Investigation of the electrochemical activity of chromium tolerant mutants of *Geobacter metallireducens*

Gabriele Pastorella
B.Sc. in Biotechnology (University of Palermo)

Thesis submitted for the degree of Doctor of Philosophy
Ph.D.
Dublin City University
School of Biotechnology

Under the supervision of Dr. Enrico Marsili

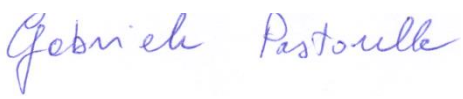
September 2013

Declaration

I hereby certify that this material, which I now submit for assessment on the program of study leading to the award of Ph.D. is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

ID No.: 58119019

Date: 17/09/2013

Signed: 

ACKNOWLEDGEMENTS

I would like to thank the School of Biotechnology and Dublin City University for allowing me to use all the laboratory facilities and services.

I would like to thank everyone involved in this project for their knowledge and support. In particular I would like to thank Giulio and Ilaria for their positive contribution to the project.

I would like to thank my advisor, Dr. Enrico Marsili, for its constant support and guidance.

Special thanks to my family for their support, especially to my parents for

their support. Special thanks also to my lovely girlfriend, Valentina, for her constant support and love throughout this process.

Gabriele Pastorella

Abstract

Dissimilatory metal reducing bacteria (DMRB) can reduce insoluble electron acceptors, such as metals and electrodes, through extracellular electron transfer. The DMRB *Geobacter metallireducens* can reduce Cr(VI) to Cr(III) via direct electron transfer through transmembrane cytochrome complexes. Extracellular electron transfer is relevant to both energy production and Cr(VI) detoxification in subsurface environments and groundwater. However, the toxicity of Cr(VI) to *G. metallireducens* limits its application to field bioremediation, where Cr(VI) concentration may exceed the toxicity threshold (few mg L^{-1}).

In this study, we improved the *G. metallireducens* electroactivity and its tolerance to Cr(VI) through genome shuffling. The parent strain was exposed to nitrosoguanidine (NTG) for 60 minutes. Then, protoplasts obtained by lysozyme recombined their genomes.

Following protoplast recombination, the mutants obtained are screened by biological, molecular, and electrochemical assays, to select those with higher Cr(VI) tolerance and electroactivity. After one round of genome shuffling, the best performing mutants could grow at 5 mM of Cr(VI), that is, two fold the Cr(VI) concentration tolerated by the wild type *G. metallireducens*.

Most of the Cr(VI)-tolerant mutants obtained by genome shuffling showed a two-fold decrease of electroactivity when grown in potentiostat-controlled electrochemical cells. This suggests that resistance to Cr(VI) and electroactivity may be partially overlapping pathways, therefore a screening method based on resistance to Cr(VI) is not adequate to select only electroactive strains. Out of ten Cr(VI)-tolerant mutants, we select the M23 mutant that shows two-fold increase in electroactivity with respect to the wild type strain.

In this study we show for the first time that genome shuffling can be applied to strict anaerobes, metal-reducing bacteria, to improve metal resistance and electroactivity. The mutants obtained could be applied to Cr(VI) bioremediation in highly contaminated soil and groundwater.

Table of contents

Publications

Articles in refereed journals

1) Jain A, Zhang X, **Pastorella G**, O'Connolly J, Barry N, Woolley R, Krishnamurthy S, Marsili E. (2012) Electron transfer mechanism in Shewanella loihica PV- 4 biofilms formed at graphite electrode. *Bioelectrochemistry* **87**, 28-32.

2) Connolly J, Jain A, **Pastorella G**, Krishnamurthy S, Mosnier J-P, Marsili E. (2011) Zinc oxide and indium tin oxide thin films for the growth and characterization of *Shewanella loihica* PV-4 electroactive biofilms. Virulence **2**(5), 479-482.

Chapters in refereed volumes

1) **Pastorella G**, Gazzola G, Guadarrama S, Marsili E (2012). Ch5: Biofilms: Applications in Bioremediation. In Microbial Biofilms: Current Research and Applications, ed Lear G and Lewis GD. Caister Academic Press, Norfolk, UK.

2) Arora S, **Pastorella G,** Byrne B, Marsili E, O'Kennedy R (2009) Microbial Cells and Biosensing: A Dual Approach - Exploiting Antibodies and Microbial Cells as Analytical/Power Systems. Reviews in: Pharmaceutical and Biomedical Analysis, ed. O'Kennedy R (E-book series). Bentham Science Publishers, UK.

