Chapter 2
Extracellular electron transfer

* Adapted from Hernandez & Newman, 2001. *Cell Mol. Life Sci*., Vol 58, p.1562-1571.

2.1 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 (15, 58).

Given that the most productive routes for energy generation typically involve the establishment of a proton-motive force across a membrane (38) it is not surprising that most studies of electron transfer metabolisms have focused on molecules that are embedded in or associated with membranes. Recent work on electron transfer 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 (48, 50). In this review, we summarize the evidence that supports this type of electron transfer 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, it seems likely that bacteria in diverse settings (both natural and clinical) may benefit from this type of metabolism.

2.2 How do microbes breathe minerals?

At a fundamental molecular level this question remains unanswered, despite the fact that it has been more than a decade since environmental microbiologists 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 (28). 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, it is only recently that the genomes of two organisms that engage in mineral respiration (*Shewanella oneidensis* strain MR-1, formerly *Shewanella putrefaciens* strain MR-1, and *Geobacter sulfurreducens*) became available, and a genetic approach to studying the mechanisms of their electron transfer pathways became more practical. 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 (32, 45, 60).

Unlike in 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 I), it is one of the most important electron acceptors for microorganisms in anaerobic environments (28). 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, *Geobacter sulfurreducens* uses a membrane-bound NADH dehydrogenase coupled to a *c*-type cytochrome to transfer electrons to iron (36). In *Shewanella* species, a series of electron carriers such as cytochromes and menaquinones are required to transfer the electrons to iron down a long respiratory chain (52). Although the specifics of energy generation have not yet been determined, a proton-motive force presumably is established by proton pumping by electron carriers such as menaquinone and/or the NADH dehydrogenase (43) (42). Whether in *Geobacter* or *Shewanella* species, all of these pathways assume direct contact between microbes and minerals.

It is now known that a diversity of microorganisms engage in electron transfer to iron minerals (27) and moreover, that 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 (57). 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 (45). These organisms have been associated with corroding steel pipelines (51)

and are capable of forming thick biofilms (2, 44). Not surprisingly, therefore, the molecular mechanisms of mineral respiration appear to be different for these groups. While it appears that direct contact between microbe and mineral is required for *Geobacter* species to reduce iron in the absence of exogenous electron shuttles (see discussion below) (47) direct contact is not required for *Shewanella* species (48) contrary to previous reports (2). 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 (4, 5, 11, 14, 26, 36, 41), it now appears that, 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.

2.3 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 I) could serve the purpose (35). Organic quinone-containing compounds have been shown to stimulate iron mineral reduction most effectively (49). Although inorganic molecules like sulfide and reduced uranium also can function as shuttles to iron minerals in the laboratory (49), they will not be discussed in this review.

We primarily focus on the identity (Table II) 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).

2.3.1 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 (33). 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 (37). 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 (29). 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 that was produced when they were placed in contact with iron oxides (54). Furthermore, small quinoid compounds like anthraquinone-2,6-disulfonate (AQDS) were confirmed to function as electron shuttles [Table II] (30). In experiments with soil microcosms, the addition of extracellular quinones enhanced the rate of iron reduction (49). All iron-reducing Bacteria and Archaea tested in the laboratory to-date, as well as some fermenting bacteria (6), have been found to reduce humics (30, 31, 59). Accordingly, it seems likely that the indirect reduction of iron via humic shuttles may 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 I], the fact that AQDS is highly soluble and bioavailable likely provides the cell with comparatively more energy gained per unit time.

Table 2.1. Iron solubility and redox potentials of shuttling compounds.

