6

Genetically modified microorganisms for bioelectrochemical systems

Miriam Rosenbaum and Largus T. Angenent

6.1 INTRODUCTION

Biotechnological utilization of microbial processes normally starts with the discovery, investigation, and understanding of naturally occurring microbial reactions. Biomolecular tools can help us, not only to gain better understanding of protein reactions; they also enable us to influence reaction properties. For efficient practical applications, modern biological techniques allow us to tailor these microbial reactions to optimize the desired functionality.

We are still in the infancy of our understanding of microbial reactions in microbial fuel cells (MFCs), or more generally in bioelectrochemical systems (BES). One major pathway of electron transfer to a MFC anode is the direct

 Bioelectrochemical Systems: From Extracellular Electron Transfer to Biotechnological Application. Edited by Korneel Rabaey, Largus Angenent, Uwe Schröder and Jürg Keller. ISBN: 9781843392330. Published by IWA Publishing, London, UK.

extracellular electron transfer by means of cell membrane bound redox-proteins. Thus far, *Shewanella oneidensis* and *Geobacter sulfurreducens* have been extensively studied for extracellular respiration with an anode. Both are known for their ability to respire with solid extracellular electron acceptors, such as mineral oxides of Fe, Mn, and U, and are model representatives of the group of dissimilatory metal-reducing bacteria (DMRB) (Nealson *et al.* 2002; Weber *et al.* 2006). Common to those organisms is a chain of redox-proteins (mostly cytochrome *c*-type) to conduct electrons across the cell envelope. The true pathway of the electrons and the importance of specific proteins is still under investigation, but researchers have identified strong suspects that may be involved in this respiration process. However, a genomic screen for both species shows a vast number of coded *c*-type cytochromes, which hinder standard biomolecular knock-out studies to reveal the function of certain proteins. The possibility of expressing the most promising assembly of *c*-type cytochromes in a heterologous host (*Escherichia coli*) will be discussed with regard to both scientific (investigative) and technological (improvement) interest.

Biomolecular techniques help us clarify the essential electron transfer reaction steps; the first approaches are under way to engineer and design microorganisms that can utilize complex fuels and convert them into electricity in much more efficient ways. This chapter will discuss recent work on the investigation and improvement of these extracellular respiration reactions using genetic tools and give an outlook of what may be possible in the near future.

6.2 EXTRACELLULAR RESPIRATION IN *SHEWANELLA ONEIDENSIS* AND *GEOBACTER SULFURREDUCENS*

Shewanella or *Geobacter* spp. respiration with extracellular electron acceptors requires the transport of catabolic electrons across the cell envelope. Proposed electron transfer pathways for both organisms are shown in Figure 6.1. Common to extracellular respiration and aerobic or other anaerobic respiration pathways is reoxidation of NAD(P)H by cell-membrane bound dehydrogenases. Protons are pumped over the cell membrane to generate a proton motive force for subsequent ATP generation, while electrons are fed into a quinone/menaquinone pool located in the cell membrane. For both species, the subsequent steps of the electron pathway are not yet completely proven, and several possible pathways are discussed in the literature. As a first step in this chapter, we will reflect on the most probable reaction steps.

In *S. oneidensis*, cell membrane bound CymA, which is a tetraheme cytochrome, takes up electrons from the quinone/menaquinone pool to reduce

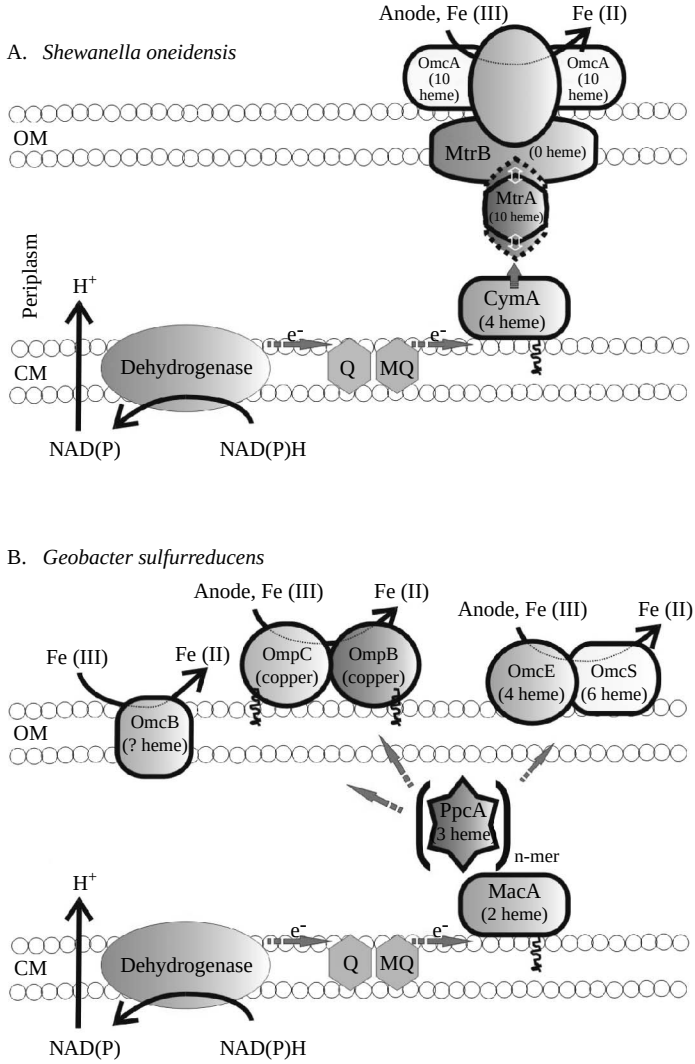

Figure 6.1. Proposed electron transfer steps for extracellular respiration with mineral oxides or MFC anodes in *Shewanella oneidensis* MR-1 (A) and *Geobacter sulfurreducens* (B). OM – outer membrane, CM – cell (inner) membrane, Q and MQ – quinone/menaquinone pool; all other proteins are discussed in the main text