Conference presentations

Invited talks

1) Metodi genetici per migliorare il biorisanamento delle acque sotterranee at Magellano – idee dal mondo, IRER, 18-12-2008 Milan. (Genetic methods to improve groundwater bioremediation at Lombardy research meeting).

2) Genetic methods for *G. metallireducens* strain improvement, oral presentation at the second research day of School of biotechnology - Dublin City University. 30-01-2011.

Poster presentations

1) Genetic technology to improve biofilms electroactivity for bioremediation applications at 8th annual meeting of Environmental Protection Agency (EPA), 12 November 2010, Dublin, Ireland.

2) Genetic technology to improve biofilms electroactivity for bioremediation applications at 61th annual meeting of International Electrochemical Society (ISE), 26 September-1 October 2010, Nice, France.

3) Genetic methods to improve Cr(VI) bioremediation in groundwater at 1st research day of School of biotechnology - Dublin City University (first prize poster award), 30 January 2009, Dublin, Ireland.

Introduction

1.1 Biofilms

Most microorganisms in the environment reside in dynamically structured communities of multiple species embedded in a polymeric matrix, which are termed biofilms. Biofilms are heterogeneous microstructures that microorganisms form under certain conditions, such as mechanical and chemical stress, lack of nutrient, exposure to antibiotics, etc. (Flemming & Wingender, 2010). The microorganisms in biofilms live in a self-produced matrix of hydrated extracellular polymeric substances (EPS). EPS are mainly composed of polysaccharides, proteins, nucleic acids and lipids; they provide the mechanical stability of biofilms, mediate their adhesion to surfaces and form a cohesive, three-dimensional polymer network that interconnects biofilm cells. In addition, the biofilm matrix acts as an external digestive system by keeping extracellular enzymes close to the cells, enabling them to metabolize dissolved, colloidal and solid biopolymers.

Biofilms are formed in most natural environments, such as surface water, seawater, and groundwater. Environmental biofilms are composed of multiple bacterial species, and are in general much more complex than the single species biofilms commonly encountered in laboratory research. The structure of environmental biofilms depends on several factors such as the nutrient availability, solid phase morphology, and physicochemical parameters of the microenvironment. The complex interactions between mechanical and chemical constraints/stress in the environment result in a large variety of biofilm growth forms, including cluster- and protrusion-type structures, cell aggregate bridging, and thick bioweb-type growth (Rodríguez & Bishop, 2007).

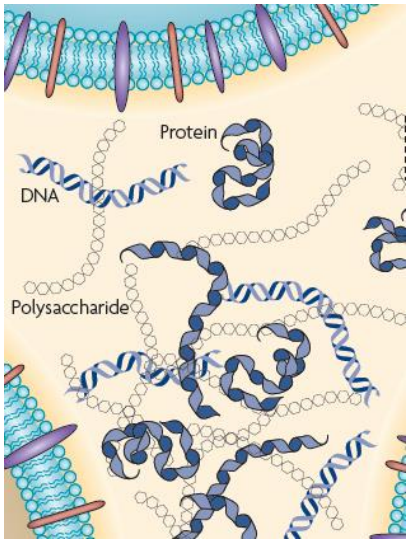


Figure 1.1: The major matrix components — polysaccharides, proteins and DNA — are distributed between the cells in a non-homogeneous pattern, setting up differences between regions of the matrix. Reproduced with permission from Magnuson et al., (2001).

Biofilm is the preferred growth mode (Ramage et al., 2010) for those microorganisms capable of interfacing with solid conductive surfaces, such as metals. These microorganisms are termed dissimilatory metal reducing bacteria (DMRB) and are relevant to biogeochemical processes (D. Lovley, 2013). In fact, they are responsible for the formation of reduced mineral ores, such as Fe(II), and contribute to the chemical cycles of Fe, Mn, and S (D. Lovley, 2013). A subset of DMRB biofilms is capable of interacting with conductive surfaces, such as electrodes. The latter biofilms are conventionally termed electroactive, electrochemically active, or exo-electricigens biofilms (Rittmann, 2013).

1.1.2 Subsurface

Conventionally, the subsurface extends from the crust to the nucleus. However, we define subsurface with respect to the ecological niche of DMRB, which extends from a few centimetres below the surface, where compact soil prevents oxygen permeation, to a few kilometres below the surface, where the high temperature and extremely low nutrient concentration do not permit microbial life.

