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Review

Energetic problems faced by micro-organisms growing or surviving on parsimonious energy sources and at acidic pH: I. *Acidithiobacillus ferrooxidans* as a paradigm

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ABSTRACT

The mitochondrial paradigm for a chemiosmotic energy transduction mechanism requires frequently misunderstood modifications for application to microbes growing and surviving at acidic pH values and/or with relatively weak reductants as energy sources. Here the bioenergetics of the iron oxidiser *Acidithiobacillus ferrooxidans* are reviewed and analysed so as to develop the general bioenergetic principles for understanding organisms that grow under these conditions. Extension of the principles outlined herein to organisms that survive (as opposed to grow) under these conditions is to be presented in a subsequent article.

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1. Introduction

Many species of bacteria grow under challenging conditions, for example at extremes of external pH or on sources of electrons that, for even aerobic respiration, yield scarcely sufficient energy for ATP synthesis and other endergonic processes vital for the cell. These challenges present significant problems for the understanding of cellular energetics. Furthermore, even organisms that grow at pH 7, and thus are neutrophiles, have mechanisms that allow for transient adaptation for survival at extremes of pH. Respiration implies that the fundamental chemiosmotic mechanism of energy transduction will apply in these organisms, but with modifications. The aim of this article is to explain and clarify the frequently misunderstood mechanisms whereby bacteria and archaea can adapt to difficult energetic conditions, for instance living parsimoniously on a substrate whose oxidation can yield only a small amount of energy, or living at extremes of pH which impose restrictions on the transmembrane parameters of the chemiosmotic mechanism.

The familiar textbook chemiosmotic mechanism describes the oxidation of NADH or succinate by oxygen with concomitant outward proton translocation to generate a transmembrane proton-motive force (pmf). Mitochondria in the cell have an external (cytoplasm of the cell in which they reside) pH of around 7 and the pmf is generally considered to comprise a membrane potential of around 150 to 170 mV plus a small pH gradient of approximately −0.4 U (i.e. matrix alkaline), the latter contributing approximately 30 mV to the total pmf of approximately 200 mV. The pmf comprises the membrane potential ($\Delta\psi$) and the transmembrane pH difference (ΔpH), i.e. the two forces which can act directly on the transmembrane distribution of a proton (electrical and concentration difference). These two forces, when converted to the same units, can be summated: pmf (mV) $=\Delta\psi-59\Delta pH$ (note the ΔpH is expressed pHout–pHin and the 59 is the conversion factor at $T=298$ K for ΔpH into millivolts). The mitochondrial pmf is often (although an oversimplification) considered to be consumed solely by the synthesis of ATP which occurs as protons move back into the mitochondrial matrix via the ATP synthase; in fact a proportion of the proton current is consumed in driving transport processes.

The same broad description of chemiosmotic processes, and use of the proton current, applies to suspensions of many respiring microbes when the external pH is approximately 7 and the energy source heterotrophic material. However, in the microbial world this is only one narrow condition out of a much wider range of possibilities. One of the organisms facing a big 'energetic challenge' is the bacterium *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans*). This organism has frequently proved to be a source of much confusion, both in the literature and amongst students of microbial energetics, because it requires understanding of the consequences of both growing at very acidic external pH values and of using a relatively weak reductant, the ferrous ion, as the sole source of electrons for respiration. Thus we begin this article with a description of the current state of understanding of the energetics of this organism. It was a challenge to evolve bioenergetic strategies to cope with the growth mode and today understanding these strategies continues to prove a challenge to biochemists and microbiologists.

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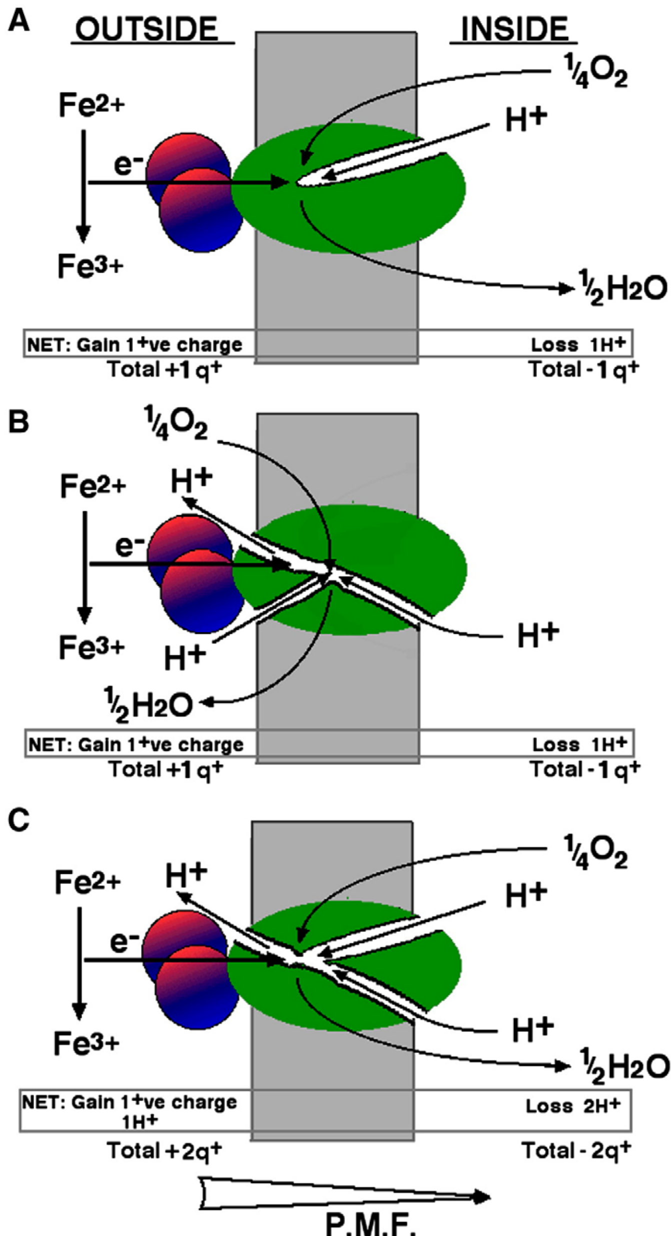
2. The bioenergetic challenge posed to *A. ferrooxidans*