periplasmic reductases (Myers and Myers 2000) (Figure 6.1A). It is involved in a broad range of respiration reactions, for example with Fe(III), Mn(IV), nitrate, nitrite, fumarate, and DMSO (Gescher *et al.* 2008; Murphy and Saltikov 2007; Myers and Myers 1997; Schwalb *et al.* 2002, 2003). From this point on, the electron transport chain for extracellular respiration is uncoupled from proton transport across the inner membrane. CymA reduces the periplasmic decaheme protein MtrA. MtrA can act as a terminal reductase to reduce soluble Fe(III) (e.g. Fe(III)-citrate), and it most likely functions as a linking protein in the periplasm to shuttle electrons between CymA at the inner membrane and outer-membrane proteins MtrB and MtrC (Pitts *et al.* 2003). Very recent studies have shown that MtrA is weakly associated with MtrB, and thus anchored to the periplasmic side of the outer membrane (Ross *et al.* 2007). Interaction and complex formation studies also revealed a strong complex being formed between the noncytochrome *c* protein MtrB and decaheme cytochrome MtrC (Ross *et al.* 2007). MtrC (also called OmcB), on the other hand, has been found to form a complex with an OmcA dimer (Shi *et al.* 2006). MtrC and OmcA have been proven to be terminal reductases that are crucial for the reduction of insoluble iron (hydr)oxides and electron transfer to MFC electrodes (Beliaev *et al.* 2001; Bretschger *et al.* 2007; Hartshorne *et al.* 2007; Myers and Myers 2001; 2003). MtrB is crucial for the correct localization of MtrC and OmcA in the outer membrane (Myers and Myers 2002). This information, together with the complex formation studies, suggests that non-cytochrome MtrB forms a sleeve around MtrA and MtrC and enables the electron transfer (or tunnelling) from periplasmic MtrA to the outer membrane MtrC, while MtrC either directly reduces external electron acceptors or passes the electrons on to OmcA (Figure 6.1A).

In *Geobacter sulfurreducens*, MacA, an inner membrane anchored diheme cytochrome, receives electrons from the quinone/menaquinone pool (Butler *et al.* 2004; Kim and Lovley 2008) (Figure 6.1B). MacA reduces the periplasmic triheme cytochrome PpcA (Lloyd *et al.* 2003). While in *S. oneidensis* the electron transfer is most probably uncoupled from proton transfer beyond the reactions in the inner membrane, Pessanha *et al.* (2006) have shown that PpcA has the potential to contribute to the cells' proton motive force, and thus energy (ATP) generation by transferring electrons coupled to trans-membrane proton transport. It was proposed that PpcA polymerizes in a chain-like conformation to conduct electrons as a periplasmic 'nanowire' from the inner membrane to outer membrane reductases (Pokkuluri *et al.* 2004). Several different terminal reductases are crucial for either metal oxide reduction only, or electron transfer to metal oxides and anode electrodes. Thus, the cytochromes OmcE and OmcS with four and six heme groups, respectively, which are located on the outside of

the outer membrane of *G. sulfurreducens,* seem to be essential for respiring when using a MFC anode as the terminal electron acceptor (Holmes *et al.* 2006; Mehta *et al.* 2005). Both, also are necessary for efficient reduction of Fe(III) (hydr)oxides. On the other hand, a deletion mutation of outer membrane cytochrome OmcB only affects the reduction of extracellular Fe(III) and not the electron transfer to an anode (Holmes *et al.* 2006; Leang *et al.* 2003). Another pair of reductases, which are even less understood but apparently important, are multi-copper proteins OmpC and OmpB (both being crucial for Fe(III) reduction and electron transfer to anodes) (Holmes *et al.* 2008). Thus, the specific protein functions can be very different and leave much room for investigation.

6.3 SCIENTIFIC MOTIVATION FOR HETEROLOGOUS GENE EXPRESSION

The genomes of *S. oneidensis* MR-1 and *G. sulfurreducens* PCA have been completely sequenced and are available to the public (see reference to Comprehensive Microbial Resources). A genome wide search for both organisms predicts a total of 42 and 111 membrane-associated *c*-type cytochromes for *S. oneidensis* and *G. sulfurreducens,* respectively (Methe *et al.* 2003; Meyer *et al.* 2004). The high numbers of proteins involved in potential anaerobic respiration processes, make functional studies within these organisms very complicated. The enzymatic pathways presented in Section 6.2 are not completely proven. One reason being that deletion mutations often do not lead to an overall loss of function in the mutant because other cytochromes take over and allow the reaction to happen. It is also becoming more and more obvious that results we gain from iron or manganese oxide reduction experiments are not necessarily similar for electron transfer to anodes. For example, Bretschger *et al.* (2007) showed that *S. oneidensis* knock-out mutations of the outer membrane reductases OmcA/MtrC only repress iron oxide reduction and electron transfer rates to the anode, but not manganese oxide reduction rates; while knock-outs of periplasmic proteins MtrA and MtrB limit the ability to reduce all three types of electron acceptors. Obviously, some proteins participate in a broad range of enzymatic pathways, while others are very specific for certain reactions.

Knock-out studies also demonstrate that *c*-type cytochromes, which are crucial for Fe(III) reduction or electron transfer to anodes (Figure 6.1), often require the expression of other cytochromes that are not directly involved in the respiration reaction to allow full functionality. For example, *G. sulfurreducens* *c*-type cytochromes OmcG and OmcH are hypothesized to post-translationally

affect OmcB. As mentioned earlier, OmcB has been proven to be essential for Fe(III) reduction in *G. sulfurreducens*, and therefore knocking out OmcG and OmcH will reduce reduction rates indirectly (Kim *et al.* 2006). In addition, the small outer membrane cytochrome OmcF from *G. sulfurreducens* influences expression levels of essential cytochromes for Fe(III) reduction: in an OmcF-deficient strain no transcripts of OmcB could be detected, while OmcS was upregulated (Kim *et al.* 2005). Recently, a possibly similar function of expression control of OmcB, and thus indirect involvement in the reduction reaction, has also been described for MacA, which is a protein that had been considered a key-player in the electron transfer chain of *G. sulfurreducens* (Kim and Lovley 2008).