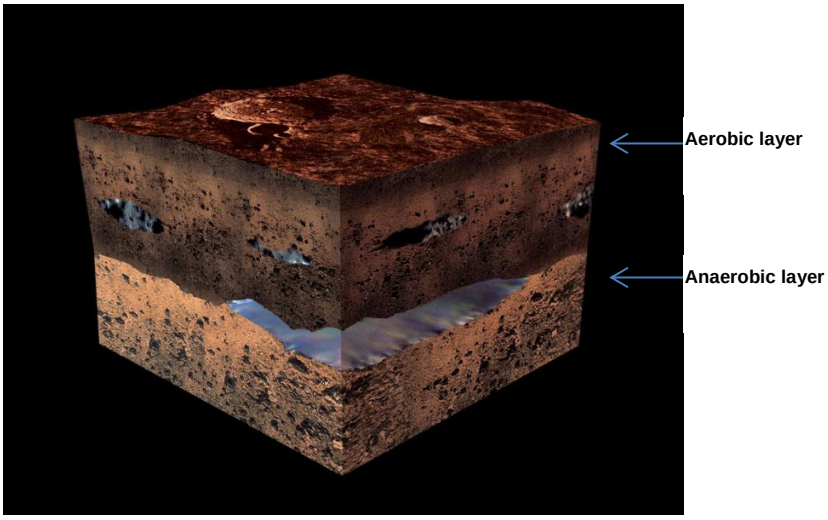


Figure 1.2: Imagine of subsurface: anaerobic bacteria are predominant in deeper soil as well the oxygen content decreases. Dissimilatory metal reducing bacteria are predominant in Fe and Mn reach zones. Modified by Medialab. Copyright : ESA 2001.

Life extends far deeper into the Earth's subsurface than believed until a few years ago. In the past, it was assumed that life is a surface phenomenon, and that even ‘hardy prokaryotic types’ were not capable of living deeper than tens of meters below the surface (Jannasch et al., 1971) . In the 1990s, it became apparent that genetically and metabolically diverse microbial communities existed under highly reducing conditions in the deep subsurface (R. J. Parkes et al., 1994). Today we know that life in the deep

subsurface is ubiquitous and comprises a large proportion of the biomass on Earth (Fry et al., 2008). However, many questions concerning life in the deep remain unanswered; for example: What is the lower depth limit of the deep biosphere? Which energy sources (i.e., electron donors and acceptors) are sustaining these communities? How are genetic diversity and functional activity linked to geochemical and geological factors? What we know is that the deep subsurface is an extreme environment and that the microorganisms living here have developed numerous mechanisms to deal with high pressure and temperature, limited energy and nutrient availability, extreme acidity and alkalinity, metal toxicity, and radioactivity (Kerr, 1997; Wentzel et al., 2013).

Also, there are many evidences that in extreme ecosystems microbial communities not only adapt to these conditions, but also change these environments to suit their needs (Reith, 2011). For example, a study has demonstrated that prokaryotes can stimulate mineral H_2 formation for the deep biosphere promoting thermogenic activity (R. J. Parkes et al., 2011).

1.2 Dissimilatory metal reducing bacteria

Dissimilatory metal reducing bacteria (DMRB) are a class of microorganism coupling the metal reduction with their metabolism (Seeliger et al., 1998)(Liu et al., 2002). DMRB use metals as extracellular terminal electron acceptors, in contrast to metal internalization for metabolic reasons (Jain et al., 2012)(D. R. Lovley, 1993). The metal reduction reactions of environmental relevance are Fe(III) →Fe (II) and Mn(IV) → Mn(II). The redox potential of these reactions (respectively, 0.77V and 1,51V) is lower than that of O_2 (2.42V) reduction. Therefore, DMRB are most competitive in anaerobic conditions. DMRB played a key role in the formation of the modern ores and atmosphere, e.g., banded iron formation in the Cambrian era, when the reducing atmosphere conditions were not permissive for plants and animals life (Konhauser et al., 2002). Several genera and families have been investigated as DMRB: here we cite *Clostridium* (H. S. Park et al., 2001), *Aeromonas* (Pham et al., 2003), *Geobacter* (D. R. Lovley, 1991)(Bond & Lovley, 2003) and *Shewanella* (Nealson & Saffarini, 1994)(L. Sh et al., 2007). The next section summarizes the best known DMRB and their application.

