Chapter 4
Microbial Electron Transport in the Deep Subsurface

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Abstract The deep ocean may be one of the largest microbial habitats on the planet. Hence, high hydrostatic pressure is a feature of microbial life. We know very little about the deep biosphere because simulating deep ocean conditions in the laboratory whilst simultaneously monitoring microbial processes is difficult. Changes in pressure can inhibit some reactions, whilst simultaneously accelerating others. Assumptions about how biochemical reactions proceed under ambient conditions may lack validity in the deep biosphere. In extreme environments, microbes often exploit metabolic strategies that yield slim energetic margins. How these occur under pressure is an interesting thermodynamic puzzle. Extracellular electron transfer (EET) is a process whereby microbes respire solid substrates in their surrounding environment. For an electron to move outside of the cell, it must transit the microbial envelope through a series of membrane bound electron carriers each of which will have a unique pressure response. EET most likely evolved in the deep biosphere and therefore makes an excellent model system for studying microbial energetics in high pressure environments. In this chapter, the reader can explore the fundamentals of thermodynamics, the discovery of EET, theoretical implications of pressure effects on the relevant biochemical apparatus, and learn about a proposed system for studying the interesting phenomenon of EET under high pressure.

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4.1 Introduction

Covering two thirds of the Earth's surface and with an average depth of 3800 m the ocean exhibits tremendous diversity and activity and represents a considerable proportion of the biosphere yet it remains largely unexplored. The piezosphere, the proportion of the ocean that is below 1000 m water depth and where pressures are therefore greater than 10 megapascals (10 MPa), is the largest microbial habitat on the planet in terms of volume, perhaps accounting for 30% of total global biomass and representing *c.a.* 60% of the earth's surface (Meersman et al. 2013; Picard and Daniel 2013). Whilst other physiochemical conditions, such as temperature, are recorded at extremes in deep oceans, away from hydrothermal vents the temperature in the deep ocean below the thermocline is uniformly close to 2–3 °C (Picard and Daniel 2013; Daniel et al. 2006). Thus high hydrostatic pressure is the ubiquitous and defining feature of life in deep marine habitats.

For each km of water depth the hydrostatic pressure increases by 100 atmospheres (10 MPa). Lithostatic pressure in the sedimentary column increases by 15–25 MPa for each km of depth and in the oceanic crust by 27–32 MPa dependent on the prevailing geology. The average hydrostatic pressure in the ocean is 38 MPa and because the deepest recorded depth in the ocean is 11 km, the current known maximum pressure at the sediment water interface is around 110 MPa. Confirmation exists of prokaryotic life extending down to depths of at least 1.6 km in sediments, and oceanic crusts represent a rich deep-biosphere (Roussel et al. 2008; Salas et al. 2015). The depth and pressure maxima of the deep biosphere have yet to be constrained, but based on collective observations of high carbon turnover in the Mariana Trench, evidence of life in both deep sediments and ocean crusts, and confirmed growth of *Moritella yayanosii* at 130 MPa, it is likely that hydrostatic pressures approaching 200 MPa are biologically relevant (Meersman et al. 2013; Picard and Daniel 2013; Roussel et al. 2008; Salas et al. 2015; Yayanos et al. 1981). Evidence that microbes can survive under hydrostatic pressures up to 80 GPa exists although it is not clear if microbes can grow under these conditions.

The majority of the carbon input into marine systems is derived from primary production that occurs in the photic zone. Immediately below the photic zone, heterotrophic bacteria aerobically degrade the majority of organic carbon with only 1% being deposited in the sea floor as either dissolved or particulate organic matter (Picard and Daniel 2013). The refractory nature of dissolved organic matter (DOM) is known to increase with depth in marine and sedimentary environments hence, the main energy source and the building blocks for prokaryotic benthic lifeforms is difficult to degrade (Aparicio et al. 2015). Carbon turnover in bathypelagic (200–4000 m) and abyssopelagic (>4000 m) sediments necessarily includes the recycling of dead microorganisms or necromass (Meersman et al. 2013). Additionally, in benthic environments, oxygen is quickly depleted, sometimes in the upper few centimetres of sediment, making the job of breaking down available DOM less energetically rewarding (Picard and Daniel 2013). However, microbes

are versatile and anaerobic benthic organisms can exploit energy margins of only -4.5 kJ mol^{-1} (the oxidation of glucose to CO_2 yields -2870 kJ mol^{-1}) by growing slowly (Meersman et al. 2013; Willey 2014; Jackson and McInerney 2002). Accordingly, organic carbon turnover in sedimentary environments occurs over millennia suggesting that there is sufficient organic material in the marine subsurface to drive microbial metabolism for millions of years. However, the turnover rate of volatile acids like acetate happens much more quickly; on a decadal timeframe (Wang et al. 2010). Like oxygen, alternative anaerobic terminal electron acceptors (e.g. nitrate) are depleted quickly in the upper layers of sediment in a thermodynamically predicable order with those that are the most energetically rewarding being utilised first (Meersman et al. 2013). However, this textbook-case of thermodynamic stratification does not reflect the reality in deep benthic systems where sulphate, iron and inorganic carbon simultaneously undergo microbial reduction (Wang et al. 2010).

Despite these constraints, there is surprising activity and diversity in the deep ocean and microbial respiration can be coupled to oxidised iron and manganese containing minerals which are abundant on the seafloor (Liao et al. 2011). Iron reduction has been shown to occur over a range of pH but biological acidophilic iron reduction is poorly understood. At circumneutral pH, iron speciation in marine sediments is varied but even though iron is mostly present as solid oxides or oxyhydroxides, like goethite or haematite, it is still biologically available (Bird et al. 2011). In this chapter we will look at a special case of how organisms can obtain energy by using iron as a terminal electron acceptor—a process known as extracellular electron transfer (EET)—to thrive in marine benthic environments. We will describe what is currently known about EET under pressure extremes concluding that the pressure element has largely been overlooked in the study of an exciting group of organisms which evolved from benthic lifeforms and which deploy a metabolic strategy that likely has primordial origins.