A. ferrooxidans typically grows at an external pH of 2 or lower using the oxidation of ferrous ions by oxygen, producing ferric ions and water. It is not the only procaryote which can utilise these conditions, the bacterium *Leptospirillum ferrooxidans* and the archeon *Ferroplasma acidurans* can also grow solely on the oxidation of ferrous iron by oxygen at acid pH, but *A. ferrooxidans* is by far the best characterised of these microbes, so much of the discussion will refer to this organism [1–3]. There are a number of reasons why ferrous iron oxidation must occur at such a relatively low pH value. Two of these are that (i) the aerobic auto-oxidation of ferrous ion by oxygen is slower at acidic pH; and (ii) the solubility of ferrous ions is greater at lower pH values. A third reason stems from energetic considerations. Whilst the mid-point potential of the FeII/FeIII couple is pH independent and is reported in the region of 0.78 V, that of the oxygen/water couple under the biological standard state conditions of pH 7 is 0.82 V whereas it is 1.1 V at pH 2.0. Although these thermodynamic values need some qualification, because standard states can only serve as a guide and the FeII/FeIII value is determined by the anionic composition of the media, it is clear that only at acid pH is there a significant amount of energy available from the oxidation of ferrous ions by oxygen. Thus the energetics 'improve' at lower pH because the potential of the oxygen/water couple becomes more positive. At pH 2 the energy available from the passage of one electron from ferrous ion to oxygen is circa 300 mV, the exact value depending on the ferrous to ferric ratio, the anions present and the partial pressure of oxygen (not 1 atm but 0.2 atm or lower). In acidophiles that oxidise ferrous iron the predominant anion in the media is sulphate which lowers the potential of the FeII/FeIII couple but the bacterium can still grow in the chemostat when the concentration of FeIII exceeds that of FeII and the measured oxidation/reduction potential (for FeII/FeIII) is as high as 770 mV [1] and coincidentally similar to the standard state value. Thus between this and the oxygen/water couple at pH 2.0 is a redox drop of not more than 330 mV. This is still small relative to the redox drop occurring for the oxidation of typical mitochondrial respiratory chain substrates (approximately 1100 mV), but must be sufficient to provide energy for the growth of *A. ferrooxidans*.



A redox drop of 330 mV needs to be viewed in the context of the chemiosmotic mechanism of energy transduction which can be taken axiomatically to apply to *A. ferrooxidans*. Energetic considerations mean that if the pmf were hypothetically to be 330 mV in a respiring cell then the transfer of one electron from ferrous ion to oxygen could be associated with the movement of maximally one positive charge out of the cell or equivalent (one negative charge into the cell). On the other hand, if the pmf were to be measured at 165 mV then the stoichiometry would be maximally two charges per electron. These maximum values for charge translocation would require that equilibrium is reached between the charge translocating electron transport process and the pmf. In practice, equilibrium will not be reached and so if the pmf were to be measured at a typical value for bacteria of around 200 mV energetic considerations would predict a probable charge translocation stoichiometry of one per electron (we assume throughout that non integral charge translocation stoichiometries do not occur for mechanistic reasons). Fig. 1A shows the model a version of the model proposed by Ingledew et al. [4], for energy conservation in *A. ferrooxidans*, an alternative arrangement with similar energetics is shown in Fig. 1B [5]. In model A a proton-motive force is established by the transmembrane separation, involving a cytochrome *c* oxidase, of the two half reactions (FeII

Fig. 1. Schematic cytochrome *c* oxidase-dependent pmf generation schemes for *A. ferrooxidans* respiring on FeII. In all cases the site of oxidation of ferrous ion is in the periplasm (outside): the two spheres in the periplasm collectively represent the FeII oxidizing enzyme together with *c*-type cytochromes and a copper protein called rusticyanin and the oval in the membrane represents the cytochrome oxidase. For simplicity the diagrams show delivery of a single electron to a nominal 1/4 O_2. The structure of cytochrome oxidase shows the site of oxygen reduction is positioned towards the periplasmic side of the membrane. In most cytochrome oxidases there are two channels which deliver protons to the site of oxygen reduction (see text). (A) Cytochrome oxidase without a proton pumping activity such that outward movement of positive charge is achieved through the inward movement of the electrons meeting the protons taken up from the cytoplasm (inside or N-phase) at the site of oxygen reduction (equivalent of one positive charge moved outward per electron). The minimal net charge ($1q^+$ per e) translocation of this scheme, and the driving force available, are energetically compatible with the large measured values of pmf (see text). The model includes the idea that *A. ferrooxidans* oxidase has only one proton delivery channel (see text). (B) Cytochrome *c* oxidase in which the protons for oxygen reduction enter from the external phase and proton pumping is retained, the net transmembrane charge generation is $1q^+$ per electron (same the scheme above). As with the model above the driving force available is energetically compatible with the large measured values of pmf (see text). (C) Cytochrome *c* oxidase with a 'standard' (as in mitochondria) proton pumping activity (two charges moved per electron). The net charge ($2q^+$ per e^-) is not energetically compatible available energy in *A. ferrooxidans* and the measured value of the pmf (see text). Two proton delivery channels are shown, but as explained in the text the proton supply rates through the two channels are not equal. As discussed in the text, the analysis of the energetics of the cytochrome oxidase reaction must be calculated for pH 2.0 even though the protons used for the reduction of water come from the cytoplasm (pH 6.5).

oxidation and O_2 reduction), without additional vectorial proton pumping. In model B the same result is achieved by not separating the to half reactions by the membrane but by pumping protons across the membrane. Models A and B contrast with that shown in Fig. 1C. Fig. 1C shows the typical cytochrome *c* oxidase which effects more charge separation by both proton pumping and separation of the half reactions, the reason for the difference is that the latter enzyme has more redox potential energy at its disposal. Some confusion can arise when charge translocation is considered. It can be achieved by separation of the two (half) reactions by the membrane (Fig. 1A), this leads to the loss of a proton from the inside and the gain of a positive charge at the outside per electron received from substrate (FeII) — thus generating a pmf. The cytochrome *c* oxidase (cytochrome aa_3 in this case) superfamily is a large family of terminal respiratory oxidases which are highly homologous in sequence and function; there are variants in reductant or in the type of haem utilised by the enzyme (e.g. haem o and o_3 instead of the 'classical' haem *a* and a_3). Herein we will refer to all the oxidases as 'cytochrome oxidase' as a generic label.