DMRB have both direct and indirect effects on the geochemical cycling of metals. Since many of them are biofilm forming, they may uptake metals into the EPS, in cell walls or interiors (Comte et al., 2008). Moreover, they facilitate electron transfers in metals as well as in those elements having multiple oxidation states, such as sulphur and carbon. This capability leads

to formation of new minerals, dissolution of existing ones, and formation of aqueous complexes that can enhance the solubility of some metals. As many other environmental microorganisms, DMRB can be found in the high potential iron-reducing zone (Nealson & Myers, 1992). These tend to be facultative, since they thrive close to the aerobic soil where atmospheric gas can diffuse driving oxygen. In deeper soil the oxygen content decreases as anaerobic species increase their predominance.
I refer the interested reader to some excellent reviews published on the microbial ecology and diversity of DMRB (Klimes et al., 2010; Reguera et al., 2005b), (Liu et al., 2002) In this thesis only concise information on the *G. metallireducens* (Chapter 1) and the rationale of its choice for Cr(VI) bioremediation (Chapter 2) are provided.

While electron transfer processes are usually intracellular, DMRB are capable of reducing insoluble extracellular electron acceptors, such as metals and electrodes. In the next paragraph, the most known DMRB species will be introduced.

1.2.1 *Geobacter* spp.

Geobacter species are specialized in making electrical contacts with extracellular electron acceptors and other organisms (Gorby et al., 2006). This permits *Geobacte*r species to fill important niches in diverse of anaerobic environments. *Geobacter* species appear to be the primary agents for coupling the oxidation of organic compounds to the reduction of insoluble Fe(III) and Mn(IV) oxides in many soils and sediments, a process of global biogeochemical significance (D. R. Lovley, 1991; D. Lovley, 2013). Some *Geobacter* species can anaerobically oxidize aromatic hydrocarbons and play an important role in aromatic hydrocarbon removal from contaminated aquifers (T. Zhang et al., 2010). The ability of *Geobacter* species to reductively precipitate uranium and other lantanide contaminants has led to the development of bioremediation strategies for contaminated environments (E. S. Shelobolina et al., 2008).

When applied in bioelectrochemical systems (BES) like Microbial Fuel Cells (MFCs), *Geobacte*r species produce higher current densities than any other single species and are common colonizers of electrodes harvesting electricity from organic wastes and aquatic sediments (Rotaru et al., 2011). Direct interspecies electron exchange between *Geobacter* species and syntrophic partners appears to be an important process in anaerobic wastewater digesters (Galushko & Schink, 2000).

Functional and comparative genomic studies have begun to reveal important aspects of *Geobacter* physiology and regulation, but much remains unexplored. Quantifying key gene transcripts and proteins of subsurface *Geobacter* communities has proven to be a powerful approach

to diagnose the in situ physiological status of *Geobacter* species during groundwater bioremediation (Mouser et al., 2009). The growth and activity of *Geobacter* species in the subsurface and their biogeochemical impact under different environmental conditions can be predicted with a systems biology approach in which genome-scale metabolic models are coupled with appropriate physical/chemical models.

The proficiency of *Geobacter* species in transferring electrons to insoluble minerals, electrodes, and possibly other microorganisms is due to the high concentrations of multi-haem membrane cytochromes but also to the pili-like structures termed “microbial nanowires” that conduct electrons along their length with metallic-like conductivity (Reguera et al., 2005a). Cytochromes are important for making the terminal electrical connections with Fe(III) oxides and electrodes and also function as capacitors, storing charge to permit continuous respiration when extracellular electron acceptors are temporarily unavailable (Leang et al., 2010).

The high conductivity of *Geobacter* pili and biofilms and the ability of biofilms to function as super capacitors are novel properties that might contribute to the field of bioelectronics (Malvankar & Lovley, 2012; Rotaru et al., 2011). The study of *Geobacter* species has revealed a remarkable number of microbial physiological properties that had not previously been described in any microorganism.

All the known *Geobacter* isolates are Gram-negative rods capable of oxidizing acetate with the reduction of Fe(III). Other commonly conserved features include the ability to reduce Mn(IV), U(VI), elemental sulphur, and humic substances or the humic substance analogue anthraquinone-2,6-disulfonate (AQDS). Many isolates have the ability to use other small molecular weight organic acids, ethanol, or hydrogen as an electron donor. The two most heavily studied *Geobacter* species have been *G. metallireducens* and *G. sulfurreducens. G. metallireducens* was the first *Geobacter* species recovered in pure culture (D. R. Lovley & Phillips, 1988). It was with this isolate that many of the novel physiological attributes listed above were discovered.