Iron solubility and chelation		log K	Reference
Alcaligin	$Fe_2L_3 = 2\ Fe^{3+} + 3L^{2-}$	-64.8	39
Goethite	$\alpha\cdot FeOOH_{(S)} + H_2O = Fe^{3+} + 3OH^-$	-41.5	37
Ferric hydroxide	$am\cdot Fe(OH)_{3(S)} = Fe^{3+} + 3OH^-$	-38.8	37
Hematite	$1/2\ \alpha\cdot Fe_2O_{3(S)} + 3/2\ H_2O = Fe^{3+} + 3OH^-$	-42.7	37
Siderite	$FeCO_{3(S)} = Fe^{2+} + CO_3^{2-}$	-10.7	37
Pyrrhotite	$FeS_{(S)} = Fe^{2+} + S^{2-}$	-18.1	37
Vivianite	$Fe_3(PO_4)_2.8H_2O_{(s)} = 3\ Fe^{2+} + 2\ PO_4^{3-} + 8\ H_2O$	-36.0	37

Redox potentials	E_0' (mV)	Reference
Alcaligin acid ox/red	-588	39
$NAD^+/NADH$	-320	26
$S°/HS^-$	-270	26
2,6-AQDS/ 2,6-AHDS	-184	33
Humics	-200 to +300	35
1/2 $\alpha \cdot Fe_2O_3/Fe_2^+$	-159	a
$\alpha \cdot FeOOH/Fe^{2+}$	-88	a
Menaquinone ox/red	-75	26
ACNQ ox/red	-71	42
Pyocyanin ox/red	-34	49
$am \cdot Fe(OH)_3/Fe^{2+}$	+59	a
Ubiquinone ox/red	+113	26
O_2/H_2O	+820	26

[a] Redox potentials were calculated from ref. 39 assuming [Fe(II)]=1 μM

2.3.2 Endogenous shuttles

A similar extracellular electron transfer mechanism could employ a microbially-produced shuttle. In fact, *Shewanella oneidensis* strain MR-1 was found to excrete a quinone-like molecule that was hypothesized to serve this purpose (50). This extracellular factor is recovered from cell-free supernatants of a *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, that encodes o-succinylbenzoic acid synthase, an enzyme involved in the biosynthesis of menaquinone. Menaquinone is a lipophilic napthoquinone that can freely diffuse in membranes and shuttles electrons between proteins of low redox potential in the respiratory chain [Table II]. The fact that the *menC* mutant is unable to make the extracellular factor shows that the factor and menaquinone share at least part of a common biosynthetic pathway and suggest 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 daltons. 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], which suggests that there might also be interspecies cycling of shuttles. Although direct proof that this molecule is a shuttle is not yet available, there are other lines of evidence that 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 [$Fe(OH)_3$] trapped inside alginate beads. Because the bacteria cannot directly contact the majority of the $Fe(OH)_3$ 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 (48). 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, α-FeOOH) (2). Although no Fe(III) reduction was observed under these conditions, when AQDS was used as a positive control to repeat these experiments, no Fe(III) reduction was observed either (47). 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 (CCl_4) to carbon dioxide (25). 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 CCl_4, thereby reductively degrading it. Whether this shuttling mechanism is used to generate energy for growth is not known. Pyridine-2,6-thiocarboxylate 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 it is also possible that its metal-binding capacity relates to its redox activity.

2.3.3 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. They are small molecules that are 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 (39)). Siderophores are well known to play an important role in iron assimilation in both natural and clinical settings (46). Whether bacteria produces siderophores under metal-reducing conditions has just recently begun to be investigated. If indeed siderophores are 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 organisms were grown on amorphous ferric hydroxide and soluble Fe(III) and Fe(II) were measured in solution (47). Second, experiments with *S. putrefaciens*, that used dialysis membrane to separate the bacteria from goethite, did not yield any Fe(II) in solution (2). Because iron complexed to the synthetic chelator, nitrilotriacetic acid (NTA), was able to pass through the membrane and was subsequently