Against the above theoretical analysis we can now consider experimentally determined values for the pmf in *A. ferrooxidans*. It should be kept in mind that quantitation of the pmf for any system has to be by indirect methods and is therefore subject to inaccuracies. For cells respiring on FeII the pmf was measured as 256 mV at both pH 1 and pH 2, larger than the typical value for bacteria [6,7]; this higher value may be a consequence of the energy conservation mechanism in this bacterium (Fig. 1A). At a typical external pH of 2 the cytoplasm of *A. ferrooxidans*, respiring on FeII oxidation, is measured to be pH 6.5, meaning that the pH gradient is 4.5 U, equivalent to 266 mV. The membrane potential is 10 mV, positive inside the cell, so as to give a net pmf of 256 mV. At pH 1.0, the internal pH was measured at approx. 6.7 and a membrane potential, positive inside, of 70 mV. The striking point here is that the membrane potential in respiring cells is small in magnitude and, at these pH values, of opposite polarity to normal. This is a necessary consequence of the requirement that the cytoplasm be kept at close to pH 7 whilst the external pH is low. We defer for the moment consideration of what maintains this pH gradient. First, we consider the implications of a respiration-dependent pmf of 256 mV. As explained above, a pmf of 256 mV implies that the transfer of one electron from ferrous ion to oxygen can be associated with movement of only one (equivalent) net positive charge outwards across the cytoplasmic membrane; this is the net result of the mechanisms shown in Fig. 1A and B. The molecular basis is clear. Ferrous iron is oxidised in the periplasm and the electron passed via *c*-type cytochromes and/or a copper protein called rusticyanin, to a cytochrome oxidase. Fig. 1A and B, show this process, a proton is lost from the inside and a positive charge gained outside for each electron consumed, thus work is done against the pmf (reduction of O_2 requires four electrons but the arguments are more simply presented by considering a single electron transfer). However, there is potentially a problem with this straightforward description; all cytochrome oxidases are thought not only to catalyse the combination of electron and proton movement shown in Fig. 1A but also the pumping of one additional proton from the inside to the outside of the membrane per electron, giving a loss of two protons from the interior and the gain of a proton and an additional positive charge outside (i.e. two positive charges and the chemical entity of a hydrogen ion), as shown in Fig. 1C [8–10]. Fig. 1C is clearly incompatible with the current explanation of the energetic constraints when *A. ferrooxidans* is oxidising ferrous iron, and yet the *A. ferrooxidans* gene sequence shows that its oxidase is a member of the cytochrome oxidase superfamily. The pumping of protons by cytochrome oxidase is well established. There are at least two possible escapes from this conundrum of the apparent incompatibility of the more usual cytochrome oxidase with the energetics of *A. ferrooxidans*. The first is that the cytochrome aa_3 oxidase of *A. ferrooxidans* might be atypical by not having a lower stoichiometry of charge separation per turnover (Fig. 1A or B). The second, see below, is that the thermodynamics are not as constrained as the discussion above implies, i.e. pmf is lower and/or the available redox energy is larger than estimated.

3. No net proton pumping by *A. ferrooxidans* cytochrome oxidase?

The structures of bovine, *Paracoccus denitrificans*, *Rhodobacter sphaeroides* and *Thermus thermophilus* cytochrome oxidases and the *Escherichia coli* quinol oxidase are known [11–15]; however, the mechanism of their proton pumping is still hotly debated [8,16–19]. A variety of studies on the enzyme have implicated specific amino acid residues as important for proton pumping activity, amongst which are conserved residues which can be seen in the structure of the protein to lie in two putative proton conducting channels, D and K. These channels are thought to conduct protons from the inside (cytoplasm in a bacterium) phase to the site of oxygen reduction in the centre of the protein [16–18], although there is evidence for plasticity [20].

The gene sequence of the *A. ferrooxidans* cytochrome aa_3 oxidase is known [21] and a comparison with a consensus sequence for the enzyme shows some interesting differences. A genome sequence of a second iron oxidiser is now also available, that from the phylogenetically quite distinct archeon *Ferroplasma acidarmanus* [http://www.genome.ornl.gov/microbial/faci/], which also contains a member of the cytochrome oxidase superfamily (although the *F. acidarmanus* enzyme may be a variant containing haem O rather than haem A). The *A. ferrooxidans* enzyme is only the 381st closest homologue to *F. acidarmanus* (as assessed by WU-Blast2 alignment, unpublished) but the two converge in sharing some unique features, described below, and which appear to present an example of how the protein structure–function relationship can be tuned to the particular energetic demands on the energy transducing system.

3.1. *The proton conducting 'K-channel' is absent from the cytochrome oxidase of the iron oxidisers*

The *A. ferrooxidans* and *F. acidarmanus* cytochrome oxidases appear to be unique in lacking most of the essential residues of the proton conducting K-channel, including the mechanistically crucial lysine K354, without any apparent alternative compensatory adaptations to allow proton access [16,18,19]. The K-channel takes its name from the conserved lysine (K354 in the numbering for the prototype *P. denitrificans* enzyme). In the *P. denitrificans* enzyme a proton channel leads from serine S291, which is in contact with external water molecules, via lysine K354 to tyrosine Y280. There is some variety in the putative K-channel but in 500 sequences examined the conservation of the lysine is remarkable, there were five cases of alternative hydrophilic residues in the K position; these can presumably compensate. In *A. ferrooxidans* and *F. acidarmanus* the K is replaced by hydrophobic leucine and isoleucine respectively. The two closest homologues of *F. acidarmanus*, *Aeropyrum pernix* K1, an aerobic hyperthermophilic archaeon isolated from a solfataric thermal vent (Archaea — Crenarchaeota), and *Haloferax volcanii*, a Dead Sea halophile (Archaea — Euryarchaeota), also lack the lysine and other components of the K-channel, but they have other modifications which could compensate — they are the sole examples of loss of the adjacent PTG motif and have instead a PFD/E motif (Fig. 2).

The normal K-channel leading up to the crucial linking lysine K354 incorporates other highly conserved residues; both tryptophan W358 and serine S291 are also absent in *A. ferrooxidans* and *F. acidarmanus*. In the *A. ferrooxidans* enzyme the W358 is replaced by an alanine, in *F. acidarmanus* by a leucine. This is shown in Fig. 2A. Out of the 500 sequences aligned the W358 is also replaced in *A. pernix* K1 and *H. volcanii*, in another it is replaced by arginine (this is one in which K354 is replaced by a hydrophilic residue and has a string of alternative hydrophilic residues forming an alternative 'K-channel').