The recent development of a genetic system for *G. metallireducens* (Tremblay et al., 2012) is likely to refocus attention on this organism to elucidate the physiology of important novel properties, such as anaerobic benzene degradation. *Geobacter sulfurreducens* was the first *Geobacter* species for which methods for genetic manipulation were developed (Aklujkar et al., 2009; Coppi et al., 2001)(Rollefson et al., 2009) and therefore it has served as the *Geobacter* of choice for functional genomic studies designed to understand its metabolism, gene regulation, and

extracellular electron transfer. It was the first *Geobacter* species found to use hydrogen as an electron donor, or to grow with elemental sulphur as an electron acceptor. The originally isolated strain was referred to as strain PCA (Caccavo et al., 1994).

A commonly used strain of *G. sulfurreducens* derived from strain PCA is frequently referred to as strain DL-1 (Coppi et al., 2001) because this culture was maintained for many transfers in the laboratory and may have accumulated mutations that were not present in the originally isolated PCA strain. For example, the DL-1 strain only poorly reduces Fe(III) oxide unless it is adapted for growth on Fe(III) oxide for long periods of time. The capacity for effective Fe (III) oxide reduction was recovered via adaptive evolution (Tremblay et al., 2011).

Another valuable strain of G. sulfurreducens is strain KN400, which was recovered in a study designed to adaptively evolve G. sulfurreducens for growth on electrodes (Li et al., 2011). Although the KN400 and DL-1 strains have an identical 16S rRNA gene sequence, they have some important physiological differences. In addition to producing more current than DL-1 (Butler et al., 2012), KN400 also reduces Fe(III) oxides much faster.

One reason for this may be greater expression of pili in KN400, which, as discussed below, is thought to be a major conduit for electron transfer to Fe(III) oxide. Further, strain KN400 is motile, whereas strain DL-1 is not. This can be attributed to interruption of the gene for the master regulator for flagella gene expression, FrgM, in DL-1 (Ueki et al., 2012).

Motility is important in Fe(III) oxide reduction, as described below, and flagella could play a role in biofilm formation on electrodes. Some *Geobacter* isolates have been isolated in studies focused on novel physiological properties such as the ability to use aromatic compounds (*G. toluenoxydans*; (Kunapuli et al., 2010)) or reduction of Fe(III) in clays (*G. pickeringii, G. argillaceus*; (E. Shelobolina et al., 2007; Sung et al., 2006). *G. lovleyi* (Sung et al., 2006) is the only *Geobacter* species that has been shown to reductively de-chlorinate the chlorinated solvents tetrachloroethylene (PCE) and trichloroethylene (TCE) that are common groundwater contaminants and 16S rRNA gene sequences closely related to the pure culture have been recovered in dechlorinating enrichment cultures (Daprato et al., 2007) as well as subsurface environments contaminated with chlorinated solvents (Penning et al., 2010).

1.2.2 *Geobacter metallireducens*

G. metallireducens is a Deltaproteobacterium, member of Geobacteraceae. It is a rod shaped Gram-negative, strict anaerobic bacteria. When *G. metallireducens* senses metal oxides it can develop

flagella and pili (see Figure 1.3) (Childers et al., 2002). *G. metallireducens* was isolated the first time from freshwater sediment by Derek Lovley. Metabolic analysis showed its ability to obtain energy through dissimilatory reduction of Fe and Mn (D. R. Lovley, et al., 1987).

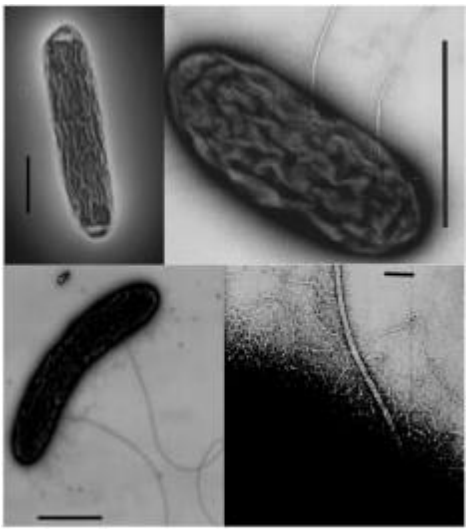


Figure 1.3 Electron micrographs showing the absence of flagella on *G. metallireducens* cells grown with Fe(III)-citrate (top left), in contrast to cells grown in Fe(III) (top right) or Mn(IV) (bottom left) oxides as the terminal electron acceptor. Scale bars, 1 mm. The bottom right panel is a higher resolution electron micrograph of pili on cells. Scale bar, 0.1 mm. The contrast of the image was increased to enhance the visibility of pili. Cells were stained with 4% uranyl acetate and viewed with a JEOL 100S microscope. Reproduced with permission from (Childers et al., 2002).

