal-binding capacity possibly relates to its redox activity (e.g., the metal becomes the redox-active center).

### Siderophores as shuttles?

Iron chelators make iron more accessible to the cell by providing a soluble complex with a metal center as the terminal electron acceptor. Bacteria can make their own chelators, called siderophores. These are small molecules produced and sent out by bacteria to scavenge Fe(III) in low-iron environments (e.g., aerobic habitats where free iron concentrations are typically  $10^{-18}$  M [37]). Siderophores are well known to play an important role in iron assimilation in both natural and clinical settings [38]. Whether bacteria produce siderophores under metal-reducing conditions has just recently begun to be investigated. If siderophores are indeed produced by bacteria to acquire iron for the purpose of respiration, we would predict that such a mechanism would not require direct contact between microbes and minerals. Yet two independent sets of experiments argue that this is not the case. First, no evidence could be found for the release of chelators by *G. metallireducens* when the organism was grown on amorphous ferric hydroxide, and soluble Fe(III) and Fe(II) were measured in solution [19]. Second, experiments with *S. putrefaciens* that used dialysis membrane to separate the bacteria from goethite did not yield any Fe(II) in solution [17]. That iron complexed to the synthetic chelator nitrilotriacetic acid was able to pass through the membrane and was subsequently reduced to Fe(II) by the bacteria could be taken as evidence that microbially produced chelators (e.g., siderophores) would have been detected had they existed. However, as in the case of AQDS (discussed above), siderophores could possibly

not make it through this membrane and, thus, would not have been detected by this assay. In principle, a mechanism where iron minerals could be solubilized by siderophores and the resulting metal-complex used as a terminal electron acceptor with subsequent liberation of the siderophore without the metal upon reduction could be a means of transferring electrons to insoluble iron minerals during respiration. It is noteworthy that hydroxamate siderophores, like the putrebactin of *S. oneidensis* (table 2) [39], are known to be reduced and recycled in this way during iron assimilation. The low redox potential of these siderophores (see alcaligin in table 1) [40], however, makes them unlikely candidates as terminal electron acceptors for energy generation. Furthermore, we would expect that the higher the affinity of a siderophore for Fe(III), the poorer a shuttle it would make. Overall, there is little evidence that siderophores play a role in iron mineral respiration. If Fe(III)-containing shuttles participate in electron transfer to iron minerals at all, they seem more likely to bind iron permanently, using it only as a redox-active center (as is the case for cytochromes) [41].

### Shuttling between species

Small molecules excreted by one organism can be taken up and used for intracellular electron transfer by other organisms. An example of this is the quinoid compound, 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ), which has been shown to stimulate growth strongly by a shuttling mechanism in a beneficial population of bacteria in the human gastrointestinal tract [42]. ACNQ is produced and excreted by *Propionibacterium freundenreichii*, and plays a role as an electron transfer mediator from NAD(P)H to oxygen and hydrogen peroxide in the cytoplasm of the obligate anaerobe *Bifidobacterium longum*. *B. longum* does not have a respiratory chain and normally regenerates NAD(P)<sup>+</sup> by a lactate dehydrogenase-catalyzed reaction, consuming pyruvate and excreting lactate. This process consumes an important intracellular metabolite (e.g., pyruvate), and becomes thermodynamically unfavorable if lactate accumulates in the environment. ACNQ offers an alternative pathway catalyzed by cytosolic diaphorase and peroxidase. By coupling the regeneration of NAD(P)<sup>+</sup> to the reduction of  $\text{O}_2$  and  $\text{H}_2\text{O}_2$ , ACNQ stimulates growth and is thought to protect *B. longum* from oxygen toxicity. The hydrophilicity of ACNQ increases upon reduction, promoting its accumulation inside the cell. Therefore, only very low concentrations of extracellular ACNQ (i.e., 0.5 nM) are required for it to stimulate the growth of *B. longum*. Whether the release of ACNQ by *P. freundenreichii* directly benefits its metabolism is not yet known. If so, ACNQ release and uptake could be considered a form of syntrophy between



the two species, as is the case for interspecies  $H_2$  transfer [43]. Also possible is that ACNQ is simply a secondary metabolite excreted by the cell as a waste product, and the ability of *B. longum* to benefit from ACNQ is fortuitous.