4.2 Electron Acceptors and Microbial Respiration

Most lifeforms obtain energy from coupling the oxidation (i.e. loss of electrons) of an electron donor to the reduction (i.e. gain of electrons) of a terminal electron acceptor. The compound that is oxidised (also called reductant or fuel) is often organic carbon although certain specialist prokaryotes, known as chemolithotrophs, can use inorganic reductants such as hydrogen and sulphur. The terminal electron acceptor is also known as the oxidant and for many organisms, including mammals, this is molecular oxygen which, being ubiquitous in air and water as a by-product of photosynthesis, is the most oxidised, widely-available terminal electron acceptor. For chemical reactions to proceed spontaneously (with the release of energy), the reductant must have a lower reduction potential than that of the oxidant. The free energy available to microbes when catabolising a given reaction is directly proportional to the difference in reduction potential between the reductant and that of

the oxidant and is commonly known as Gibbs free energy and annotated in biochemical treatise as $\Delta G'$—the prime symbol denotes biological conditions that are assumed to be representative of those prevailing in the cytoplasm. If the activities of all chemical species are known then $\Delta G'$ can be calculated with precision.

The electron tower is commonly used in microbiology to illustrate the concept of bioenergetics (Fig. 4.1). The first thing to note is that reductants or electron donors have a negative reduction potential ($E^{\theta'}$) whilst compounds that have a tendency to accept electrons have a positive reduction potential ($E^{\theta'}$). Microbes can obtain the most energy from coupling the oxidation of reduced organic compounds (such as glucose) to oxygen because the potential difference between the electron donating reductant ($E^{\theta'}$ − 0.43 V for glucose) and the accepting oxidant (+0.82 V for oxygen) is large ($\Delta E^{\theta'}$ = 1.24 V). Electrons spontaneously flow from carriers having a negative potential to those with a positive potential and the greater the potential difference between the reductant and the oxidant, the larger the $\Delta G'$ is for a given reaction and the more metabolic energy the microbe can extract.

Note from the electron tower that the reduction potential for the ferric iron (Fe (III)) to ferrous iron (Fe (II)) redox couple has a large positive value (E^{θ} = +0.77 V). In a thermodynamic sense, ferric iron is a good oxidant and therefore potentially a good biological terminal electron acceptor. The reduction potential for Mn (IV) to Mn (II) (E^{θ} = +0.47) is close to that of the nitrate (NO_3^-) nitrite (NO_2^-) redox couple ($E^{\theta\prime}$ = +0.42 V) suggesting that Mn (IV) makes as good a candidate terminal electron acceptor as nitrate based purely on its reduction potential (Logan 2008). The free energy of coupling acetate oxidation to iron and manganese reduction ranges between −712 and −814 kJ mol^{-1} at neutral pH which is very close to the energy that can be obtained from coupling acetate degradation to the oxygen reduction reaction

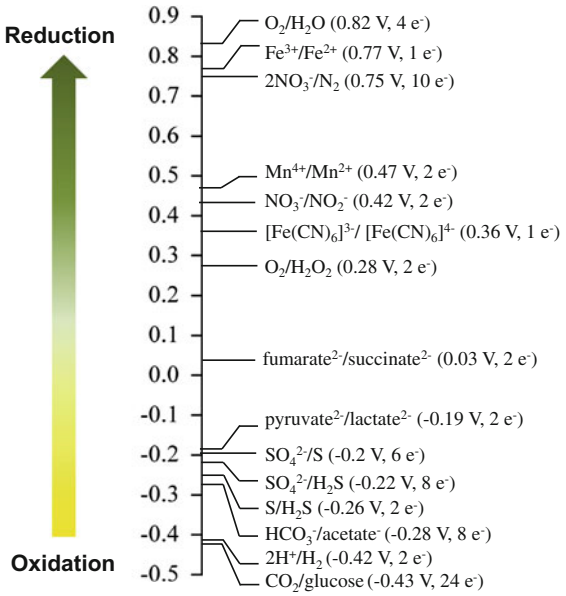

Fig. 4.1 The electron tower concept is useful to illustrate energy yielding redox reactions in biology. Theoretically, any reaction at the *bottom* of the electron tower can be coupled to a reaction at the top. The amount of energy ($\Delta G'$) available from a given reaction is directly proportional to the difference in the reduction potential of the redox couples in question

($\Delta G' = -849$ kJ mol^{-1}), meaning microbes can gain significant energy from using iron or manganese as terminal electron acceptors (Lovley and Phillips 1988).

However, ferric iron and manganese (IV) are present primarily as insoluble oxides and oxyhydroxides in marine sediments which complicates the thermodynamic rationale for their suitability as terminal electron acceptors when compared to dissolved species like oxygen or nitrate (Bird et al. 2011; Nealson and Myers 1992). Additionally, iron is polymorphic, with structural order ranging from amorphous ferric oxides to highly crystalline goethite (α-FeOOH) and akaganeite (β-FeOOH) each of which have considerably different kinetic properties from dissolved terminal electron acceptors. The range of crystallinity apparent in iron containing minerals gives rise to different midpoint reduction potentials ranging from $E^{0'} = +0.38$ V for ferric citrate to −0.31 V for magnetite (Lovley and Phillips 1988). It not surprising therefore that the significance of metal oxides as biological terminal electron acceptors was not fully appreciated until the late 1980s when, almost simultaneously, Lovley and Phillips (1988) and Myers and Nealson (1988) reported the phenomenon of dissimilatory iron and manganese reduction by benthic isolates (Lovley and Phillips 1988; Myers and Nealson 1988). This was a watershed in microbiology because, until that point, biological terminal electron acceptors where though to be soluble species that diffused freely in and out of the cell and that cellular redox reactions occurred intracellularly. The in situ free energy of coupling acetate oxidation to haematite reduction at neutral pH is −41.6 (±12.4) kJ mol^{-1}, much smaller than the $\Delta G'$ determined from standard conditions (−738) meaning that the energetic margins in the deep biosphere are curtailed by prevailing environmental conditions (Wang et al. 2010; Lovley and Phillips 1988; Roden 2003). The reduction of iron and manganese oxides yields sufficient energy to support microbial life allowing organisms that adapt this metabolic strategy to thrive as is evident from the extent of metal oxide reduction in some sedimentary systems which has been reported to account for up to 78% of anaerobic organic carbon degradation (Canfield et al. 1993).