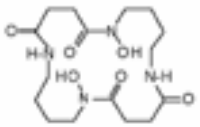
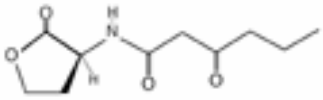
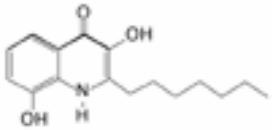
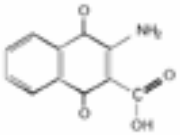
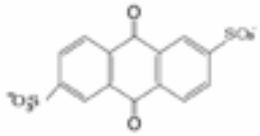
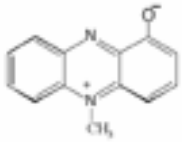
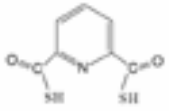
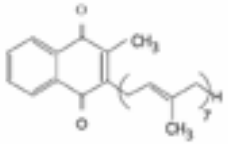
reduced to Fe(II) by the bacteria, this could be taken as evidence that microbially-produced chelators (e.g., siderophores) would have been detected had they existed. However, as in the case of AQDS (discussed above), it is also possible that siderophores could not make it through this membrane, and 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 *S. oneidensis'* putrebactin [Table II] (24), are known to be reduced and recycled in this way during iron assimilation. The low redox potential of these siderophores (see alcaligin in Table I) (18), however, makes them unlikely candidates as terminal electron acceptors for energy generation . Indeed, we would expect the higher the affinity a siderophore has 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, it seems more likely that they would bind iron permanently, using it only as a redox active center (as is the case for cytochromes) (55).

2.3.4 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-naphtoquinone (ACNQ), which has been shown to strongly stimulate growth by a shuttling mechanism in a beneficial population of bacteria

in the human gastrointestinal tract (63). 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)+ 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 $NAD(P)^+$ to the reduction of O_2 and H_2O_2, ACNQ stimulates growth and is thought to protect *B. longum* from oxygen toxicity. ACNQ's hydrophilicity 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*. It is not yet known whether the release of ACNQ by *P. freundenreichii* directly benefits its metabolism. 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 (7). It is also possible that it is simply a secondary metabolite excreted by the cell as a waste product, and *B. longum*'s ability to benefit from ACNQ is fortuitous.

Table 2.2. Structures and proposed functions of siderophores, signals and shuttles.

Name	Structure	Function	Reference
Putrebactin		siderophore	39
3-OC_6-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

2.3.5 Shuttling by phenazines

Some evidence suggests that pyocyanin [Table II], a phenazine blue pigment produced by *P. aeruginosa* and several other strains (19) can function as an extracellular electron shuttle (13). 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 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 (13). From these experiments it was hypothesized 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 (13). Moreover, the production of pyocyanin was shown to be regulated by the energy status of the cell, presumably by intracellular ATP levels (62). 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 virulence factor, whose mechanism of action is the generation of toxic byproducts during its oxidation (e.g., superoxide radical (O_2^-) and H_2O_2) (16). 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 concomitant liberation of Fe(II) (9). 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 suggest 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 I] (12). Interestingly, phenazine derivative with a polyisoprenoid side chain has recently been shown to function as a key membrane bound electron-shuttle during methanogenesis (1) 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 *Methanosarcina thermofila* (40). 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* (61). Yet whether pyocyanin plays such a respiratory role in high density cultures remains to be shown.

2.4 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 and its utilization by any cell; a relatively closed system where the shuttles could be efficiently recycled; and an abundant external oxidant (the iron mineral) to regenerate it. In the attempt to integrate some of the data described earlier in this review, we have drawn a cartoon of a single-species *Shewanella oneidensis* biofilm developing on the surface of an iron mineral [Figure 1]. Our focus is on the differences we might expect to see in any given cell's activity 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 also would 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 metabolism 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 (3), if we assume an initial basal level of production of shuttles, 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 mileau (8). 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 at this level of the biofilm anaerobic conditions prevail (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 regenerated 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, it is possible that under the appropriate physiological conditions (e.g., depressed ATP synthesis) shuttles could be used to mediate respiration.