### Shuttling by phenazines

Some evidence suggests that pyocyanin (table 2), a phenazine blue pigment produced by *Pseudomonas aeruginosa* and several other strains [44] can function as an extracellular electron shuttle [45]. Cultures of pyocyanin-producing bacteria change from blue to colorless upon reaching anaerobic conditions and shaking the cultures restores the original blue color. In this process, the blue pigment undergoes a reversible two-electron reduction to a colorless product called leukopyocyanin, which, in turn, is readily oxidized by oxygen. Early ideas about its function held that pyocyanin was an accessory respiratory pigment due to its ability to increase the oxygen consumption of *P. aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae* cells, as well as erythrocytes. In particular, significant differences in the rates of oxygen consumption were observed in buffer-resuspended cells in the presence or absence of pyocyanin when no carbon source was added [45]. These experiments led to the hypothesis that pyocyanin was involved in the oxidation of intracellularly stored molecules like lipids or polysaccharides and transferred electron to oxygen. Further evidence for a role for pyocyanin in electron shuttling came from observations that respiratory quotients (e.g.,  $CO_2$  produced per  $O_2$  consumed) increased in the presence of pyocyanin and that cyanide and carbon monoxide inhibited pyocyanin-mediated respiration [45]. Moreover, the production of pyocyanin was shown to be regulated by the energy status of the cell, presumably by intracellular ATP levels [46]. Together, these results suggest that pyocyanin may play a role in energy metabolism under non-optimal growth conditions, but whether bacteria actually gain energy from pyocyanin reduction has not yet been demonstrated. Once the antimicrobial activity of pyocyanin was recognized, attention shifted away from its possible role as a mediator of respiration. Most of the current literature on pyocyanin emphasizes its role as a virulence factor, whose mechanism of action is the generation of toxic byproducts during its oxidation (e.g., the superoxide radical ( $O_2^-$ ) and  $H_2O_2$ ) [47]. Its extracellular release, therefore, may simply be a form of chemical warfare against competitors and have nothing to do with energy generation per se.

Other evidence for electron-shuttling activity by phenazines derives from studies of iron acquisition by *P. aeruginosa* and electron transport in methanogens. Pyocyanin was shown to mediate iron acquisition from transferrin by reducing transferrin-bound Fe(III) with con-

comitant liberation of Fe(II) [48]. Because this process was inhibited by cyanide, it appeared to utilize a respiratory pathway. Furthermore, the fact that electron transfer from pyocyanin to Fe(III) was also inhibited by oxygen suggests that iron and oxygen compete as final oxidants of pyocyanin. Pyocyanin behaves as a reversible dye of the quinoid type with a redox potential similar to menaquinone (table 1) [49]. Interestingly, a phenazine derivative with a polyisoprenoid side chain has recently been shown to function as a key membrane bound electron shuttle during methanogenesis [50], and water-soluble 2-hydroxy-phenazine plays the same role in in vitro reactions with other components of the electron transport chain of *Methanozarcina mazei* Go1 and *M. thermofila* [51]. It is tempting to speculate, therefore, that pyocyanin-mediated respiration is important in high-density, poorly mixed cultures with low oxygen concentrations, a condition that characterizes many bacterial infections. Consistent with this hypothesis, quorum-sensing signals were recently found to induce phenazine biosynthesis genes in *P. aeruginosa* [52]. Nevertheless, whether pyocyanin plays such a respiratory role in high-density cultures remains to be shown.

### Perspectives on extracellular electron transfer in biofilms

Extracellular electron transfer is most likely to take place in an environment where a terminal oxidant is not easily accessible (e.g., poorly soluble and/or diffusion limited) and where excreted shuttles can be efficiently recycled. A biofilm community on an iron mineral surface is a good example of such an environment as it would provide a high cell density that would allow the build-up of the shuttle, a relatively closed system where the shuttles could be efficiently recycled, and an abundant external oxidant (the iron mineral) to regenerate it. In an attempt to integrate some of the data described earlier in this review, we have drawn a cartoon of a single-species *S. oneidensis* biofilm developing on the surface of an iron mineral (fig. 1). Our focus is on the differences we might expect to see in the activity of any given cell at different layers in the biofilm. We assume that organic carbon inputs to the system are sufficient to sustain a dense community of cells, and that the biofilm develops in an oxygenated environment. In the real world, more than one species would comprise the biofilm and the environmental chemistry would also be significantly more complex. This figure is merely a thought experiment. The predictions generated by it, of course, must be tested in the laboratory.