4.3 Extracellular Electron Transfer

4.3.1 Background

The thermodynamic case for biological metal oxide reduction is strong and evidence that several microbial genera can mediate extracellular transfer of electrons from the cytoplasm to a solid acceptor is unequivocal.

Much of what we know about EET in microbes comes from the field of bioelectrochemical systems (Wang et al. 2013; Harnisch and Schröder 2010; Logan et al. 2006; Allen and Bennetto 1993). Bioelectrochemical systems have been a benchtop curiosity for over a century and EET has been applied in microbial fuel cells to recover waste from sewage, in microbial electrosynthesis and, more recently, in bioelectroanalytics (Seviour et al. 2015; Hinks et al. 2016; Kim et al. 2002; Potter 1911).

The first dissimilatory metal oxide reducing isolates, GS-15 reported by Lovley and Phillips (1998) and MR1 reported by Myers and Nealson (1998), were later designated as the novel species *Geobacter metallireducens* and *Shewanella oneidensis* MR1 (formerly *Shewanella putrefaciens* and *Alteromonas putrefaciens*) respectively (Nealson and Myers 1992; Lovley et al. 1993; Venkateswaran et al. 1999). The unusual nature of a solid phase terminal electron acceptor invited speculation as to how the terminal electron acceptor was rendered biologically available. Hypotheses included: (1) direct electron transfer exchange via cellular attachment to the solid phase electron acceptor, (2) a method of solubilising the solid substrate, and a mechanism to transport solid particles into the cell where they could be reduced (Nealson and Myers 1992). The role of membrane bound electron transport chains in carrying out dissimilatory Fe (III) reduction was soon appreciated, but the exact mechanisms were still not completely understood (Gorby and Lovley 1991).

We now know that EET can occur either directly through contact between the microbe and the solid terminal electron acceptor or indirectly through redox carriers called electron shuttles (Gorby et al. 2006; Roller et al. 1984). EET is well documented although certain aspects of direct EET, particularly the role of and conductive nature of nanowires, are still controversial (Yan et al. 2015; Malvankar et al. 2011, 2012; Strycharz-Glaven and Tender 2012). EET may occur through a number of mechanisms and is not limited to *Geobacter spp*. and *Shewanella spp.* but appears to be a relative common phenomenon. However, in this chapter we will focus on EET mechanisms known in *Shewanellaceae* since EET has been well studied in this group. In addition, one of its members, *S. oneidensis*, was the first dissimilatory metal reducing organism whose genome was fully sequenced (Heidelberg et al. 2002).

4.3.2 Mechanisms of EET

Like many organisms, the most efficient energy generation in *Shewanella* is achieved by establishing a proton motive force across the biological membrane during oxidative metabolism. A proton motive force is established by transferring electrons from reduced carriers such as NADH and $FADH_2$, produced during the cytoplasmic catabolism of organic compounds, to the quinone pool. The reduced quinones must be continually reoxidised through a number of plasma-membrane bound electron carriers (quinone dehydrogenases) with sequentially increasing reduction potential in a manner analogous to the electron tower (Richardson et al. 2012). The protons generated during this process are translocated across the membrane and sequestered in the periplasmic space setting up a chemiosmotic gradient that is used in the oxidative phosphorylation of ADP to ATP, with a free energy change proportional to the difference in potential of the quinone oxidase and the terminal oxidase (Richardson et al. 2012; Mitchell 1961). The specific details of generic electron transport chains (ETCs) can be found in any good standard introductory text to microbiology (Willey 2014).

The electron transport chains of *Shewanellacea* and other dissimilatory metal reducers, and indeed many organisms capable of anaerobic respiration, can be highly branched. This means electrons can enter and exit at different points in the ETC effectively permitting interactions with a broad range of electron donors and acceptors with different midpoint reduction potentials. The branched nature of *Shewanella*'s ETC underpins its metabolic versatility as it allows electrons to exit the ETC at different reduction potentials, explaining why the organism can utilise a number of different terminal electron acceptors (Heidelberg et al. 2002).

Extracellular electron transport is achieved in *Shewanella oneidensis* via the metal reduction (Mtr) pathway—a modular, multicomponent protein system that creates an electron conduit between the cytoplasm and the outer membrane to effect the reduction of an extracellular terminal electron acceptor (Fig. 4.2). Electrons from cytoplasmic electron carriers, which have been reduced by cytoplasmic catabolic processes, are captured by menaquinone or ubiquinone and then transferred to CymA, a plasma-membrane bound c-type cytochrome with four haem units. In the periplasmic space, another c-type cytochrome, MtrA, is the next electron carrier in sequence that finally passes the electron to the outer membrane associated c-type cytochrome, MtrC. Both MtrA and MtrC are multi-haem proteins containing 10 haem units. Fully functioning Mtr pathways also contain an additional component, a β-barrel protein (MtrB), which functions as a pore and which ensures the steric accessibility and transmembrane arrangement necessary for MtrA and OmcA to conduct electrons through the outer membrane to directly reduce metal oxides or intermediate shuttling compounds such as a riboflavins (Coursolle and Gralnick 2012).

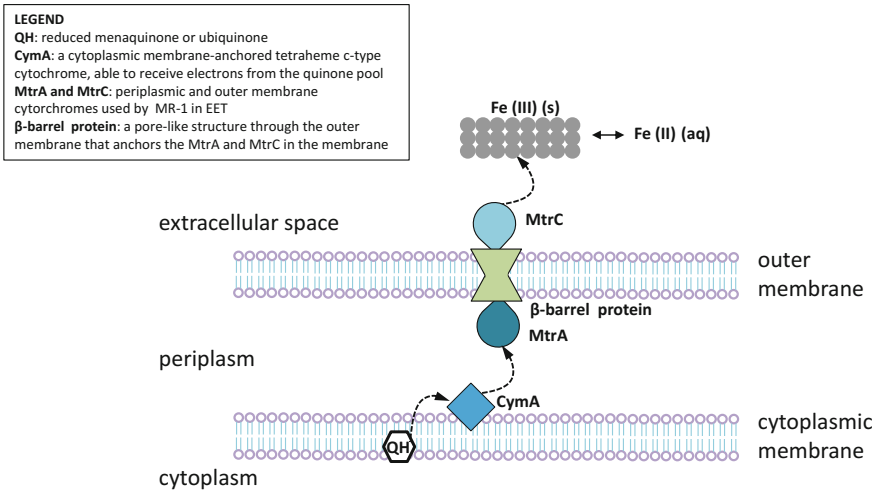

Fig. 4.2 A schematic of the Mtr pathway in Shewanellacae, the conduit through which EET is achieved. Note there are number of paralogs of each of the components in this transmembrane electron conduit