The ability to reduce Cr (VI) was found later as well by Lovley together with manganese, uranium and other metals (D. R. Lovley, 1993). This metal reduction (besides Fe and Mn) occurs through metabolism or co-metabolism. *G. metallireducens* was the first organism found to be able to completely oxidize organic compounds to carbon dioxide using iron oxides as the electron acceptor (D. R. Lovley et al., 2004). *G. metallireducens* can oxidize short chain fatty acids, alcohols and mono-aromatic compounds such as toluene and phenol using iron as its electron acceptor (D. R. Lovley, 1993). *G. metallireducens* also takes part in carbon, nutrient and geochemistry cycles and in metal bioremediation, transforming soluble and harmful contaminants into insoluble and harmless forms (Childers et al., 2002).

The genome of *G. metallireducens* has been sequenced and is available in gene data bank (Copeland et al., 2005); it is a 4.01 Mbp genome consisting of 1) a 3,997,420 bp circular chromosome encoding 3,621 genes with a GC content of 59.51% and 2) a 13,762 bp plasmid encoding 13 genes with a GC content of 52.48% (Aklujkar et al., 2009). In the

chromosome are encoded genes of housekeeping pathways like metabolism enzymes, cell structure proteins, chemotaxis sensor, flagella and pili (Rotaru et al., 2011). In the plasmid are encoded a gene for a toxin and the related protein resistance, known as RelE/StbE system. The genes encoding the flagella synthesis are induced only when iron oxide is sensed and soluble electron acceptors are in low concentration (H. S. Park et al., 2001; Childers et al., 2002). *G. metallireducens* was originally thought to be immotile because they were grown under laboratory and favourable conditions. When soluble metals were replaced with less favourable iron oxide *G. metallireducens* synthesized flagella (Childers et al., 2002) to move towards and reduce the metal through direct electron transfer by outer membrane cytochromes (D. R. Lovley et al., 2004) and pili (Reguera et al., 2005a). As cited before, the chromosome of *G. metallireducens* contains genes that allow the ability of chemotaxis and flagella synthesis. Chemotaxis allows *G. metallireducens* to detect compounds and evaluate the environment and together with motility, allows *G. metallireducens* to move where the environmental conditions have higher nutrient and metal concentrations (H. S. Park et al., 2001; Childers et al., 2002).

G. metallireducens shows greater metabolic versatility than other *Geobacteraceae* species, and comparative genomic analysis suggests that metabolism, physiology and regulation of gene expression in *G. metallireducens* may be dramatically different from other *Geobacteraceae* (Aklujkar et al., 2009)**.** For this and other reasons that will be discussed in the next chapter, we have chosen *G. metallireducens* for our work.

G. metallireducens shows greater metabolic versatility than other *Geobacteraceae* species, and comparative genomic analysis suggests that metabolism, physiology and regulation of gene expression in *G. metallireducens* may be dramatically different from other *Geobacteraceae* (Lloyd et al., 2003)**.** For this and other reasons that will be discussed in the next chapter, we have chosen *G. metallireducens* for our work.