Our main prediction is that the mechanisms employed for energy generation at different layers in a biofilm are likely to be different from one another, even for a single-species biofilm. As examples, we compare the metabo-

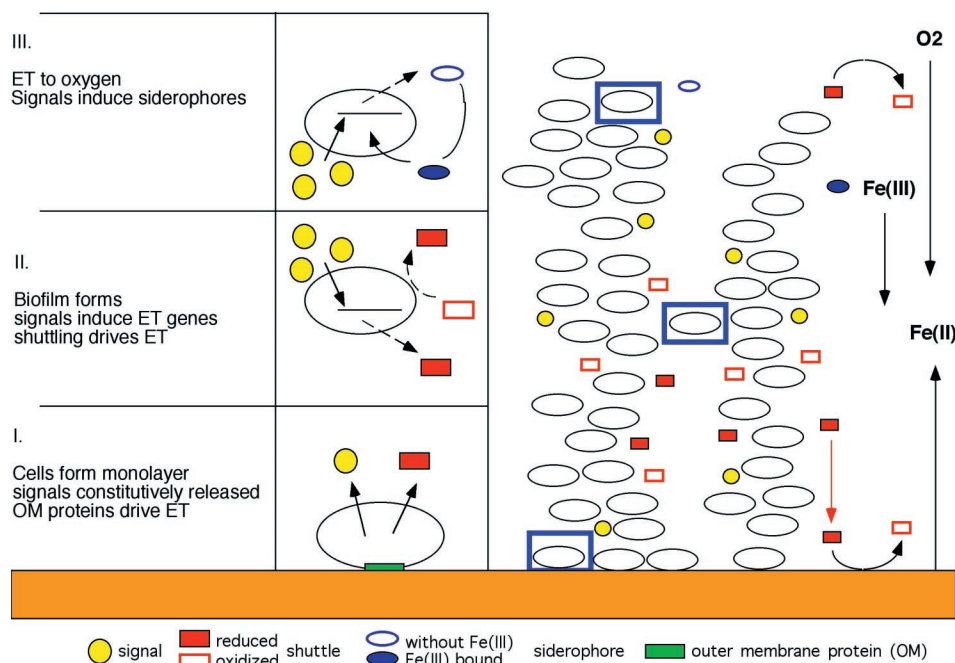


Figure 1. A model of electron transfer (ET) pathways within a biofilm of *S. oneidensis* growing on an iron mineral surface. Representative cells from three different layers of the biofilm (surface-associated, middle, and top) are shown. The hypothetical role of signals, shuttles, and siderophores are drawn for each layer. Details are provided in the text.

lism of three cells at discrete places in the biofilm: one at the mineral surface (I), one in the middle of the biofilm (II), and one on the top of the biofilm (III). At the surface, we would expect electron transfer, at least initially (e.g., in the colonization stage, where cells form a monolayer prior to the establishment of a three-dimensional structure), to be dominated by outer-membrane proteins that directly contact the mineral. We hypothesize that extracellular electron transfer is not a dominant mechanism of energy generation at this point, as sufficient concentrations of endogenous shuttle molecules would not be present (i.e., any given shuttle could easily be lost by diffusion into the ambient environment). As is the case for quorum-sensing signals [53], if we assume an initial basal level of shuttle production, as the biofilm develops, we would expect local concentrations to rise (the exopolysaccharide matrix that characterizes established biofilms is known to slow down the diffusion of small molecules between the biofilm and the external milieu [54]). Such conditions would then be favorable for cells that cannot directly contact the mineral surface to use the shuttles for energy generation. An important question then becomes, what is the final oxidant for the shuttles used by these cells? Assuming that anaerobic conditions prevail at this level of the biofilm (due to low  $O_2$  solubility, low  $O_2$  diffusivity, and rapid  $O_2$  utilization by aerobically respiring cells), if soluble  $Fe(III)$  is not available and no other diffusible oxidants – such as humic substances – are present, we must assume the shuttles are re-