A number of nonspecific proteins are essential to ensure proper post-translational folding and membrane localization of the terminal reductases. First, the respiratory proteins require transport over the inner membrane of the bacteria by the evolutionary conserved Sec pathway (most proteins designated for export leave the cytoplasm via this translocation system). Second, the proteins mature with the help of cytochrome *c* maturation complexes, which assist the correct attachment of heme molecules to the proteins within the periplasmic space (Feissner *et al.* 2006). Third, if necessary, they are then transported over the outer membrane, which is most probably facilitated by the Type II secretion system (Shi *et al.* 2008).

Overall, due to the large number of *c*-type cytochromes and the special processing requirements of the proteins, electron transfer reactions to extracellular electron acceptors in *S. oneidensis* and *G. sulfurreducens* are very complex, which makes functional studies of individual enzymes difficult.

One way to study the minimal enzymatic requirement for respiration with extracellular electron acceptors is the heterologous expression of candidate genes in a host strain without the machinery to reduce external electron acceptors. Genetic engineering star organism *E. coli* is perfectly suitable for this task, because the wild-type strain has no intrinsic ability to reduce external electron acceptors and does not posses outer membrane *c*-type cytochromes. More importantly, a broad range of biomolecular tools are already available for manipulating and screening *E. coli* cell lines. The genes of potential enzyme candidates can be transferred into *E. coli* and studied for expression, folding, localization, and *in-vitro* functionality. Via step by step addition of the most promising proteins into the heterologous host, we may be able to build the minimum chain of proteins that will allow *E. coli* to respire with extracellular terminal electron acceptors. Once this minimal required protein chain is established in one *E. coli* working cell line, the protein expression can be tuned and optimized to maximize product yield (current production). The gene

assembly can then also be transferred into a broad variety of available *E. coli* cell lines that have been optimized for certain purposes, such as specific substrate degradation or product formation to broaden the application range of these new highly productive strains.

Although our toolkit for genetic engineering in *E. coli* is tremendous, our existing techniques to delegate post-translational modifications are still very scarce, making such a research effort difficult. Membrane-associated or secreted proteins often require extensive post-translational maturation to become functional. It is a scientific challenge to express one membrane protein in a heterologous host while ensuring correct maturation and localization. Expressing a series of proteins, which all need to be functionalized in very specific spaces within the cell envelop and, in addition, to allow functional interaction between the individual proteins, is an even greater task.

6.4 METHODS AND CHALLENGES FOR HETEROLOGOUS GENE EXPRESSION IN *E. COLI*

As mentioned in the last section, the scientific motivation for heterologous expression of *c*-type cytochromes is considerable. Following from this scientific interest, some first attempts to express cytochromes from both organisms – *S. oneidensis* and *G. sulfurreducens* – in *E. coli* have been published. The strategies and successes of the different approaches will be reflected in this section.

One general requirement for the functional expression of *c*-type cytochromes is the successful attachment of heme with the help of a cytochrome *c* biogenesis system in the periplasm. In the microbial world this maturation process is realized by either System I (α- and γ-proteobacteria and archea) or System II (gram-positive bacteria, ε- and β-proteobacteria and cyanobacteria) (Kranz *et al.* 1998; Thony-Meyer 1997). *E. coli*'s own cytochrome *c* maturation complex System I (coded by the gene cluster *ccmABDCEFGH*) is not expressed under aerobic conditions and, under normal expression rates, most probably would also not have the capacity to allow the maturation of several multi-heme cytochromes (up to 12 heme molecules per cytochrome). Successful over-expression of System I and System II in *E. coli* has been shown before (Arslan *et al.* 1998; Feissner *et al.* 2006) and co-expression with the target cytochrome *c* is regarded as an important requirement for the heterologous expression of multi-heme cytochromes (Herbaud *et al.* 2000).

To the best of our knowledge, only individual recombinant cytochrome *c* expressions in *E. coli* have been published, indicating how challenging and complex the functional expression of cytochrome *c* assemblies is.

For *S. oneidensis* the expression of the following cytochromes has been studied:

MtrA – was the first *Shewanella* cytochrome *c* to be heterologously expressed in *E. coli* (Pitts *et al.* 2003). The protein was correctly targeted to the periplasm and matured with System I cytochrome *c* assembly complex, which was co-expressed from the pEC86 vector (Arslan *et al.* 1998). Analysis with magnetic circular dichroism gave evidence that all ten heme molecules were covalently attached. *In-vivo* functional assays showed that MtrA was able to receive metabolic electrons from the inner membrane and reduce the soluble electron acceptor Fe(III)-NTA in the periplasm or donate electrons to other host cell oxidoreductases (e.g. the nitrite reductase NafA).

CymA – was cloned and expressed in *E. coli* by Gescher *et al.* (2008) using a pBAD202 vector and likewise co-expressed with the pEC86 plasmid containing the *E. coli* System I cytochrome *c* maturation genes. The protein was functionally expressed in a Δ*napCDEF* mutant as periplasmic NapC could perform similarly to CymA. The CymA-expressing strain was able to grow on non-fermentable glycerol and with Fe(III)-nitrilotriacetic acid (Fe(III)-NTA) as soluble terminal electron acceptor, showing that CymA itself is not only a electron-mediating enzyme, but also has terminal reductase activity. However, *in-vivo* respiration could only be shown for Fe(III)-NTA. Other soluble electron acceptors that can be reduced by *S. oneidensis,* such as AQDS, could not be used.