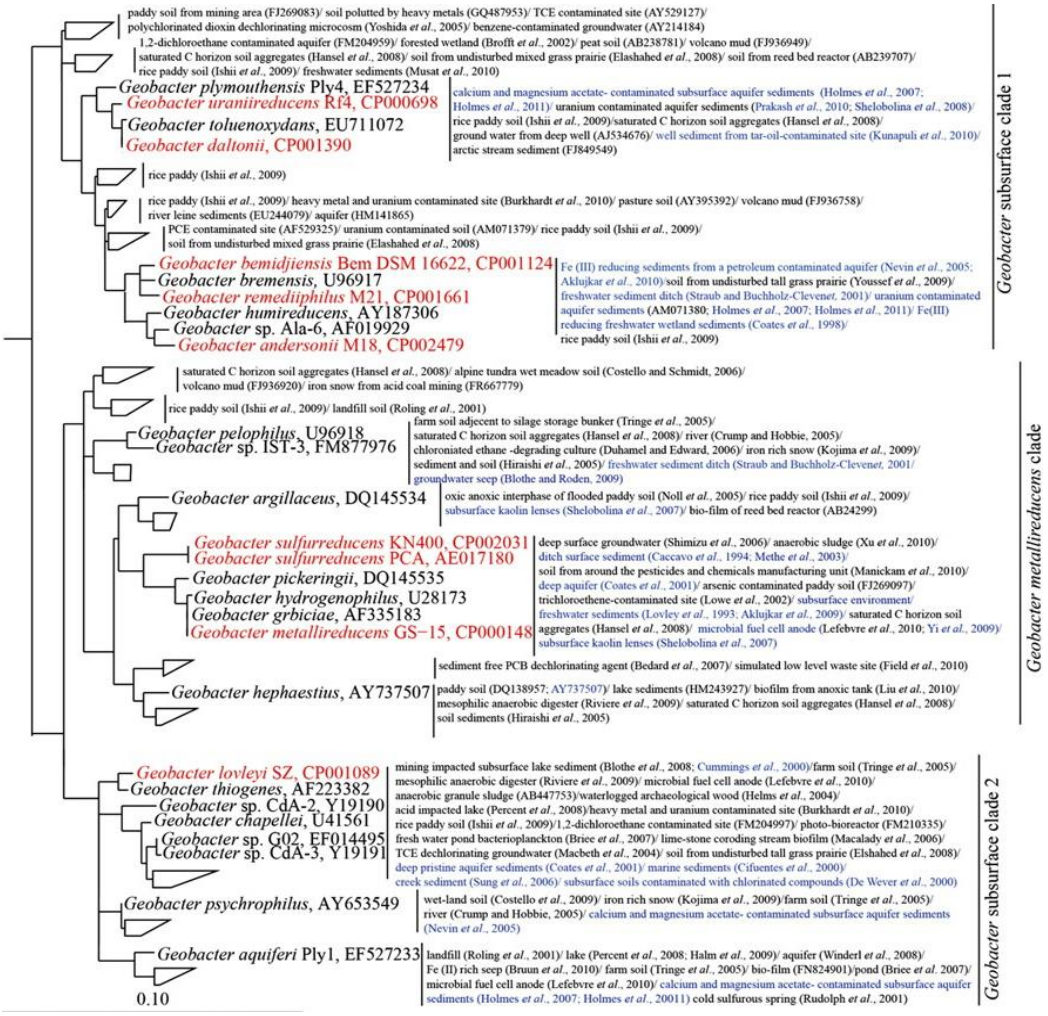


Figure 1.4 Neighbour-joining tree showing the phylogenetic relationship within the genus Geobacter based on 16S rRNA gene sequences. The clone sequences having >98% 16S rRNA gene sequence identities were grouped into a single cluster. Cultured representatives (black), including isolates whose genomes are fully sequenced (red) are shown in the figure. Isolation source and the reference for both pure culture isolates (blue) and representatives environmental clone sequences (black) are also shown at the right side of the tree. The sequences assigned as unpublished in the NCBI and SILVA databases are presented with their accession number. The scale bar represents 10% sequence divergence. Imagine reproduced from Rotaru et al., (2011).

1.2.3 *Geobacter sulfurreducens*

Geobacter sulfurreducens is an important model of DMRB isolated for the first time in 1994 in a soil contaminated by hydrocarbons in Oklahoma (Caccavo et al., 1994). *G. sulfurreducens* are found in the anaerobic subsurface and are one of the predominant metal-reducing bacteria. *G. sulfurreducens* can oxidize organic compounds coupling this activity to the reduction of metals such as Fe and Mn. *G. sulfurreducens* can completely oxidize acetate to carbon dioxide and water while reducing compounds such as sulphur, fumarate, and some metals including iron oxides (Caccavo et al., 1994).

The genome of *G. sulfurreducens* is 3.8 million base pairs long. It contains genes conferring surprising capabilities including evidence of aerobic metabolism, one-carbon and complex carbon metabolism, motility, and chemotactic behavior. These characteristics, coupled with the possession of many two-component sensors and more than 100 c-type cytochromes, reveal an ability to create alternative, redundant, electron transport networks and offer insights into the process of metal ion reduction in subsurface environments (Methe et al., 2003).