The majority of c-type cytochrome in *S. oneidensis* (80%) is located on the outer membrane; there are at least 39 different multi-haem cytochromes in its genome (although reports have suggested the genome encodes for 42 different multi-haem containing cytochromes) whereas *S. piezotolerans* WP3 reportedly contains 55 c-type cytochromes (Richardson et al. 2012; Heidelberg et al. 2002; Meyer et al. 2004). The outer membrane association of c-type cytochromes is consistent with the concept of EET and the large number of cytochrome variants is consistent with functional redundancy of the classical Mtr pathway described above. Each unit in the Mtr pathway, with the exception of CymA, contains a number of paralogs in many species of *Shewanellacea* (Coursolle and Gralnick 2012). In *S. oneidensis*, there are four paralogs each of both MtrA and MtrB and three of MtrC giving 11 putative Mtr components and 48 possible Mtr pathways (Coursolle and Gralnick 2012). However, only nine pathways have been confirmed to function in Fe (III) reduction (Coursolle and Gralnick 2012). This is because in *S. onedenis*, mutants with Mtr pathways reconstructed from different combinations of MtrABC paralogs, functional Fe (III) reduction was not demonstrated with two of the β-barrels, two of the MtrA paralogs, and for one outer membrane associated MtrC paralog. Given that the Mtr paraglogs are highly conserved in *Shewanella* species, it is likely that they have distinct functions that are yet to be discovered (Coursolle and Gralnick 2012).

The outer membrane associated MtrC has been show to bind ionically to iron oxides in a manner that is dependent on ionic strength and pH. Additionally, MtrC may interact with a number of intermediate electron carriers before depositing an electron to solid oxides (Richardson et al. 2012). Crystallographic analysis of *Shewanellacea* multi-heam cytochromes shows that they have a conserved CX_8C disulphide motif that alters its conformational state in response to redox conditions which in turn governs its binding affinity for riboflavin (Edwards et al. 2015). Experimental studies suggest that under anaerobic conditions, MtrC is configured as an outer-membrane flavocytochrome that is important for Fe (III) reduction, and that upon exposure to oxygen the disulphide bridge reforms, causing riboflavin to dissociate, presumably so that the reductive unit can utilise oxygen (Edwards et al. 2015). In silico reconstruction of the MtrC protein structure showed the spatial arrangement of haems in a 'staggered cross' formation with 4–10 Å intermolecular spacing flanked by β-barrel hydrophobic domains, suggesting that precise spatial arrangement is maintained by an equilibrium between the proteins and the supporting lipid bilayer (Edwards et al. 2015).

Conductive pili play a putative role in long range EET of *Shewanellacea* (Strycharz-Glaven et al. 2011). Whilst the mechanism of electron transport in nanowire based EET is unknown, the nanowires are thought to be an adjunct to the Mtr pathway rather than a separate conductive pathway. Nanowires are thought to directly interface with the outer membrane associated cytochromes and to be rendered conductive through a series of π–π couplings in the aromatic residues of the main structural protein, pilin (Yan et al. 2015). Alternatively, the length of the nanowires may be studded with multi-haem containing cytochromes which conduct electrons, and it has been show that two outer membrane associated deca-haem

c-type cytochromes, MtrC and OmcA are crucial in maintaining nanowire conductivity. However these details and the electron tunnelling mechanism are still the subject of fierce debate (Yan et al. 2015; Strycharz-Glaven et al. 2011; El-Naggar et al. 2010).

EET in *Shewanellacea* is, therefore, achieved through a specific spatial relationship of at least four proteins that are associated with both the plasma and outer membrane as well as a diffusible shuttle such as riboflavin and, putatively, a conductive appendage and possibly bound flavins in the form of outer membrane associated flavoproteins. The primary function of the β-barrel MtrB is thought to be in maintaining the orientation of these proteins with one another and within the lipid bilayer via hydrophobic interactions between the hydrophobic residues in the β-barrel and the surrounding lipid bilayer. Pressure exerts known effects on biomacromolecules, in particular lipids and proteins, yet the pressure effect of this essential respiratory pathway in benthic organisms remains largely unexplored. Before exploring what is known about EET and dissimilatory Fe (III) reduction under pressure, it is necessary to revise general considerations regarding the effect of pressure on biopolymers.

4.3.3 Pressure Effects

Temperature exerts a predictable and monotonic effect on the reaction rates and equilibria in chemical systems. Pressure, on the other hand, can accelerate, inhibit or have a neutral effect on chemical reactions depending on the sign and magnitude of a fundamental parameter, the volume change, which is described by Le Châtelier's principle, when pressure is exerted on a system. The equilibrium of a reaction with a positive volume change is shifted to the left (inhibited) when pressure is exerted whilst reactions that yield a negative volume change will be shifted to the right (facilitated). In the absence of a volume change the equilibrium remains unaffected as does the rate constant for a given reaction, and can be described as follows (Meersman et al. 2013; Abe 2007; Bartlett 1999):

$$K_p = K_1 exp(-P\Delta V/RT) \tag{4.1}$$

$$k_p = k_1 exp(-P\Delta V^{\neq}/RT) \tag{4.2}$$

where K and k represent the equilibrium and rate constants respectively with subscript '1' denoting the constant at atmospheric pressure and subscript 'p' its value at elevated pressure. The volume change established during equilibrium is ΔV and the volume change associated with the formation of the activation products of a given reaction is $\Delta V^{\neq}$, where R is the gas constant and T is the absolute temperature. It is interesting to note that the relationship between pressure and rate constants is exponential, therefore small changes in volume may exert an effect on the

rate constant that is large in magnitude (Bartlett 1999). Volume changes in the order of 20–100 cm^3 mol^{-1} are biologically relevant. A reaction with a 100 cm^3 increase in volume at atmospheric pressure would be inhibited by 35% at 10 MPa and by 99% at 100 MPa. We know that microbes thrive in environments exceeding 100 MPa and must conclude that their biochemistry favours reactions with low volume changes or that they have developed strategies that counter this fundamental thermodynamic constraint.