OmcA – was co-expressed with pEC86 in two *E. coli* cell lines: a K12 derivative and a B-type strain (Donald *et al.* 2008). Both strains produced comparable amounts of OmcA, which was located in inner and outer membrane fractions, but not in the soluble fraction (cytoplasm and periplasm). In both strains, the heterologously produced OmcA is functional for soluble Fe(III) reduction, however, only the B-type strain BL21 was able to reduce insoluble amorphous iron (hydr)oxide. These findings indicate that only in the B-type strain is OmcA exposed to the outside of the cell, while in the K12 cell line it is stuck on the inner side of the outer membrane. This is consistent with previous reports on a cryptic type II protein secretion pathway across the outer membrane in *E. coli* K12 strains (Francetic and Pugsley 1996). Deletion mutations within the type II secretion pathway of the B-type strain resulted in a similar phenotype to the K12 strain, confirming that the type II secretion pathway is a crucial factor for the successful expression of outer membrane cytochromes. The successful secretion of recombinant proteins with the host secretion system has been found to be rather rare as the substrate recognition of the secretion complex is very specific

(Filloux 2004). Thus, successful trans-membrane transport in the B-type strain without the requirement for the synthesis of a fusion protein to simplify recognition (as described by d'Enfert and Pugsley 1987), is a crucial piece of information for the functional expression of other potentially important outer membrane cytochromes.

Besides these individual protein expression approaches, Londer *et al.* (2008), recently published six new expression vectors that allow high-throughput parallel expression of cytochrome *c*. The potential of the new vectors was shown with the exemplary expression of 30 hypothetical *S. oneidensis c*-type cytochromes with four or less heme molecules (predicted via genome sequence analysis). The authors reported a high success rate with 26 of the 30 cytochromes being expressed. However, their cloning effort only incorporated the soluble part of the proteins to allow for best purification. The correct localization in the membrane was not the goal of this study.

For *G. sulfurreducens* the expression of the following c-type cytochromes has been studied:

PpcA – was first cloned and heterologously expressed in 2002 by Londer *et al.* (2002). This early work resulted in a fully matured (co-expression of System I genes) and *in-vitro* functional enzyme. Since then, PpcA has been extensively studied in *E. coli* and *G. sulfurreducens*. The structure of PpcA has been resolved by X-ray diffraction (Pokkuluri *et al.* 2004) and, recently, it has been studied via nuclear magnetic resonance (Morgado *et al.* 2008). Both works suggest that PpcA linearly polymerizes, with the three heme molecules of each monomer forming a chain within the polymer resulting in an electron-transferring 'nanowire' in the periplasm. Recently, a new expression system was developed, which allows isotopic labelling of the cytochrome and promises further elucidation of structural functions (Fernandes *et al.* 2008). Since PpcA is one of the hypothesized key-proteins in the centre of the electron transport chain of *G. sulfurreducens* (see Figure 6.1), the knowledge of the *in-vivo* function is of high interest.

Further, **two dodecaheme cytochromes (GSU1996 and GSU0592)** – representing a new class of cytochromes – have been expressed in and purified from *E. coli* (Londer *et al.* 2006). Processing was performed by System I expressed from pEC86. Mass spectrometry analysis indicates that all 12 hemes were attached. These cytochromes' true function, to date, is unknown, but sequence analyses predict that their structure could be a one-dimensional chain of four homologous domains, each containing three heme molecules. Thus, these dodecaheme cytochromes could be the 'one-protein' version of the predicted polymerized PpcA chain and form a biological 'nanowire' through the cell membrane.

From the expression of these individual cytochromes in *E. coli* we can see that: (I) the co-expression of a cytochrome *c* biogenesis system is an important and successful way to produce multi-heme proteins in a heterologous host; (II) inner membrane or periplasmic cytochromes can be expressed and localized correctly within the heterologous host organism; and (III) B-type *E. coli* strains open a promising new alternative to the construction of fusion proteins for the proper localization of outer membrane proteins via the type II secretion pathway. The big remaining challenge is the application of these strategies to express multiple cytochromes in the same host cell and to allow their functional interaction.

One further issue for the recombinant expression of multiple cytochrome *c* proteins and their accessories that has not been addressed yet, but is well known for the expression of other large enzymatic complexes, are crowding conditions within the cell. Diffusion processes within the cell are three to fourfold slower for small molecules and even slower for large proteins (Conrado *et al.* 2008). Thus, we have to consider if expressed proteins experience a 'traffic jam' in the cytoplasm and how we can overcome this challenge.

6.5 BIOTECHNOLOGICAL APPLICATIONS – DESIGNING THE 'SUPER BUG'

6.5.1 The 'super bug' for BES applications

In section 6.4, we broadly discussed scientific motivations for the transfer of the enzymatic machinery for extracellular respiration into *E. coli*. Most of the previous work has been performed because of scientific objectives. However, with growing interest in *S. oneidensis* and *G. sulfurreducens* as biocatalysts in BES, the incentive for recombinant expression of the transmembrane cytochrome *c* assembly has changed. This section will discuss different approaches to use biomolecular engineering to improve BES performance. Hereby, we will not only discuss the heterologous expression of the proteins required for extracellular respiration, but also other possible metabolic changes that will help to design highly efficient and versatile biocatalysts for BES applications.

Beginning with cytochrome *c* proteins: for biotechnological applications it is desirable to respire extracellularly in an easy-to-grow, easy-to-maintain, and easy-to-manipulate host microbe. With the successful over-expression of the minimum required set of cytochrome *c* proteins, we wish to maximize direct electron transfer to a BES anode. While extracellular respiration of *S. oneidensis* is mainly based on the oxidation of lactate (oxidation to acetate and CO_2 gives 4 electrons) and that of *G. sulfurreducens* is based on acetate oxidation

(delivering a theoretical maximum of 8 electrons), engineered *E. coli* could presumably completely oxidize glucose to CO_2 and generate 24 electrons. Thus, more complex and efficient 'fuels' could be directly converted into electricity and the improved fuel conversion efficiency would result in better BES performance. The biocatalyst potentially could be designed based on the fuel stream for the BES to further improve efficiency. In addition, if biocatalysts are specifically designed to convert certain fuels (or chemical species) with a high efficiency, BES could also find new applications as environmental or industrial biosensors. Thus, BES current generation could be used as a qualitative and quantitative measure for one specific chemical substrate that is available to the genetically designed biocatalyst during a sensing event.