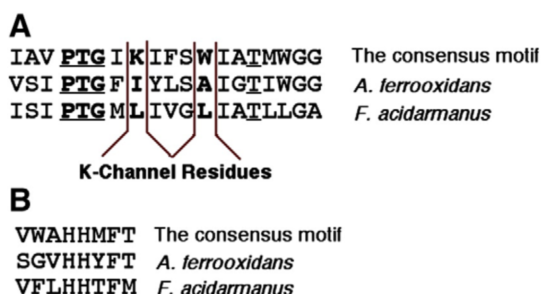


Fig. 2. Sections of the alignment of the cytochrome oxidase sequences from two very distinct (one a Eubacterium and one from an Archaea) iron oxidisers. (A) The absence of the consensus motif for the K-channel. The consensus sequence shown is from I347 to G365 (*Paracoccus denitrificans* numbering). (B) Section of the alignment of the cytochrome oxidase sequence from iron oxidisers showing the consensus motif containing residues adjacent to the double histidine motif involved in Cu(B) binding. The consensus sequence shown is from V322 to T329 (*P. denitrificans* numbering).

In *A. ferrooxidans*, *F. acidarmanus*, *Aeropyrum pernix* K1 and *H. volcanii*, serine (S291) is also absent. In an alignment of the 500 closest sequences to the *F. acidarmanus* sequence S291 is occasionally replaced by a threonine or a tyrosine. The two closest homologues to *F. acidarmanus* appear to lack much of the K-channel but have a unique adjustment amongst all the sequences studied which introduces an acidic residue and could switch the access to an alternative H^+-translocation route.

3.2. The proton conducting 'D-channel' is present in the cytochrome oxidases of the Iron oxidisers

The other proton conducting channel, the D-channel, appears to be present in *A. ferrooxidans* and *F. acidarmanus*. The D-channel is usually formed by a chain of water molecules leading from aspartate D124 to glutamate E278 (Paracoccus numbering) and incorporating, threonine T203, asparagine N199, and serines S134 and S193 [16,18,19]. In the *A. ferrooxidans* oxidase the D124 is replaced by an asparagine, the other residues are present. In *F. acidarmanus* the sequence data is truncated towards the N-terminus, it starts just after the D124, the S134 is replaced by a tyrosine, the S193 is replaced by a threonine, the N199 is replaced by a glutamate and T203 and E278 are both present. None of the latter replacements is unique and all are hydrophilic so it is likely that the channel is functional. Parenthetically, it is known that the D pathway can exhibit some plasticity and certain alterations (e.g. S197D) allow turnover (35%) but the reaction is no longer coupled to net proton pumping, and a fraction of the substrate protons are taken from the outer phase (c.f. Fig. 1B) [20].

During recent years a consensus has emerged which envisages that for every oxygen molecule reduced by cytochrome aa_3 oxidase, 6 or 7 of the 8 protons involved (including all 4 which are regarded as pumped across the membrane) travel via the D pathway and only 1 or 2, each required for reduction of oxygen to water, are routed via the K pathway. At first sight, therefore, the retention of the D pathway in the oxidases from FeII oxidising acidophilia would suggest that proton pumping is retained. However, there is still considerable uncertainty about the functions and reasons for the differential roles of the D and K pathways. This key to whether model 1A or 1B is appropriate for *A. ferrooxidans*, if the D-channel carries the pumped (vectorial) protons then model 1B is favoured, if channel K carries the pumped protons then model 1A is favoured.

3.3. Further modifications of the A. ferrooxidans and F. acidarmanus cytochromes oxidase

The loss of the K-channel may require further adaptations of the *A. ferrooxidans* and *F. acidarmanus* cytochrome oxidases as turnover is inhibited in 'cytochrome oxidases' when the K-channel is blocked by mutation [18], thus suggesting that proton access through the K-channel needs to be de-coupled from oxygen reduction in these iron oxidisers. It may be that compensation for the absence of the K pathway means that the D pathway can only be used for the supply of protons needed for oxygen reduction to water, or that proton pumping is retained but proton access for the oxygen reduction is from the periplasmic phase (model 1B). An interesting aside is a comparison with the cytochrome oxidase orthologue *nitric oxide reductase*; this enzyme differs from cytochrome oxidase in that the copper at the catalytic site is replaced by an iron and the preferred substrate is nitric oxide, the enzyme does have oxygen reducing activity. This enzyme does not appear to have either a K or a D-channel [22] and has be shown not to be electrogenic during turnover. The enzyme in membrane vesicles of *Rhodobacter capsulatus* was monitored using a carotenoid bandshift assay to indicate the formation of a transmembrane electrochemical gradient and the authors concluded that the enzyme is not electrogenic [23]. This implies that the proton access to the catalytic core must be from the outside, such as proposed in model 1B. Cytochrome oxidases in general must have a proton pathway leading from the catalytic core to the external phase, but this is gated so as to be the exit pathway.

Apart from the principal haem and copper ligating residues and the proton-channel residues, there are a number of other highly conserved residues in the cytochrome oxidase superfamily which are not retained in either the *A. ferrooxidans* or *F. acidarmanus* the enzyme, for example close to the consensus Cu_b binding sequence at the catalytic site, a region implicated in most models of proton pumping and oxygen reduction (Fig. 2B).

The VWxHHMF sequence is highly conserved in the superfamily except, as shown in Fig. 2B, in the two FeII oxidising organisms. The sequence is integral to the catalytic site. The conserved HH provide the bidentate histidine ligation to the copper center Cu_b, in all sequences. The VW is present in all other examples (one LW). The M327 is less highly conserved. The F is occasionally replaced by a Y. Could the loss of the *VW* impact on the functioning of the catalytic site, enabling either proton access from the outside or the decoupling from proton pumping? At the very least the foregoing sequence analyses are suggestive that *A. ferrooxidans* and *F. acidarmanus* cytochrome oxidases might be variants, adapted to be only partially electrogenic compared to other cytochrome oxidases. Parenthetically, one might note that respiratory chain bacterial *nitric oxide reductases*, also do not contain the W of the WxHH motif.

4. An analysis of the magnitude of the thermodynamic parameters

The alternative solution to the apparent incompatibility of a proton pumping cytochrome oxidase and the thermodynamics of ferrous iron oxidation by *A. ferrooxidans* and *F. acidarmanus* is that the thermodynamics have been estimated inaccurately, and that there is, after all, enough energy for the oxidase to pump additional protons. In this case it would have to be argued that either the pmf has been overestimated, the driving force of FeII oxidation has been underestimated, or a combination of the two. For illustrative purposes, if the redox drop was greater than 360 mV and the value of the pmf was lower than180 mV, there would be enough driving force to pump protons as shown in Fig. 1C.