The implications of pressure effects on various macromolecules has already been covered in Chap. 3 but we will briefly review the implications of pressure for the biochemistry relevant to EET in this chapter, namely: Fe (III) reduction, membrane phospholipids, and membrane proteins.

4.3.4 Fe (III) Reduction

The ionic radius of the more oxidised ferric (Fe (III)) ion is smaller (≈0.64 Å) than the radius of the reduced ferrous ion (≈0.74 Å) the larger ionic radius resulting from the additional electron carried by Fe (II) (Slichter and Drickamer 1972). Le Châtelier's principle therefore predicts that, given the apparent positive ΔV associated with iron reduction, equilibrium favours a larger ratio of Fe (III) speciation to Fe (II), and—assuming that this positive ΔV translates into a positive $\Delta V^{\neq}$—that the rate constant would decrease under increasing hydrostatic pressure. However, the ΔV of various Fe (III)/Fe (II) systems has been experimentally determined to be smaller than expected based on the ionic radii alone. Whilst ΔV for Fe (III)/Fe (II) redox couples is positive in many instances, it is usually less than 10 cm^3 mol^{-1} in aqueous solutions (Giovanelli et al. 2004). While volume changes are invariably positive for dissimilatory Fe (III) reduction under high pressure the ΔV is within the lower range expected for biochemical reactions (Slichter and Drickamer 1972; Sachinidis et al. 1994). The specific ΔV and $\Delta V^{\neq}$ vary depending on the redox couple in question and in many instances of Fe (III)/Fe (II) couples with organic ligands, the observed ΔV is negative, which points towards strategies to counter thermodynamic constraints at high pressure. Given the multicomponent nature of EET pathways as described above, predicting volume changes at pressure is complicated. To date no sufficiently quantitative treatment of EET volume changes at high pressure has been experimentally determined and therefore the response of the ETC to increasing pressure and therefore the thermodynamics of the system in benthic environments is not well described. In studies conducted over a lithostatic gradient of 900 m (between 25 and 28 MPa) the ΔV was considered as a constant in determining ΔG although modelling studies have shown that of all possible microbial respiratory strategies, Fe (III) reduction was the only one when which became energetically more favourable with increasing hydrostatic pressure (Macdonald 1997; Fang et al. 2010; Fang and Bazylinski 2008). However, the problem is sufficiently multifactorial and situation depended that it would benefit

from experimental clarification that will shed light on the energetics of these processes under environmentally relevant conditions and for a range of organisms.

4.3.5 Lipids

By virtue of their ability to maintain the cell membrane in the homeoviscous state necessary for biological function across a range of physicochemical conditions, lipids are the most compressible of the common biopolymers. Hence their pressure response is large (Daniel et al. 2006). When subject to increased pressure, lipids undergo an increase in order (a more closely packed and linear arrangement) and a decrease in rotational motion which is manifest by a phase transition from the fluid-like liquid crystalline mesophase to a gel state (Winter and Jeworrek 2009). As a general rule, many biologically relevant lipids respond to pressure in a similar way as they do to temperature and a 100 MPa increase in pressure is equivalent to an 18–21 °C drop in temperature (Bartlett 1999; Watanabe et al. 2009). Thus at a depth of 10 km and a temperature of 3 °C, the lipid mesophase state will be equivalent to that predicted at −15 to 18 °C under ambient pressure conditions.

The increase in lipid order brought about by pressure means the membrane tends toward the solid gel phase at elevated pressures and microbes respond by increasing the proportion of both mono-unsaturated and poly-unsaturated fatty acids in their membranes at elevated pressures (Bartlett 1999). The double bonds in the acyl chain are associated with a 30° bond angle which increases both the apparent volume and the packing parameter of the phospholipid resulting in less membrane order and an increased tendency towards liquid crystalline mesophases. The liquid crystalline to gel transition temperature of mono-unsaturated and saturated fatty acids observed in the pressure response of microbes is low. The transition temperature for stearoyl-arachidonoylphosphatidylcholine, for example, is −13 °C and will therefore remain in a physiologically relevant gel mesophase in deep sea conditions (Daniel et al. 2006; Usui et al. 2012). Additional lipid based adaptations include increased fatty acid chain length and branching which, in combination, serve to counter the pressure related tendency towards increased ordering of the acyl chains (Bartlett 2002). Additionally, hopanoids have been postulated to extend the liquid crystalline to gel transition of lipid mixtures over a pressure gradient and are analogous in function to cholesterol found in eukaryotic membranes (Daniel et al. 2006).

It has been shown that various *Shewanellacea* respond to pressure by increasing the proportion of eicosapentaenoic acid in their membranes (Usui et al. 2012; Sato et al. 2008). Eicosapentaenoic acid is a 20 carbon polyunsaturated fatty acid with five double bonds (C20:5) and was shown to be critical for maintaining membrane fluidity close to that expected at atmospheric pressure across a pressure gradient. However, it is not fully understood whether fluidity must be globally maintained across the membrane or only in localised regions, called rafts, to maintain biological function (Usui et al. 2012).

4.3.6 Proteins

Pressure has a tendency to denature proteins in a specific way compared to temperature. Elevated pressure favours hydrogen bonding compared to hydrophobic interactions. Hence pressure denatured proteins exhibit a globular conformation compared to the completely unravelled state that occurs upon temperature denaturation. Pressure induced protein denaturation is, therefore, thought to occur through incursion of water into the protein interior because of impaired hydrophobic interactions and results in preferentially denatured secondary structures like β-sheets and α-helices. The volume change for protein unfolding is estimated to be in the region of $\Delta V = -80\ cm^3\ mol^{-1}$ indicating their tendency to unfold at elevated pressure (Daniel et al. 2006). Monomeric proteins are more stable than oligomeric proteins and typically undergo unfolding at 200 MPa compared to 100 MPa for many multimeric proteins (Daniel et al. 2006; Bartlett 1999). However, solute effect plays a major role in membrane stability and pressure adapted organisms have been shown to modulate the solute concentration of their membranes in such a manner that ΔV becomes independent of pressure over a physiologically relevant range (Daniel et al. 2006). Cytochromes undergo a negative volume change upon oxidation and therefore their tendency to reduce an electron acceptor would be favoured at high pressure (Giovanelli et al. 2004). Studies with horse heart cytochrome c determined ΔV to be $-24\ cm^3\ mol^{-1}$ accompanied by a positive shift in midpoint reduction potential of around 25 mV (Cruanes et al. 1992). Incidentally, the redox activity of cytochrome c could be maintained at 500 MPa suggesting that the tertiary protein structure may not be pressure sensitive (Cruanes et al. 1992).