It should be noted that although this chapter examined the recombinant expression of proteins involved in extracellular respiration in *E. coli*, other host organisms, with other desirable substrate degradation abilities, may also be considered for this purpose. However, *E. coli* is the model organism that is most suitable for breaking ground in this field, because the biomolecular tools for heterologous expression first need to be developed for the other microbes.

While the protein machinery for extracellular respiration would allow *E. coli* to use an anode as the terminal electron acceptor when in direct cell-to-electrode contact, other microorganisms can transfer electrons even when they are not in direct contact. Several bacteria are known to produce soluble electron shuttle molecules (mediators) to pass metabolic electrons to electron acceptors that are not in close cell proximity. For example, *S. oneidensis,* in addition to its cell wall cytochromes, produces soluble riboflavin molecules that can shuttle electrons to external electron acceptors (Marsili *et al.* 2008; von Canstein *et al.* 2008). In Addition, *Pseudomonas aeruginosa* produces phenazine-type redox shuttles to dispose of electrons (Pham *et al.* 2008; Rabaey *et al.* 2005; Rabaey *et al.* 2004). Thus, it is conceivable to further engineer *E. coli* to produce its own specific mediators and use an electrode at a distance as terminal electron acceptor (recently, it was published that *E. coli* could use certain metabolites as redox-shuttles, however, the proof that the suggested compounds actually mediate electrons to an electrode is still lacking (Zhang *et al.* 2008)).

A completely different objective would be to engineer a host microbe that can accept electrons from an electrode rather than donate. Such a bacterium would use the cathode as electron (energy) donor to perform biochemical reactions. In classical BES this would correspond to the reduction of oxygen or alternative electron acceptors (inclusive contaminants) (He and Angenent 2006; Rabaey and Keller 2008). More recently, in microbial electrolysis cells (MECs) this can be the production of valuable chemicals. Thus far, the production of chemicals with MECs mainly focused on cathodic hydrogen production (Rozendal *et al.* 2006). However,

the reaction rate of microbial electrolysis processes at this point is greatly limited by the bioelectrochemical process. This lower rate of chemical production may render bioelectrochemical systems (both MFCs and MECs) economically feasible when high-value products, such as fatty acids or vitamins are generated on the cathode with engineered microbes as biocatalysts. One example for a cathodic electron-accepting microorganism is *G. sulfurreducens* (Gregory *et al.* 2004). Thus, a genetic engineering effort could again start with heterologous cloning of *G. sulfurreducens* genes into other host organisms that produce certain valuable chemicals, followed by engineering towards higher efficiency of the electron accepting process. With this approach the objective of BES would switch from a power generating device to a biochemical synthesis reactor.

6.5.2 The 'super bug' for bioremediation applications

Before *S. oneidensis* and *G. sulfurreducens* were discussed as model organisms for direct electron transfer in MFCs, they were intensively studied because of their potential importance in bioremediation processes. *Shewanella* spp. are of great interest due to their ability to respire with a great variety of soluble and insoluble electron acceptors (Nealson *et al.* 2002). Their capacity to reduce a broad range of often toxic or radioactive heavy metal compounds into less environmentally threatening insoluble states, or to reduce halogenated organics is of high interest at contaminated sites (Hau and Gralnick 2007). However, the natural abundance and activity of *Shewanella* spp. is limited to aquatic habitats, while bioremediation applications are also desirable in soil environments. In addition, the rates of bioremediation are low even in aquatic habitats owing to the relatively low quantity of the biocatalysts.

Genetically modified bacteria could be tailored to exclusively live off the reduction of the contaminating species. These organisms could be applied in high concentrations to contaminated sites of, for example, mines, military land, volcanoes, or flooded areas. In a comparably short time, toxic species could be detoxified or converted into less harmful states (e.g. reduction of U(VI) to insoluble U(IV) or reduction of Cr(VI) to oxide forming Cr(III)). If we could force such bacteria to exclusively live off the contaminating species, the controlled host organism would simply die off once the site is clean.

Besides the scientific challenge, these ideas challenge the legal and ethical boundaries of today's society. However, the application of such clean up biocatalysts in more industrial settings (e.g. in closed systems) is conceivable. Heavy metal or radioactive industrial waste waters could be treated with specialized microorganisms to reduce and precipitate heavy metal salts in a new, effective and inexpensive way.

6.6 CLOSING REMARKS

In this chapter we presented the possibilities and opportunities of genetic engineering tools to the scientific investigation of physiological processes in BES. In addition, we discussed the design of desired microbial biocatalysts for biotechnological applications. We mainly focused on the recombinant expression of the cytochrome *c* apparatuses, which allow *S. oneidensis* and *G. sulfurreducens* to respire with extracellular electron acceptors in *E. coli*. Due to the complexity of multi-protein expression, post-translational modification, and localization, the scientific challenges of this approach are tremendous. Yet, some important breakthroughs with single protein expressions during recent years have delivered us the important biomolecular tools to get closer to this goal. It should be noted that the application of biomolecular tools to study or improve bacteria for BES applications are certainly not limited to the proteins of extracellular electron transfer and to recombinant expression in *E. coli*. Genetic engineering approaches can target any desired metabolic pathway and there are no theoretical limits to overexpression in the target microbe or recombinant expression in any host organism, albeit the necessary genetic toolkits may need to be developed. This very young field of biological engineering leaves a lot of room for dreaming. Scientific reality will reveal what is practically possible and in what time frame we can achieve our goals.