4.1. Measurement of the proton-motive force in A. ferrooxidans

As mentioned earlier, the values for the membrane potential in respiring (energised) cells of *A. ferrooxidans* have been measured and are positive inside in strongly acid media [5]; the same polarity is found in the absence of respiration [7]. In the absence of substrate and the presence of uncoupler the internal pH was estimated at 5.6 and the membrane potential at 136 mV (the external pH was 3.0), giving an apparent pmf of 17 mV (within experimental error of 0 mV).

4.2. How can the membrane potential be positive inside during respiration when positive charge is being translocated from the inside?

At first encounter, having a respiratory system which translocates positive charge out of the cell is difficult to reconcile with the idea that the membrane potential during coupled respiration is small and positive inside. However, the situation becomes clearer when the nature of the pmf in non-respiring cells or uncoupled cells is analysed. Here experimental analysis [6,7] has shown that the pH gradient can be maintained for substantial periods in the absence of respiration. However, the pH gradient, is accompanied by a membrane potential, positive inside, of similar magnitude, resulting in a net pmf close to zero. This situation has been shown to hold in other acidophiles [24,25]. The absence of respiration, and therefore also of oxidative phosphorylation, implies that maintenance of the positive inside membrane potential cannot be a result of ATP-dependent inward cation movement, a mechanism that has been suggested elsewhere. Set in this context we can immediately see that the effect of respiration is to translocate positive charge outwards and thus to decrease the magnitude of the membrane potential, positive inside. A further important point is that an acidophile such as *A. ferrooxidans* cannot vary the pH gradient because it is constrained by the typical medium pH of 2 (or lower) and a requirement that cytoplasmic pH must not deviate too much from 7. We return below to some considerations as to the origin of the large inside positive membrane potential and how the cytoplasmic pH is maintained in non-respiring cells. In the context of this section we must ask, how reliable are these measurements of proton-motive force? It is arguable that the indirect methods used are less reliable at the extreme values, particularly at high accumulation ratios for the probes, but remember that the two components of the pmf are measured independently. The larger of these in coupled respiring cells is the pH difference, which, although large, is logically correct; the internal pH must be maintained close to neutrality. Because of the predicted and measured high accumulation ratios for the probes in measuring the cytoplasmic pH, any error is most likely to be in under-estimating the gradient. The variable of the two parameters contributing to the pmf is the membrane potential, and if there is an over-estimation of the pmf logic suggests it must be through an under-estimation of the internally positive membrane potential. The probe used to estimate the membrane potential depends on the polarity of the membrane potential, an anionic probe is used when the membrane potential is positive inside and a cationic probe used when the membrane potential is negative inside (as only accumulation can be accurately measured). The measurement of a small internally positive membrane potential in respiring cells at pH 1.0 and 2.0 is internally consistent with a large increase in the value of this potential when the cells are treated with protonophore, with the dependence of the measured potential on the pH of the suspending media; a cross-over of the polarity of the membrane potential is observed (where one probe is no longer accumulated the other begins to be accumulated, just above pH 2,0) and with the controls used in the original publication [6,7]. Thus there are grounds for accepting a respiration-dependent pmf of approximately 250 mV, much larger than for neutrophiles. Why might the measured pmf be larger than that measured in other bacteria? Possibly because of the direct coupling of the redox process, with no proton pumping, to the generation of the pmf (see legend to Fig. 1A and B).

4.3. Some possible wider implications of a positive inside membrane potential

The transfer of proteins to the periplasm in bacterial is facilitated by the sec (unfolded) and the tat (folded) systems. Each of these relies, in a far from characterised way on the pmf and it is often assumed on the basis of model studies with *E. coli* that the membrane potential is the key factor. However, the periplasmic positive membrane potential, as found in acidophiles, would not help pull a positive charged signal peptide through the membrane; however, there are data suggesting that ΔpH is as effective as $\Delta\psi$ in driving the process [26]. Thus there is no problem in having a positive inside potential. Another aspect of the sec system is that it is responsible for the insertion of integral membrane proteins which adopt a topology according to the positive inside rule. If we assume that this rule operates also in *A. ferrooxidans* then we must conclude that the $\Delta\psi$ is not a crucial determinant in the positioning of integral membrane proteins. This point has been examined before for another acidophile and use as evidence against $\Delta\psi$ involvement in locating positively charged loops to the cytoplasmic side of the membrane [27].

4.4. Is the estimate of the available redox potential difference accurate?

The second possible error is an underestimate of the available redox potential difference. The mid-point potential of the FeII/FeIII couple is pH independent and is cited in the region of 0.78 V, but in sulphate containing growth media the value is shifted to approximately 0.65 V [1]. However, as mentioned earlier the bacterium can still grow in the chemostat when the concentration of FeIII exceeds that of FeII and the measured electrode potential is as high as 0.77 V [1]. The mid-point potential of the oxygen/water couple is approximately 1.1 V at pH 2.0 (although this will be slightly less oxidising in practice because the partial pressure of oxygen will be less than 1 atm). Thus the cells can grow when there is as little as 330 mV of redox potential energy available. This is a low value. However, this value did not apply when the measurements of proton-motive force during FeII oxidation were made; under those conditions the effective electrode potential of the FeII/FeIII couple can be (gu)estimated to be closer to 650 mV, giving up to 450 mV redox potential energy. However even this value is too small to accommodate the translocation of two charges against the measured pmf ($>2\times256$ mV, the pmf); the translocation of two charges can only be accomplished if the magnitude of the pmf is also much overestimated (which we doubt).

5. An aspect of the mechanism proposed in Fig. 1A which has lead to confusion

At this point we address another aspect of the bioenergetics of *A. ferrooxidans* that frequently causes confusion. Fig. 1 shows that the protons for the reduction of oxygen to water are taken from the cytoplasm, the pH of which is close to neutrality. This causes many to question why the oxidase would use cytoplasmic protons for oxygen reduction when the redox potential of the oxygen/water couple is much more positive, and hence favourable at pH 2.0? Alternatively, it is questioned whether using the thermodynamics of oxygen reduction at pH 2.0 is appropriate for analysis of the energetics of *A. ferrooxidans* growth. The simplest answer is that in thermodynamic terms it makes no difference which phase the protons come from (although it does matter mechanistically); ultimately everything used by the organism is taken up from, and waste products delivered to, the external medium which is at pH 2. Thermodynamics relates to beginning and end states and in this context it is clear that use of the pH 2 values for energetic analysis is appropriate even when the oxygen is reduced using protons taken from the cytoplasmic phase. The reader who seeks reassurance on this point can consult articles where this is demonstrated arithmetically [1–3].