Hydrophobic interactions are important in governing the spatial arrangement and stability of membrane inserted components including proteins like c-type cytochromes (Hinks et al. 2014, 2015). Molecular insertions into the membrane have been shown to alter membrane fluidity and microbes will respond to hydrophobic mismatch by altering their fatty acid profile. By extension, a pressure induced change in, for example, acyl chain length would result in a hydrophobic mismatch for proteins whose hydrophobic secondary structure usually results in a stable spatial configuration at atmospheric pressure such as that described for the Mtr pathway (Fig. 4.2). Positive mismatch (where the inserted component has a hydrophobic region that is greater than the thickness of the membrane), between a membrane protein and the cell membrane can be well tolerated and is achieved by a small rotation around the protein axis, effectively shortening its length, so that it fits obliquely into the bilayer (Hinks et al. 2015; Strandberg et al. 2012). Instances of negative mismatch, when the transmembrane domain is shorter than the lipid acyl chains, can induce membrane thinning and a tendency towards excessive membrane disorder or poration.

As described above, microbes respond to increased pressure by increasing the proportion of long chain fatty acids in their membranes. As a result, negative mismatch between the hydrophobic transmembrane regions that are stable at

atmospheric pressure and the lengthened fatty acids chains in the pressure adapted membrane will occur (Bartlett 2002). Indeed, the archetypal piezotolerant organism, *Photobacterium profundum* SS9, differentially expresses two outer membrane porins: OmpL is important at low pressure whereas OmpH is expressed preferentially at elevated pressure (Bartlett 1999). Neither OmpH nor OmpL are crucial for growth but it has been experimentally determined that OmpH has a larger transmembrane channel than OmpL (Bartlett 1999).

The ΔV determined for various reduction processes was shown to be highly dependent on the ordering of the supporting lipid bilayer in a model system because of its effect on the spatial arrangement of the redox active moieties supported within it (Cruanes et al. 1995). This means that steric considerations can also influence the thermodynamics of electron transfer processes under pressure further reinforcing the need for precise arrangement of the Mtr components within the membrane.

4.4 Implication of Biochemical Pressure Effects on EET Apparatus in *Shewanella*

As discussed earlier, there is speculation that the branched nature of the *Shewanella* EET chain is configured for it to respond quickly to changing conditions thus conferring a survival advantage in both stratified and dynamic environments (Coursolle and Gralnick 2012). This branching of the EET is loaded towards the terminal end, meaning that *Shewanellaceae* exhibit relative metabolic diversity in terms of electron acceptors but not electron donors when compared with many other species. Indeed the absence of paralogs of the membrane bound quinone oxidoreductase, CymA, means that the branched ETC in *Shewanellacea*, and therefore a choice of terminal electron acceptors, can be regulated by just this single protein (Coursolle and Gralnick 2012). In contrast, *E. coli* has up to eight paralogs of the CymA equivalent, NapC. This difference is in agreement with the notion of *Shewanellaceae* being biochemically equipped to respond quickly to changing environmental conditions (Jensen et al. 2010). The suggested advantage of the redox active motif in MtrC that forms a flavoprotein complex under anaerobic conditions is that it could allow rapid transition from an aerobic to an anaerobic lifestyle pointing towards transcriptionally independent ways of modulating redox pathways (Edwards et al. 2015).

Under high pressure, we can infer a negative volume change occurring when the outer membrane cytochromes become oxidised upon reducing Fe (III); therefore this reaction is likely to be stimulated by high pressure (Fig. 4.3). The ΔV for mammalian c-type cytochrome oxidation is around $-27\ cm^3\ mol^{-1}$ at ambient pressure and around $-23\ cm^3\ mol^{-1}$ at 100 MPa. Taking into account the ΔV for Fe (III) reduction of around $10\ cm^3\ mol^{-1}$, it likely that the net ΔV for biological Fe (III) reduction via c-type cytochromes is negative. This is because of the larger negative volume change associated with cytochrome oxidation relative to that of the iron reduction (Fig. 4.3). Experimental data is lacking to make this assertion with

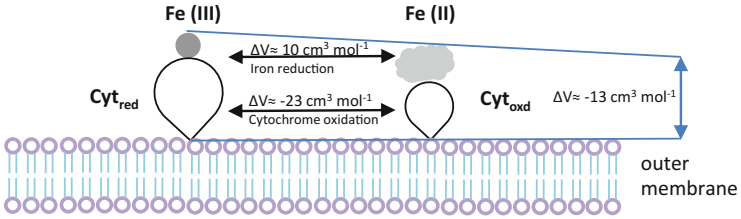

Fig. 4.3 Theoretical volume changes relevant to microbial Fe (III) reduction. The oxidation of cytochrome c can reasonably be assumed to undergo a negative volume change upon releasing an electron to Fe(III). Conversely, upon accepting an electron Fe(III) undergoes a positive volume change. Overall, the terminal step in EET undergoes a negative volume change negative because ΔV for cytochrome c has a greater magnitude than that of Fe(III) reduction

confidence although it appears to be supported by at least one modelling study of benthic environments (Fang et al. 2010). Assuming that a similar pressure-induced positive shift in midpoint redox potential as observed for mammalian cytochrome c also occurs in the membrane bound cytochromes of *Shewanellacea*, then more energy would be available to cells respiring Fe (III) under pressure. This is because the energy available to the cell is proportional to the difference in redox potential of the substrate and that of the terminal cytochrome. However, since the pressure response of the redox potential of Fe (III) minerals will more stable than cytochromes the electron transfer rate will slow as the redox potential of the cytochrome and the Fe (III) terminal electron acceptor eventually converge. Precise midpoint potentials are difficult to assign to Mtr cytochromes since the multiple redox centres mean they are active over a redox potential spanning 250 mV. Assuming the pressure response in terms of redox potential of *Shewanellaceae* cytochrome c is similar to that observed in mammalian equivalents (a positive shift of 20 mV at 100 MPa) (Giovanelli et al. 2004) we can infer that pressure is not likely to have a large effect on the kinetics of Fe (III) reduction at high pressure.