6.7 ACKNOWLEDGEMENTS

The financial support for M.R. was provided through a specific collaborative agreement between L.T.A. and the Fermentation Biotechnology Research Unit, USDA, Agricultural Research Service, Peoria, Illinois. L.T.A. acknowledges support through US NSF CAREER grant #0645021.

REFERENCES

Arslan, E., Schulz, H., Zufferey, R., Kunzler, P. and Thony-Meyer, L. (1998) Overproduction of the *Bradyrhizobium japonicum c*-type cytochrome subunits of the *cbb3* oxidase in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **251**(3), 744–747.

Beliaev, A.S., Saffarini, D.A., McLaughlin, J.L. and Hunnicutt, D. (2001) MtrC, an outer membrane decaheme *c* cytochrome required for metal reduction in *Shewanella putrefaciens* MR-1. *Molec. Microbiol.* **39**(3), 722–730.

Bretschger, O., Obraztsova, A., Sturm, C.A., Chang, I.S., Gorby, Y.A., Reed, S.B., Culley, D.E., Reardon, C.L., Barua, S., Romine, M.F., Zhou, J., Beliaev, A.S., Bouhenni, R., Saffarini, D., Mansfeld, F., Kim, B.H., Fredrickson, J.K. and Nealson,

K.H. (2007) Current production and metal oxide reduction by *Shewanella oneidensis* MR-1 wild type and mutants. *Appl. Environ. Microbiol.* **73**(21), 7003–7012.

Butler, J.E., Kaufmann, F., Coppi, M.V., Nunez, C. and Lovley, D.R. (2004) MacA, a diheme *c*-type cytochrome involved in Fe(III) reduction by *Geobacter sulfurreducens*. *J. Bacteriol.* **186**(12), 4042–4045.

Comprehensive Microbial Resource (CMR) at the J. Graig Venter Insitute http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi

Conrado, R.J., Varner, J.D. and DeLisa, M.P. (2008) Engineering the spatial organization of metabolic enzymes: mimicking nature's synergy. *Curr. Opin. Biotechnol.* **19**(5), 492–499.

d'Enfert, C. and Pugsley, A.P. (1987) A gene fusion approach to the study of pullulanase export and secretion in *Escherichia coli*. *Mol. Microbiol.* **1**(2), 159–168.

Donald, J.W., Hicks, M.G., Richardson, D.J. and Palmer, T. (2008) The *c*-type cytochrome OmcA localizes to the outer membrane upon heterologous expression in *Escherichia coli*. *J. Bacteriol.* **190**(14), 5127–5131.

Feissner, R.E., Richard-Fogal, C.L., Frawley, E.R., Loughman, J.A., Earley, K.W. and Kranz, R.G. (2006) Recombinant cytochromes *c* biogenesis systems I and II and analysis of haem delivery pathways in *Escherichia coli*. *Mol. Microbiol.* **60**(3), 563–577.

Fernandes, A.P., Couto, I., Morgado, L., Londer, Y.Y. and Salgueiro, C.A. (2008) Isotopic labeling of *c*-type multiheme cytochromes overexpressed in *E. coli*. *Protein Expr. Purif.* **59**(1), 182–188.

Filloux, A. (2004) The underlying mechanisms of type II protein secretion. *Biochim. Biophys. Acta* **1694**(1–3), 163–179.

Francetic, O. and Pugsley, A.P. (1996) The cryptic general secretory pathway (gsp) operon of *Escherichia coli* K-12 encodes functional proteins. *J. Bacteriol.* **178**(12), 3544–3549.

Gescher, J.S., Cordova, C.D. and Spormann, A.M. (2008) Dissimilatory iron reduction in *Escherichia coli*: identification of CymA of *Shewanella oneidensis* and NapC of *E. coli* as ferric reductases. *Mol. Microbiol.* **68**(3), 706–719.

Gregory, K.B., Bond, D.R. and Lovley, D.R. (2004) Graphite electrode as electron donors for anaerobic respiration. *Environ. Microbiol.* **6**(6), 596–604.

Hartshorne, R.S., Jepson, B.N., Clarke, T.A., Field, S.J., Fredrickson, J., Zachara, J., Shi, L., Butt, J.N. and Richardson, D.J. (2007) Characterization of *Shewanella oneidensis* MtrC: a cell-surface decaheme cytochrome involved in respiratory electron transport to extracellular electron acceptors. *J. Biol. Inorg. Chem.* **12**(7), 1083–1094.

Hau, H.H. and Gralnick, J.A. (2007) Ecology and biotechnology of the genus Shewanella. *Ann. Rev. Microbiol.* **61**, 237–258.

He, Z. and Angenent, L.T. (2006) Application of bacterial biocathodes in microbial fuel cells. *Electroanalysis* **18**(19–20), 2009–2015.

Herbaud, M.L., Aubert, C., Durand, M.C., Guerlesquin, F., Thony-Meyer, L. and Dolla, A. (2000) *Escherichia coli* is able to produce heterologous tetraheme cytochrome *c(3)* when the *ccm* genes are co-expressed. *Biochim. Biophys. Acta* **1481**(1), 18–24.

Holmes, D.E., Chaudhuri, S.K., Nevin, K.P., Mehta, T., Methe, B.A., Liu, A., Ward, J.E., Woodard, T.L., Webster, J. and Lovley, D.R. (2006) Microarray and genetic analysis

of electron transfer to electrodes in *Geobacter sulfurreducens*. *Environ. Microbiol.* **8**(10), 1805–1815.

Holmes, D.E., Mester, T., O'Neil, R.A., Perpetua, L.A., Larrahondo, M.J., Glaven, R., Sharma, M.L., Ward, J.E., Nevin, K.P. and Lovley, D.R. (2008) Genes for two multicopper proteins required for Fe(III) oxide reduction in *Geobacter sulfurreducens* have different expression patterns both in the subsurface and on energy-harvesting electrodes. *Microbiology* **154**, 1422–1435.