How these different energetic programs are regulated remains an open question. It is now well known that quorum-sensing, a widespread phenomenon of genetic control based on cell density, is important in biofilm communities (10, 56). Two examples of this are the induction of luciferase by acyl homoserine lactones (AHSLs) in communities of *Vibrio fischerii* that inhabit the light organ of the squid, *Euprymna scolopes* (20) and the

formation of a three-dimensional biofilm structure by *P. aeruginosa*, also in response to AHSLs (10). Could it be that signals, such as AHSLs, quinolones, or a LuxS-type signal (whose structure is currently unknown) (17), induce the expression of electron shuttles? If indeed electron shuttles are only useful in biofilm communities, we might predict their expression would be cell-density dependent. Interestingly, in a recent survey of ~7,000 mutants of *P. aeruginosa*, a set of 47 genes showed significant induction (>5 fold) in the presence of AHSLs (61). Among them were genes that are involved in the biosynthesis of pyocyanin; genes that appear to encode synthesis and resistance to an antibiotic-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 II) (23), 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 (22).

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, including: how does exogenous environmental chemistry (e.g., concentrations of O_2, Fe(III), Fe(II), etc) 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 the process of development. For example, a new generation of microelectrodes now allow us to measure pH, O_2, and various soluble iron and sulfur species at sub-micron levels of resolution (34); 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 (21). In future studies, it will be important to 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 would be much higher than they would be at the surface, where presumably O_2 would 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, it should be possible to measure iron speciation within the biofilm accurately. This 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.

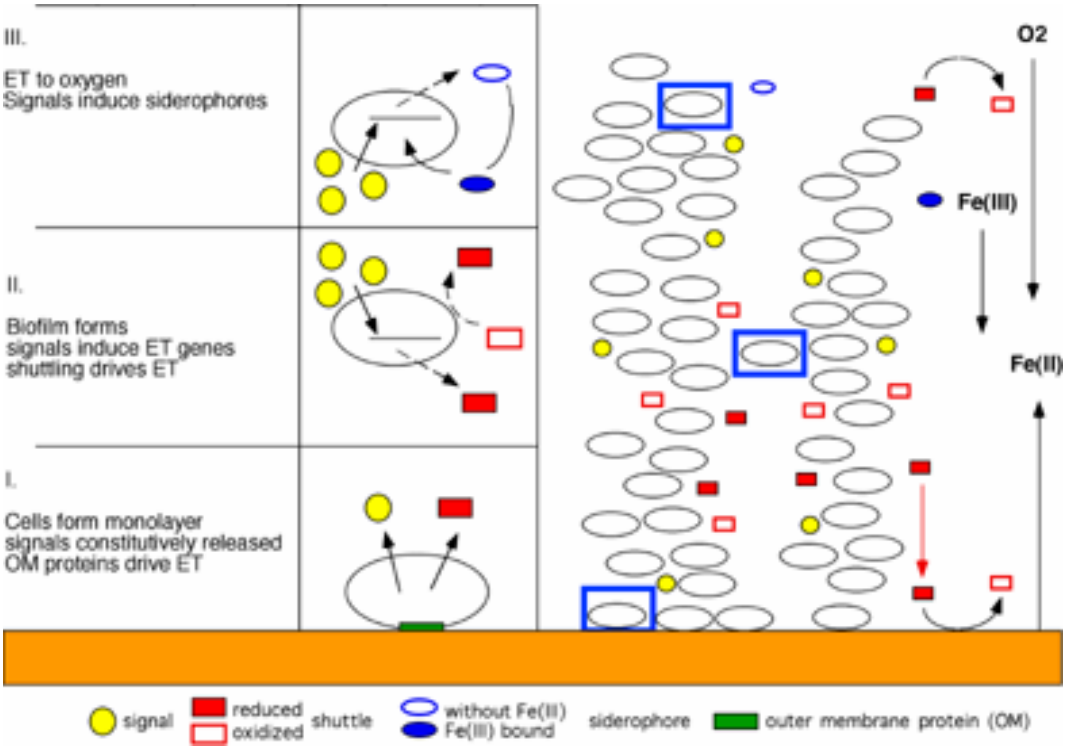


Figure 2.1. A model of electron transfer pathways at different layers within a biofilm of *S. oneidensis* growing on an iron mineral surface.

2.5 Where next?

The study of extracellular electron transfer is still in its infancy and represents a promising area for future research. 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 identify novel antibiotics from the soil (53) and it is clear that we have 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 similar pathways of uptake and efflux as antibiotics. 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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