However, changes in membrane composition and, to a lesser degree, conformational protein changes would be expected to disrupt the precise spatial arrangements of the Mtr pathway observed at ambient conditions. This spatial disruption could be overcome biochemically by the maintenance of lipid raft arrangements—localised areas of lipids having a specific, an presumably desirable, fluidity that favour a particular biochemical function (Usui et al. 2012). It could also be the case that the spatial arrangement is inconsequential or even favourable with a subtle shift bringing another of the many redox-active haem centres into service. Such passive ways of responding to pressure are consistent with the idea put forward by Edwards et al. (Edwards et al. 2015) of a similar passive cytochrome based response of *Shewanella* to changing oxygen concentrations. This does not explain the conserved functional redundancy observed in the Mtr pathway however and the need for numerous paralogs for each component of the Mtr pathway (Edwards et al. 2015).

Indeed, changes in pressure may not happen as quickly as changes in the redox conditions in the deep biosphere so a rapid coordinated response to pressure may

not be necessary. Based on documented changes in fatty acid expression in *Shewanella*, the spatial hydrophobic distribution of the membrane at elevated pressure would be expected to be different to that at ambient conditions. Evidence for pressure adapted porins in *P. profundum* SS9 exists, a response that may be to maintain a desirable spatial arrangement of the membrane inserted porin. The β-barrel paralogs in *S. oneidensis* MR1 differ in predicted size by around 55 amino acid residues. The two β-barrel paralogs that have demonstrated functionality in the Fe (III) reducing unit of *S. oneidensis*, MtrC and MtrE, are slightly larger (687 and 712 a.a respectively) than dmsF (662 a.a) and SO4359 (652 a.a)—the two putative β-barrel paralogs with hitherto unknown function in *S. oneidensis* MR1. It may be that these paralogs are expressed under pressure and that they have an organisational function in pressurised Mtr pathways. Chikuma et al. (2007) observed a high pressure respiratory component in *S. violacea* that may be membrane dependent (Chikuma et al. 2007). A systematic transcriptional analysis of *Shewanellacea* under pressure would be immensely useful in exploring such phenomena.

An additional aspect of the pressure response of EET concerns the expression of nanowires, which are thought to be membrane extruded pilus type structures (Pirbadian et al. 2015). Flagella expression is particularly sensitive to pressure and can be inhibited at pressures as low as 10 MPa. Drawing once again on observations of *P. profundum* SS9 which expresses a pressure dependent lateral flagella under elevated pressure and a polar flagellum under ambient conditions, it is likely that cell appendages exhibit specific pressure adaptations. The same lateral flagellar arrangement as on SS9 has been observed on *Shewanella benthica* DB21m2-2 (Fang et al. 2010). Pili expression has not been studied under high pressure but owing to the superficial structural similarities of type IV pili to some flagella it may be that this appendage requires specific pressure adaptations too. Recent evidence suggests that conductive nanowires in *Shewanellaceae* are not proteinaceous pili or flagella like appendages as previously thought, but rather extensions of the membrane (Pirbadian et al. 2015; Malvankar et al. 2015). To date, all studies on the conductive nature of bacterial nanowires have been under atmospheric pressure.

Finally, an unrelated high pressure study of a conductive film determined the ΔV to be negative for electron hopping conductivity (of the type that us which is thought to occur between adjacent carriers of sequentially increasing potential) and that diffusive conductivity was determined to have a positive activation volume suggesting that electron hoping would be the preferred conductive mechanism at high pressures. Again, high pressure studies may prove useful to experimentally describe mechanisms of electron transfer at high pressures (Cruanes 1995).

4.5 Experimental Studies of EET Under High Pressure

Few studies have been conducted on EET at high pressure. A study by Wu et al. (2013) with *S. piezotolerans* at pressures ranging from 0.1 to 50 MPa showed that the Fe (III) reduction rate decreased with pressure in a linear fashion and predicted

that Fe (III) reduction would terminate at 68 MPa (Wu et al. 2013). Picard et al. (2011) have shown a similar trend for Se (IV) and Fe (III) reduction at high pressures with *S. oneidensis* MR1; these reactions appeared to terminate at 150 MPa for Se (IV) and at 100 MPa for Fe (III) respectively (Picard et al. 2011, 2012). Additionally, a Fe (III) reduction rate maxima at 30-40 MPa was observed. In a similar study but with S. *profunda* LT13a Picard et al. (2015) showed Fe (III) reduction proceeding until about 110 MPa although the study was carried out using two techniques each with different starting inoculum densities (Picard et al. 2012). In one experiment with a 'low' staring inoculum (10^8 CFU ml^{-1}) a linear decrease in Fe (III) reduction rate was observed which terminated above 50 MPa. With an higher initial cell density (10^9 CFU ml^{-1}), the Fe (III) reduction rate did not appear linear but was instead maintained in steps between 0–40 and 60–80 MPa and continued to around 100 MPa (Picard et al. 2014). This stepping is consistent with different Mtr modules being deployed over a range of pressures; this hypothesis could be tested by repeating the experiment and extending it with transcriptomic data.