6. The uses of the proton-motive force

Next, we turn to an analysis of reactions that consume the pmf in *A. ferrooxidans*. Central here is ATP synthesis which requires proton movement via the ATP synthase from the periplasm to the cytoplasm. For illustrative purposes only let us assume that the *A. ferrooxidans* enzyme has 10 c subunits forming the annulus and thus a proton

translocated to ATP synthesised ratio of 3.3 (10/3) [28]. If one charge is translocated per electron passing from ferrous iron to oxygen then the P:e ratio would be 0.3, or expressed in the more conventional P:O (number of ATPs made per oxygen atom reduced, i.e. per two electrons transferred) or P:2e terminology ratio of 0.6. On the other hand if a charge translocation stoichiometry of 2 per electron flowing from FeII to oxygen were to occur then a P:e of 0.6 (i.e. P:2e or P:O = 1.2) becomes possible. It is important to realise that a lower stoichiometry of ATP synthesis does allow for a higher ratio of ATP/ADP to be achieved. The relationship is simple, the driving force ($\Delta G_{[2FeII/O]}$) is linked to the product $\Delta G_{[ATP]}$ by the P/O (gearing) ratio, thus if the P/O is smaller the $\Delta G_{[ATP]}$ can be greater, as the relationship $\Delta G_{[2FeII/O]} > [P/O] \times \Delta G_{[ATP]}$ must hold.

Fig. 1B shows a possible alternative arrangement of proton channels, in this case the protons for the oxygen reduction come directly from the exterior phase and the enzyme retains proton pumping activity, the overall transmembrane charge separation is the same as in the preceding case, thus the thermodynamics are the same.

7. Reversed electron transport

A. ferrooxidans is a chemolithotroph which means that it fixes carbon dioxide in order to build new cell material [29]. CO_2 fixation is via a Calvin Cycle and requires NAD(P)H as well as ATP. NAD(P)H will also be needed for many other anabolic reactions in the metabolism of *A. ferrooxidans*. The source of electrons for reduction of NAD(P)$^+$ to NAD(P)H has to be the ferrous ion but the latter is too weak a reductant to drive this reduction reaction unassisted (this is illustrated thermodynamically in Fig. 3 and mechanistically in Fig. 4). This assistance is by pmf-dependent reversed electron transport. What this means is that some of the electrons derived from oxidation of ferrous ion are driven 'uphill' (to lower redox potential) along the electron transfer chain from their point of entry into the chain. This is illustrated in Fig. 4, where the proton current is shown being generated by FeII oxidation by oxygen and consumed by ATP synthesis and (to a lesser extent) through reversal of the cytochrome bc_1 complex and the NADH dehydrogenase complex (both scrutiny of the genome sequence and biochemical data show that *A. ferrooxidans* has both a cytochrome bc_1 complex and an NADH–ubiquinone oxidoreductase complex [29–31].

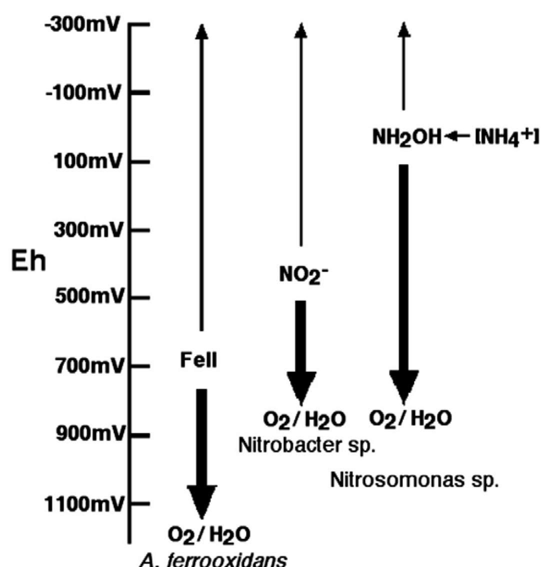


Fig. 3. A redox potential perspective of the electron transfer systems of *A. ferrooxidans*, *Nitrobacter* and *Nitrosomonas*. The approximate redox potentials of the important couples are indicated on the left-hand scale. The oxygen water couple is more positive for *A. ferrooxidans* because of the acid pH of the media, the values shown for *Nitrobacter sp.* and *Nitrosomonas sp.* are those for approximately pH7. The values for NAD$^+$/NADH are at pH7. Downwards direction indicates exergonic processes, upward direction endergonic process. The biochemistry of conversion of NH4+ to hydroxylamine which is close to being an isoenergetic process is beyond the scope of this article [33].

In vitro studies have shown that respiratory chain complexes can function in reverse of the usual direction provided that a pmf-generating reaction is also operational. For *in vitro* studies, often with mitochondrial membranes, this generation has usually been achieved by ATP hydrolysis catalysed by the ATP synthase. Such *in vitro* studies have encouraged the still common misconception that *in vivo* bacteria such as *A. ferrooxidans* also use ATP to drive reversed electron flow. However, this is most unlikely because the proton translocating ATP synthase [ATPase] could only translocate protons out of the cell if the respiration-dependent generation of pmf were to cease, a phenomena which is unlikely to occur during steady state respiration by cells. Much more likely is that the respiration-dependent pmf simultaneously acts on both the ATP synthase to drive ATP synthesis and on the cytochrome bc_1 and NADH dehydrogenase complexes to drive reversed electron transport, as illustrated in Fig. 4 The majority of electrons enter the electron transport chain from ferrous iron and pass downhill to oxygen and thus generate the pmf, which in turn drives the two endergonic processes of ATP synthesis and reversed electron transport. Quite how the relative rates of these processes might be controlled is not known, but the principal control must be thermodynamic. Just as in the steady state the pmf in, say, *E. coli* can simultaneously drive ATP synthesis, flagella motion and transport systems so *A. ferrooxidans* must be able to drive at least two processes (ATP synthesis and reversed electron transfer) at the same time. There does not seem any reason to invoke oscillations in which a fluctuating magnitude of the proton-motive force will allow transient reversed electron transfer. In the steady state the thermodynamic balance between the magnitude of the pmf, the oxidised:reduced ratio of cytochrome c_1 of the bc_1 complex and the NAD/NADH ratio will favour the coupling of inward proton movement through complexes I and III to the transfer of electrons to NAD from cytochrome c_1 and the donors to the latter. It is also not known if there are design features in the *A. ferrooxidans* cytochrome bc_1 and NADH dehydrogenase complexes that promote their optimal functioning in the reverse direction to normal [31,32]. In particular, the quinone handling site at the exterior of the cell membrane would need to operate in contact with the very acidic periplasmic phase. Dutton and colleagues have discussed in detail the factors influencing reversibility of Q-cycles [33] whilst Mitchell considered the relative effects of pH gradients and membrane potential on the directionality of electron flow through Complex III [34].