generated by transferring electrons directly to the mineral surface. At the top of the biofilm where aerobic conditions prevail, however, the shuttles could be regenerated by  $O_2$ . In this uppermost layer, electron transfer should be dominated by aerobic respiration, provided the cells are actively growing. As is the case for *P. aeruginosa* and pyocyanin biosynthesis, however, under the appropriate physiological conditions (e.g., depressed ATP synthesis) shuttles could possibly be used to mediate  $O_2$  respiration. How these different energetic programs are regulated remains an open question. Quorum sensing, a widespread phenomenon of genetic control based on cell density, is known to be important in biofilm communities [55, 56]. Two examples of this are the induction of luciferase by acyl homoserine lactones (AHLs) in communities of *Vibrio fischerii* that inhabit the light organ of the squid *Euprymna scolopes* [57], and the formation of a three-dimensional biofilm structure by *P. aeruginosa*, also in response to AHLs [55]. Could signals such as AHLs, quinolones, or a LuxS-type signal (whose structure is currently unknown) [58] induce the expression of electron shuttles? If electron shuttles are indeed only useful in biofilm communities, we might predict their expression would be cell density dependent. Interestingly, in a recent survey of ~7000 mutants of *P. aeruginosa*, a set of 47 genes showed significant induction (more than fivefold) in the presence of AHLs [52]. Among them were genes involved in the biosynthesis of pyocyanin, genes that appear to encode synthesis of and resistance to an antibi-

otic-like compound, and genes that are clearly related to electron transport (e.g., up-regulation of cytochrome p450 and a cytochrome C precursor). To the extent that pyocyanin can be considered an electron shuttle, and the fact that the structure of electron shuttles like AQDS is remarkably similar to known antibiotics (see table 2) [59], this could be taken as support for the idea that quorum sensing may help regulate the production of shuttles. In support of this hypothesis, AQDS excretion by *S. oneidensis* was recently found to depend on a TolC-mediated type I secretion pathway [J.B. Hsu and D.K. Newman, unpublished results]; TolC is well known to be involved in the transport of antibiotics [60].

The conceptual model presented in figure 1 illustrates just how complex energy generation is likely to be for even a single-species biofilm. Many questions are raised by this diagram. How does exogenous environmental chemistry [e.g., concentrations of O<sub>2</sub>, Fe(III), and Fe(II)] in conjunction with endogenous chemistry (e.g., cellular production of small molecules) affect the expression of relevant electron transfer networks? How much more complicated do these networks become when more than one species is involved? And how robust are these networks in the face of environmental perturbations? Answering these questions is a non-trivial task, and the tools with which to approach them are still in development. For example, a new generation of microelectrodes now allow us to measure pH, O<sub>2</sub>, and various soluble iron and sulfur species at submicron levels of resolution [61]; microarray technologies have recently been used to measure gene expression in biofilms of *P. aeruginosa* [M. Whiteley and E.P. Greenberg, unpublished results]; and state-of-the-art confocal laser microscopes now enable individual layers of cells to be imaged one at a time and reconstructed in three dimensions [62]. Future studies must bring these and other technologies together to begin to answer the questions posed above. For example, we might predict that at deep layers in the biofilm (close to the mineral surface), local concentrations of Fe(II) in solution are much higher than at the surface, where O<sub>2</sub> would presumably reoxidize Fe(II) back to Fe(III). Siderophore expression by the cells would, thus, be expected to be different in these two regimes (see fig. 1). Using microelectrodes, measuring iron speciation accurately within the biofilm should be possible. These data, in combination with fluorescent tagging of specific genes related to iron acquisition and direct imaging of their expression at different layers within biofilms by laser microscopy, could enable us to correlate changes in environmental chemistry with specific metabolic processes.

### Where next?

The study of extracellular electron transfer is still in its infancy and represents a promising area for future re-

search. One problem that appears likely to become increasingly important is determining the relationship between electron transfer molecules and antimicrobial compounds. Much effort is currently being directed to identifying novel antibiotics from the soil [63] and we have clearly only just begun to scratch the surface of the diversity of small molecules that are released by microorganisms. It is tempting to speculate that some small metabolites that are attractive to the pharmaceutical industry for their antibiotic activities will turn out to be related (or indeed, equivalent) to small molecules that are used by microorganisms for energy metabolisms in the environment. Pyocyanin is one example of a compound that has been studied in some detail with respect to its versatility of function (e.g., as an electron transfer agent, as an agent of iron acquisition, and as a virulence factor), and recent evidence suggests that exogenous electron shuttles, such as AQDS, may share with antibiotics similar pathways of uptake and efflux. Indeed, the underlying unity of their chemical structure and mechanism of action (e.g., redox chemistry) suggests a reason why they may have overlapping functions. The challenge we now face is to identify these compounds from organisms in nature and determine their ecological importance, be it on the surface of an iron mineral or inside the tissues of an eukaryotic host.

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