These pioneering experiments have shown the magnitude and limits of Fe (III) reduction at elevated pressure and will undoubtedly be improved in future iterations with more mindful consideration of thermodynamic considerations and with more sophisticated experimental design. One of the main problems with these data is, on account of their rarity, there is little to compare findings with and the absence of standardisation between techniques. Accordingly, the observed reduction rates, for Fe (III) at least, vary over an order of magnitude from between c.a. 80–1500 μMol h^{-1}. Additionally, for each of these experiments, it is very difficult to decouple the effect of pressure induced killing from the observed reduction rates and relate them to in situ conditions. This is due to experimental constraints and the fact that the measurements relied on extremely high inoculum densities of between 10^7 and 10^9 CFU ml^{-1} and therefore growth would not be expected in these circumstances and Fe (III) reduction could therefore only really be correlated with cell maintenance and death. In all experiments, dissimilatory metal reduction proceeded without any delay. This observation was explained by the fact that the respiratory chain was not affected by pressure. However, evidence suggests that the respiratory chains of *Shewanellaceae* are pressure sensitive and that a genetic response would be expected over the experimental pressure ranges reported (Bartlett 2002; Chikuma et al. 2007). It remains to be seen if the application of pressure to actively growing cultures would yield different Fe (III) reduction phenomena in the *Shewanellacea* family.

4.6 Horizons in the Study of High Pressure EET

Apart from the cumbersome nature and expense of high pressure equipment, one of the main problems in traditional high pressure microbiology is the difficulty of continuously monitoring cultures. To access samples grown in traditional pressure

vessels to perform measurements, the pressure vessel needs to be depressurized and opened periodically (Picard et al. 2011). This is less than ideal, as it introduces at least two unknowns into the system: (1) what happens upon depressurisation until the time of the measurement, and (2) what would the state of system be at a given point had it not been subject to continual depressurisation and repressurization events during the experimental run (Picard et al. 2011). Given enough pressure vessels individual vessels can simply be sacrificed at each time point, which while helpful, offers only a partial solution to these issues (Wu et al. 2013) (Fig. 4.4).

Two main experimental systems have been reported which allow direct measurements on pressurised cells, the diamond anvil system and an autoclave system. Both are optically accessible, the diamond anvil system by virtue of the natural properties of diamond and the autoclave system because it can be fitted with a beryllium window (Picard et al. 2011). The technique for assessing these systems has been based on X-ray Absorption Near-Edge Structure (XANES) spectroscopy, which although immensely useful, has some drawbacks. XANES requires use of a synchrotron, and beam-time is expensive. Furthermore, the radiation energy that is generated using XANES can kill bacteria, which means that continuous monitoring is not advisable. Both the diamond anvil and the autoclave system have limited experimental volumes, in the low nL and mL range respectively (Picard et al. 2014).

A recent advance at the Singapore Centre for Environmental Life Sciences Engineering that will likely prove essential in high pressure microbiology studies, in particular to study high pressure EET, is the development of a high pressure bioelectroanalytical system.

High pressure electrochemistry is a century old field, high pressure bioelectroanalytical systems are built on this experience (Giovanelli et al. 2004). They combine a compressible bioelectrochemical cell that is simply put inside a

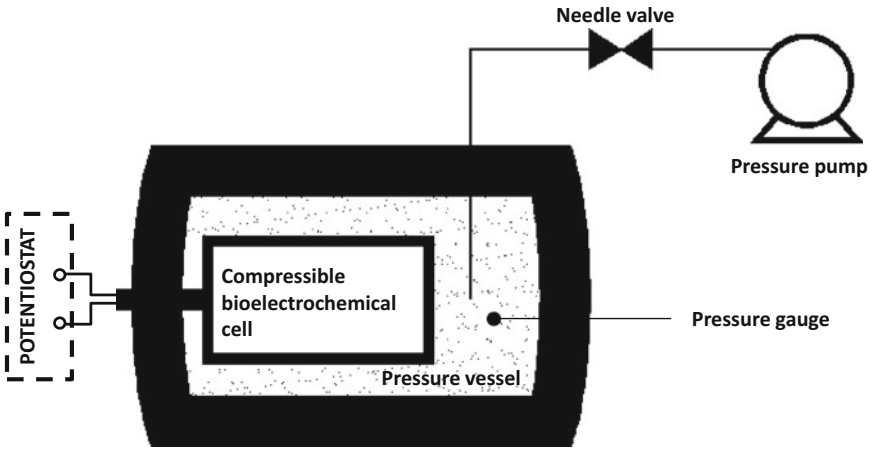

Fig. 4.4 A schematic of a high pressure bioelectrochemical reactor

traditional pressure vessel with wires passing through sealing glands in the pressure vessel wall. The compressible bioelectroanalytical cell contains either two or three electrodes and can be connected to a potentiostat, a power source or a data logger. Potentiostatic control poises the electrodes at a given potential allowing a number of amperometric, voltammetric or impedimetric analyses to be performed. Early experiments have already demonstrated a shift in redox potential at high pressure that is thought to be related to outer membrane cytochromes.

High pressure bioelectroanalytical reactors have several advantages over existing techniques. Firstly, they allow continuous, real time monitoring of microbes over durations only limited by an experimenter's budget. Several quantitative techniques, such as cyclic voltammetry or differential pulse voltammetry, can be applied in situ. The reactors are sufficiently low cost that replication can be achieved and they are simple enough they can be used in both batch and continuous flow pressure systems (Foustoukos and Pérez-Rodríguez 2015). Finally, volumes up to one litre are practical for most laboratories allowing predetermined volumes to be collected for ex situ analyses like proteomics and lipidomics. As well as fundamental questions about EET, the reactors could monitor growth and other microbial phenomena continuously and in real-time, not just of electrogenic taxa like *Shewanellaceae* or *Geobacteraceae* but, with the careful use of redox mediators, other genera such as *Photobacterium profundum*. Over the coming years these systems will be applied to explore the following:

(1) The nature of conductive nanowires and the mechanism of conductivity at high pressures
(2) The state of the membrane at high pressure using lipidomic techniques
(3) The transcriptomic response of EET under pressure including differential expression of the components of the Mtr pathway
(4) The volume changes of biologically important reactions such as Fe (III) reduction
(5) Data for a full thermodynamic analysis of EET systems under environmentally relevant conditions
(6) Performance of microbial fuel cells at high pressure
(7) High pressure biocorrosion in the deep sea.

4.7 Conclusions

There are a number of open questions in the field of microbial EET. With the development of new tools, some of which are at a stage where they can be productively used, we are now in a position to address these questions. This will give not only more insight in the effect of (high) pressure on the ecophysiology of bacteria and archaea in the deep subsurface in general and of EET in particular, but also result in a better understanding of EET at atmospheric pressure conditions too.

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