Kim, B.C. and Lovley, D.R. (2008) Investigation of direct vs. indirect involvement of the *c*-type cytochrome MacA in Fe(III) reduction by *Geobacter sulfurreducens*. *FEMS Microbiol. Lett.* **286**(1), 39–44.

Kim, B.C., Leang, C., Ding, Y.H., Glaven, R.H., Coppi, M.V. and Lovley, D.R. (2005) OmcF, a putative *c*-type monoheme outer membrane cytochrome required for the expression of other outer membrane cytochromes in *Geobacter sulfurreducens*. *J. Bacteriol.* **187**(13), 4505–4513.

Kim, B.C., Qian, X., Leang, C., Coppi, M.V. and Lovley, D.R. (2006) Two putative *c*-type multiheme cytochromes required for the expression of OmcB, an outer membrane protein essential for optimal Fe(III) reduction in *Geobacter sulfurreducens*. *J. Bacteriol.* **188**(8), 3138–3142.

Kranz, R., Lill, R., Goldman, B., Bonnard, G. and Merchant, S. (1998) Molecular mechanisms of cytochrome *c* biogenesis: three distinct systems. *Molec. Microbiol.* **29**(2), 383–396.

Leang, C., Coppi, M.V. and Lovley, D.R. (2003) OmcB, a *c*-type polyheme cytochrome, involved in Fe(III) reduction in *Geobacter sulfurreducens*. *J. Bacteriol.* **185**(7), 2096–2103.

Lloyd, J.R., Leang, C., Hodges Myerson, A.L., Coppi, M.V., Cuifo, S., Methe, B., Sandler, S.J. and Lovley, D.R. (2003) Biochemical and genetic characterization of PpcA, a periplasmic *c*-type cytochrome in *Geobacter sulfurreducens*. *Biochem. J.* **369**(Pt 1), 153–161.

Londer, Y.Y., Pokkuluri, P.R., Tiede, D.M. and Schiffer, M. (2002) Production and preliminary characterization of a recombinant triheme cytochrome *c*(7) from *Geobacter sulfurreducens* in *Escherichia coli*. *Biochim. Biophys. Acta* **1554**(3), 202–211.

Londer, Y.Y., Pokkuluri, P.R., Orshonsky, V., Orshonsky, L. and Schiffer, M. (2006) Heterologous expression of dodecaheme "nanowire" cytochromes *c* from *Geobacter sulfurreducens*. *Protein Expr. Purif.* **47**(1), 241–248.

Londer, Y.Y., Giuliani, S.E., Peppler, T. and Collart, F.R. (2008) Addressing *Shewanella oneidensis* "cytochromome": the first step towards high-throughput expression of cytochromes *c*. *Protein Expr. Purif.* **62**(1), 128–137.

Marsili, E., Baron, D.B., Shikhare, I.D., Coursolle, D., Gralnick, J.A. and Bond, D.R. (2008) *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proc. Natl. Acad. Sci. USA* **105**(10), 3968–3973.

Mehta, T., Coppi, M.V., Childers, S.E. and Lovley, D.R. (2005) Outer membrane *c*-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. *Appl. Environ. Microbiol.* **71**(12), 8634–8641.

Methe, B.A., Nelson, K.E., Eisen, J.A., Paulsen, I.T., Nelson, W., Heidelberg, J.F., Wu, D., Wu, M., Ward, N., Beanan, M.J., Dodson, R.J., Madupu, R., Brinkac, L.M., Daugherty, S.C., DeBoy, R.T., Durkin, A.S., Gwinn, M., Kolonay, J.F., Sullivan,

S.A., Haft, D.H., Selengut, J., Davidsen, T.M., Zafar, N., White, O., Tran, B., Romero, C., Forberger, H.A., Weidman, J., Khouri, H., Feldblyum, T.V., Utterback, T.R., Van Aken, S.E., Lovley, D.R. and Fraser, C.M. (2003) Genome of *Geobacter sulfurreducens*: metal reduction in subsurface environments. *Science* **302**(5652), 1967–1969.

Meyer, T.E., Tsapin, A.I., Vandenberghe, I., de Smet, L., Frishman, D., Nealson, K.H., Cusanovich, M.A. and van Beeumen, J.J. (2004) Identification of 42 possible cytochrome *c* genes in the *Shewanella oneidensis* genome and characterization of six soluble cytochromes. *Omics* **8**(1), 57–77.

Morgado, L., Bruix, M., Orshonsky, V., Londer, Y.Y., Duke, N.E.C., Yang, X.J., Pokkuluri, P.R., Schiffer, M. and Salgueiro, C.A. (2008) Structural insights into the modulation of the redox properties of two *Geobacter sulfurreducens* homologous triheme cytochromes. *Biochim. Biophys. Acta, Bioenerg.* **1777**(9), 1157–1165.

Murphy, J.N. and Saltikov, C.W. (2007) The *cymA* gene, encoding a tetraheme *c*-type cytochrome, is required for arsenate respiration in *Shewanella* species. *J. Bacteriol.* **189**(6), 2283–2290.

Myers, C.R. and Myers, J.M. (1997) Cloning and sequence of *cymA*, a gene encoding a tetraheme cytochrome *c* required for reduction of iron(III), fumarate, and nitrate by *Shewanella putrefaciens* MR-1. *J. Bacteriol.* **179**(4), 1143–1152.

Myers, C.R. and Myers, J.M. (2002) MtrB is required for proper incorporation of the cytochromes OmcA and OmcB into the outer membrane of *Shewanella putrefaciens* MR-1. *Appl. Environ. Microbiol.* **68**(11), 5585–5594.