The genome sequence for *A. ferrooxidans* shows the presence of two sets of genes for the cytochrome bc_1 complex which raises the question as to whether two slightly different complexes are expressed, one specific for the reversed electron flow discussed here and the other for downhill electron transfer. Expression of one of the bc_1 complexes is much enhanced during respiration on iron suggesting a primary role in reversed electron transfer [35]. The other might be required during the oxidation of reduced sulphur compounds, although the putative presence of a cytochrome bc_1 independent *bd*-type quinol oxidase may allow electrons originating from such compounds to bypass the bc_1 complex [36].

Demonstrating pmf-dependent reversed electron transport *in vivo* has not been an easy matter. Indeed, in the most recent paper on the subject Elbehti et al. have argued that electron transport from a *c*-type cytochrome to NAD$^+$ is driven by ATP hydrolysis in cells of *A. ferrooxidans* [32]. This conclusion is substantially based on an observation that addition of ATP to sphaeroplasts promotes the oxidation of added ferro-cytochrome *c*. The oxidation of the cytochrome *c* is presumed to be due to transfer of electrons to endogenous NAD$^+$. The ATP-dependent oxidation of the cytochrome *c* is reported sensitive to some NADH dehydrogenase and cytochrome bc_1 complex inhibitors (rotenone and myxothioazol). This is a very

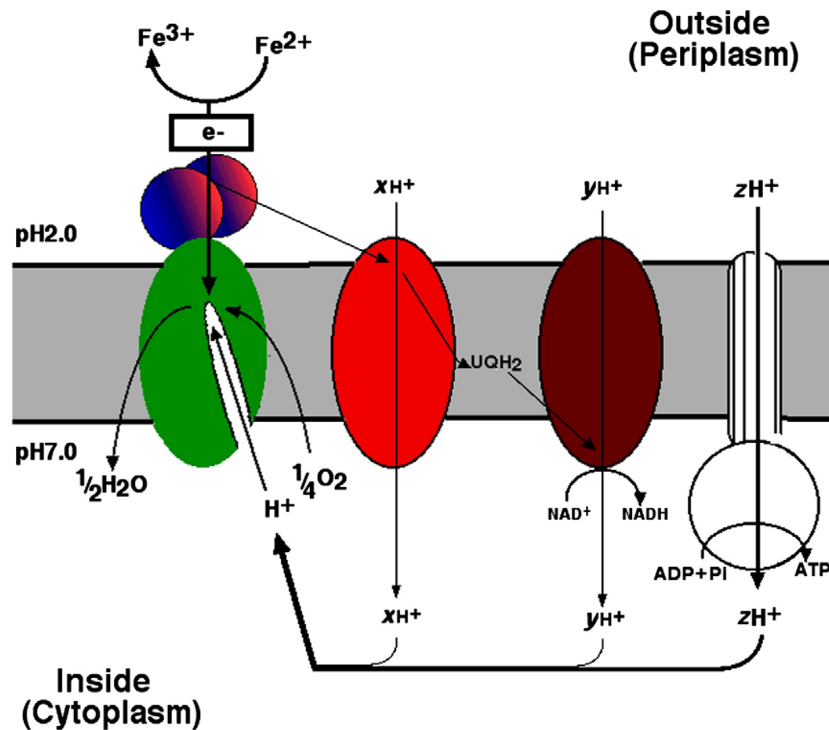


Fig. 4. Electron flow and the flux of protons during respiration on FeII. Most of the electron flow from iron goes to oxygen via cytochrome oxidase (indicated by a bold arrow) which generates pmf but <5% goes to $NAD(P)^+$, driven by the pmf via reversed electron transfer involving a complex III and a complex I (indicated by thin arrows). The majority of the inward flux of the proton current will go through the ATP synthase to make ATP (thick arrow), but some re-entry flux will be via complex III and complex I (thin arrows) to drive the reversed electron transport (x and y are the respective H^+/e stoichiometries for complexes III and I respectively, z is the H^+/ATP ratio for the ATP synthase). For simplicity a one electron transfer scheme is illustrated. The cytochrome oxidase is shown as having one proton channel (see Fig. 1). The c subunit assembly of the ATP synthase rotates and it is commonly believed that z equals the number of c subunits in the assembly divided by 3, which is the number of ATP molecules made per 360 degree rotation. This number is not known for the ATP synthase from *A. ferrooxidans* but is believed to range from 10 to 15 for synthases from other organisms so that z might be in the range of 3.3 to 5.

strange observation because added ATP should have had no access to the active site of the F_oF_1 ATP synthase or any other hypothetical ATPase on the inside of the cytoplasmic membrane of intact sphaeroplasts (the sphaeroplasts have to be intact for the coupling of energetic functions). On the basis of these observations Elbehti et al. [32] have gone on to suggest that *A. ferrooxidans* exhibits a biphasic growth pattern, with periods of respiration driving pmf-dependent ATP synthesis followed, when the ATP/ADP ratio is high, by periods of ATP-driven reversed electron transfer. This seems very implausible to the present authors.

To the best of our knowledge the strongest evidence for pmf-driven reversed electron transfer in intact bacterial cells is a study with *Rhodosprillum rubrum* [37]. It was convincingly shown that illumination of previously darkened anaerobic cells led to an immediate rise in the intracellular $NADH/NAD^+$ ratio. The effects of inhibitors and other reagents left little doubt that light-driven cyclic electron transport through the reaction centre and the cytochrome bc_1 complex was generating pmf which in turn was driving reversed electron transport from ubiquinol to NAD^+. Otherwise it has to be admitted that direct evidence for *in vivo* reversed electron transfer is lacking although we know its occurrence is an absolute requirement for the existence of certain chemolithotrophs!

This is the appropriate point to dispel the not uncommon idea that the pmf could be divided up into aliquots, with say 150 mV for ATP synthesis and 50 mV for reversed electron transport. By its very nature there can be only one value for the pmf across a given membrane and thus all consumers of pmf will experience the same steady value of that force (a feature shared with electrical circuitry). The relative rates of consumption of the force will depend on the kinetics and thermodynamics of a particular consumer in the membrane. There is no theoretical reason whatsoever why a variety of processes should not be driven simultaneously by the pmf. In addition to ATP synthesis and reversed electron transfer shown in Fig. 4, there can be transport processes and motility processes consuming part of the proton current. It is also still sometimes said that a particular proton translocating enzyme contributes 40% of the PMF to the total. This is incorrect; it can be said that the complex contributes 40% of the total proton translocation (current) associated with the substrate oxidation.