Myers, J.M. and Myers, C.R. (2000) Role of the tetraheme cytochrome CymA in anaerobic electron transport in cells of *Shewanella putrefaciens* MR-1 with normal levels of menaquinone. *J. Bacteriol.* **182**(1), 67–75.

Myers, J.M. and Myers, C.R. (2001) Role for outer membrane cytochromes OmcA and OmcB of *Shewanella putrefaciens* MR-1 in reduction of manganese dioxide. *Appl. Environ. Microbiol.* **67**(1), 260–269.

Myers, J.M. and Myers, C.R. (2003) Overlapping role of the outer membrane cytochromes of *Shewanella oneidensis* MR-1 in the reduction of manganese(IV) oxide. *Lett. Appl. Microbiol.* **37**(1), 21–25.

Nealson, K.H., Belz, A. and McKee, B. (2002) Breathing metals as a way of life: geobiology in action. *Antonie van Leeuwenhoek* **81**(1–4), 215–222.

Pessanha, M., Morgado, L., Louro, R.O., Londer, Y.Y., Pokkuluri, P.R., Schiffer, M. and Salgueiro, C.A. (2006) Thermodynamic characterization of triheme cytochrome PpcA from *Geobacter sulfurreducens*: evidence for a role played in e-/H+ energy transduction. *Biochemistry* **45**(46), 13910–13917.

Pham, T.H., Boon, N., De Maeyer, K., Hofte, M., Rabaey, K. and Verstraete, W. (2008) Use of *Pseudomonas* species producing phenazine-based metabolites in the anodes of microbial fuel cells to improve electricity generation. *Appl. Microbiol. Biotechnol.* **80**(6), 985–993.

Pitts, K.E., Dobbin, P.S., Reyes-Ramirez, F., Thomson, A.J., Richardson, D.J. and Seward, H.E. (2003) Characterization of the *Shewanella oneidensis* MR-1 decaheme cytochrome MtrA: expression in *Escherichia coli* confers the ability to reduce soluble Fe(III) chelates. *J. Biol. Chem.* **278**(30), 27758–27765.

Pokkuluri, P.R., Londer, Y.Y., Duke, N.E., Long, W.C. and Schiffer, M. (2004) Family of cytochrome *c*7-type proteins from *Geobacter sulfurreducens*: structure of one cytochrome *c*7 at 1.45 A resolution. *Biochemistry* **43**(4), 849–859.

Rabaey, K. and Keller, J. (2008) Microbial fuel cell cathodes: from bottleneck to prime opportunity? *Water Sci. Technol.* **57**(5), 655–659.

Rabaey, K., Boon, N., Hofte, M. and Verstraete, W. (2005) Microbial phenazine production enhances electron transfer in biofuel cells. *Environ. Sci. Technol.* **39**(9), 3401–3408.

Rabaey, K., Boon, N., Siciliano, S.D., Verhaege, M. and Verstraete, W. (2004) Biofuel cells select for microbial consortia that self-mediate electron transfer. *Appl. Environ. Microbiol.* **70**(9), 5373–5382.

Ross, D.E., Ruebush, S.S., Brantley, S.L., Hartshorne, R.S., Clarke, T.A., Richardson, D.J. and Tien, M. (2007) Characterization of protein-protein interactions involved in iron reduction by *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* **73**(18), 5797–5808.

Rozendal, R.A., Hamelers, H.V.M., Euverink, G.J.W., Metz, S.J. and Buisman, C.J.N. (2006) Principle and perspectives of hydrogen production through biocatalyzed electrolysis. *Int. J. Hydrogen Energy* **31**(12), 1632–1640.

Schwalb, C., Chapman, S.K. and Reid, G.A. (2002) The membrane-bound tetraheme *c*-type cytochrome CymA interacts directly with the soluble fumarate reductase in *Shewanella*. *Biochem. Soc. Trans.* **30**(4), 658–662.

Schwalb, C., Chapman, S.K. and Reid, G.A. (2003) The tetraheme cytochrome CymA is required of anaerobic respiration with dimethyl sulfoxide und nitrite in *Shewanella oneidensis*. *Biochemistry* **42**(31), 9491–9497.

Shi, L., Chen, B., Wang, Z., Elias, D.A., Mayer, M.U., Gorby, Y.A., Ni, S., Lower, B.H., Kennedy, D.W., Wunschel, D.S., Mottaz, H.M., Marshall, M.J., Hill, E.A., Beliaev, A.S., Zachara, J.M., Fredrickson, J.K. and Squier, T.C. (2006) Isolation of a high-affinity functional protein complex between OmcA and MtrC: Two outer membrane decaheme *c*-type cytochromes of *Shewanella oneidensis* MR-1. *J. Bacteriol.* **188**(13), 4705–4714.

Shi, L., Deng, S., Marshall, M.J., Wang, Z., Kennedy, D.W., Dohnalkova, A.C., Mottaz, H.M., Hill, E.A., Gorby, Y.A., Beliaev, A.S., Richardson, D.J., Zachara, J.M. and Fredrickson, J.K. (2008) Direct involvement of type II secretion system in extracellular translocation of *Shewanella oneidensis* outer membrane cytochromes MtrC and OmcA. *J. Bacteriol.* **190**(15), 5512–5516.

Thony-Meyer, L. (1997) Biogenesis of respiratory cytochromes in bacteria. *Microbiol. Mol. Biol. Rev.* **61**(3), 337–376.

von Canstein, H., Ogawa, J., Shimizu, S. and Lloyd, J.R. (2008) Secretion of flavins by *Shewanella* species and their role in extracellular electron transfer. *Appl. Environ. Microbiol.* **74**(3), 615–623.

Weber, K.A., Achenbach, L.A. and Coates, J.D. (2006) Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nature* **4**, 752–764.

Zhang, T., Cui, C., Chen, S., Yang, H. and Shen, P. (2008) The direct electrocatalysis of *Escherichia coli* through electroactivated excretion in microbial fuel cell. *Electrochem. Commun.* **10**(2), 293–297.