8. The maintenance of a large transmembrane pH difference

Next, we turn to the question as to how the large pH gradient is 'maintained' across the cytoplasmic membrane of *A. ferrooxidans*. It is reported that non-respiring cells can maintain the pH gradient for long periods in media lacking permeant anions, this is also the case in the presence of a protonophore uncoupling agent [7,38]. Under non-respiring (i.e. expected to be de-energised) conditions the pmf will be low. Under uncoupled conditions there should be no pmf; therefore, there will be no force driving a proton into the cell, as has been demonstrated (see earlier). In the presence of an uncoupler protons are free to enter down their chemical concentration gradient, but in doing so they carry a positive charge, thus establishing a membrane potential positive inside; the net entry of protons will cease when the pH difference and the membrane potential are balanced (equal in magnitude and opposed). Under the conditions of such experiments the predominant anion in the media was sulphate, which appears to be membrane impermeable in *A. ferrooxidans*, and the internal buffering capacity of the cell (which is not thought to be unusually large) is such that the internal pH only falls slightly. This situation can be quite stable. There is a misconception here that protons will leak in as it is pH 2 outside and pH 7 inside. But this will not be so in the

absence of an inflow of permeant anions (or outflow of permeant cations); the membrane potential will remain and counter balance the force of the pH difference. In theory the internal pH can remain near neutrality indefinitely, even in the presence of uncoupler, if there are no agents present which can dissipate either gradient. The two component forces of the proton-motive force may be equal and opposite with respect to a proton but they are not individually zero and a permeant ion can still move electrophoretically in response to the membrane potential and a permeant weak acid or base can still distribute in response to the transmembrane pH difference. As the membrane potential and pH difference are not being actively maintained such processes will dissipate these gradients. Collapsing the membrane potential will transiently make the pmf larger, thus there will be a force on protons to enter — which they will do. For instance, as shown experimentally [7], addition of a relatively permeable anion such as nitrate allows acidification of the cytoplasm because this permeant anion will enter at the expense of the membrane potential disturbing the counter-point between the pH difference and the membrane potential, allowing more protons to enter. The effects of the anionic composition of the media and the effects of organic acids (which can diminish the pH difference) have been measured and reported in detail in *A. ferrooxidans* [7] and support this interpretation.

9. Application of energetic principles exemplified by *A. ferrooxidans* to other organisms living on parsimonious energy sources

A. ferrooxidans is an important organism for the leaching of ores from acidic environments and has been thought to be the dominant organism is such environments [38]. However, it is now known that the flora are more complex and recently it has been shown that some such environments the oxidation of ferrous iron is predominantly by the archaeon *F. acidarmanus* [39]. In common with *A. ferrooxidans* this archaeon also respires on ferrous iron at pH values of 2 or lower. Although the molecular nature of its catalytic apparatus has not yet been described it must be constrained by the same thermodynamic principles that we have outlined here for *A. ferrooxidans*. An archaeon like *F. acidarmanus* does not have a periplasm and so it will be of interest to learn how its apparatus for catalysing the oxidation of ferrous ion is organised, also reversed electron transport is a necessity, as it is for *A. ferrooxidans*.

Growth at acidic pH is not necessarily linked to iron or sulphur respiration, in principle organisms will be able to grow on heterotrophic carbon sources at low external pH. There are, for example, organisms being described such as *Picrophilus* [40] an archaeon that can grow at an external pH of 0. Presumably they, like *A. ferrooxidans*, require a cytoplasmic pH of close to 7 and so must respire with a pH gradient of 7 U (equivalent to 420 mV) and an inwardly directed positive membrane potential of the order of 200 mV to leave a net pmf of 220 mV.

A. ferrooxidans and other iron oxidisers are by no means the only organisms which need pmf-dependent reversed electron flow. Another important example is *Nitrobacter* which relies upon aerobic oxidation of nitrite to nitrate [41]. Here the electrons are also fed into the respiratory chain at the level of *c*-type cytochrome but the site of nitrite oxidation is believed to be at the cytoplasmic side of the membrane, contrasting with the periplasmic location of FeII oxidation. For reasons discussed elsewhere, a cytochrome oxidase that does not translocate protons would be incompatible with growth of this organism [42]. Therefore, it is notable that in contrast to the putative gene sequence of cytochrome oxidase from *A. ferrooxidans*, that of *Nitrobacter* has all the conserved residues considered essential for full proton pumping activity and pmf generation. In common with *A. ferrooxidans*, *Nitrobacter* also requires reversed electron flow via complexes III and I in order to reduce NAD^+ and $NADP^+$ for use in biosynthetic reactions and illustrated in Fig. 3.

For our final example of the importance of reversed electron transfer we mention *Nitrosomonas* which obtains energy for growth from the oxidation of ammonia to nitrite [43–45]. This oxidation occurs in two stages. The first is conversion of ammonia to hydroxylamine. This is a reaction which requires molecular oxygen in a two electron oxidation and is not coupled to pmf generation. The oxidation of hydroxylamine to nitrite is a four electron reaction and a combination of biochemistry and inspection of the genome sequence indicates that these electrons are fed into the respiratory chain at the level of ubiquinone. The ubiquinol can be re-oxidised in three ways. The first is by transfer of electrons to the ammonia monooxygenase enzyme to generate more hydroxylamine (two electrons). The second is via the cytochrome bc_1 complex and cytochrome oxidase to enable generation of pmf; again it is notable that the cytochrome oxidase has all the residues believed to be important for a proton pumping activity of this oxidase (2-*x* electrons). The third, but in terms of flux minority, fate for the reducing equivalents from ubiquinol is reversed electron transport via complex I to generate NADH (*x* electrons). Thus in common with *A. ferrooxidans* and *Nitrobacter* this organism also has to use pmf generated by respiration to drive reversed electron flow as well as ATP synthesis. However, reversed electron flow is less energetically expensive for *Nitrosomonas* compared to the other two species because this organism illustrates the fairly widespread entry of electrons into the respiratory chain at the lower potential level of ubiquinone rather than at *c*-type cytochromes.

In a subsequent article we will use knowledge of how *A. ferrooxidans* copes with external acidic conditions to analyse how other bacterial species adapt for survival but not growth at acidic pH, and cope with adverse pH gradient.

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