1.2.4 *Shewanella* spp.

Members of the S*hewanella* genus are facultative anaerobes, Gram-negative, biofilm-forming soil gamma-proteobacteria. This metabolic capacity makes them an excellent model organism for biofilm electrochemistry. For this reason, the first part of the work was based on the cultivation and characterization of electron transfer mechanisms in *Shewanella loihica* PV- 4 viable biofilms formed at graphite electrodes in potentiostat-controlled electrochemical cells poised at oxidative potentials (0.2 V vs. Ag/AgCl) (Jain et al., 2012). Members of the *Shewanellaceae* family, except *S. denitrificans*, are capable of coupling the decomposition of organic matter to the reduction of the various terminal electron acceptors that they encounter in the different environments (J. K. Fredrickson et al., 2008) by Extracellular Electron Transfer (EET) as a part of their energy conservation strategy. Because of their metabolic versatility, *Shewanella* spp. are relevant to carbon cycling and have considerable potential for the remediation of contaminated environments and use in microbial fuel cells (Hau & Gralnick, 2007). Many works on the model species *Shewanella oneidensis* MR-1 and other members of this genus have provided new insights into the signal-transduction proteins, regulators, and metabolic and respiratory subsystems that govern the remarkable versatility of *Shewanella* sp. (Coursolle et al., 2010; Sophie et al., 2012).

Because of their EET properties, members of *Shewanellaceae* have been included in the group of electroactive bacteria and their biofilms are often termed electroactive biofilms (EABs) (Marsili 2008). With respect to other well-known EAB-forming bacteria, such as *Geobacter* sp., *Shewanella* sp. have a more adaptable metabolism, since they are a facultative and can grow on many substrates (Biffinger 2011). *Shewanella* sp. express numerous multi-heme cytochromes on the outer membrane that enable DET to the electrodes (Xiong et al., 2006), but also secrete flavins, extracellular redox mediators that facilitate mediated electron transfer (MET) (Marsili et al., 2008).

Shewanella oneidensis strain MR-1 is a facultative anaerobic bacterium that can respire various electron acceptors, many of which require the Mtr respiratory pathway. The core Mtr respiratory pathway includes a periplasmic c-type cytochrome (MtrA), an integral outer-membrane β-barrel protein (MtrB), and an outer-membrane-anchored c-type cytochrome (MtrC). Together, these components facilitate transfer of electrons from the c-type cytochrome CymA in the cytoplasmic membrane to electron acceptors at and beyond the outer-membrane (Coursolle et al., 2010). The genes encoding these core proteins have paralogs in the *S. oneidensis* genome (mtrB and mtrA each have four while mtrC has three) and some of the paralogs of mtrC and mtrA are able to form functional Mtr complexes.

1.2.5 *Desulfovibrio*

Desulfovibrio spp. is a model chemolithotrophic organism that are classified within the sulphate-reducing bacteria (SRB). Those species display environmentally important activities that are a consequence of the unique electron transport components or the production of high levels of H_2S: the capability of SRB to utilize hydrocarbons in pure cultures and consortia has resulted in using these bacteria for bioremediation of BTEX (benzene, toluene, ethylbenzene, and xylene) compounds in contaminated soils (Allen et al., 2008). Since SRB have displayed dissimilatory reduction of U(VI) and Cr(VI), several protocols have been proposed for using SRB in bioremediation of toxic metals(D. R. Lovley, 1993; D. R. Lovley & Phillips, 1994). Additionally, non-specific metal reductase activity has resulted in using SRB for recovery of precious metals (e.g. platinum, palladium and gold) from waste streams (Cabrera et al., 2006). Since bacterially produced sulphide contributes to the souring of oil fields, corrosion of concrete, and discoloration of stonework is a serious problem, there is considerable interest in controlling the sulphidogenic activity of the SRB. The production of bio-sulphide by SRB has led to immobilization of toxic metals and reduction of textile dyes, although the process remains unresolved, SRB play a role in anaerobic methane oxidation which not only contributes to carbon cycle activities but also depletes an important industrial energy reserve.

1.2.6 *Geovibrio*

Geovibrio spp. are Gram-negative DMRB, motile spiral-shaped cells, 0,35 µm in diameter and 2,5-6 µm long,. As other DMRB, *Geovibrio* possesses *c*-type cytochromes. *Geovibrio* spp. can use sulphur, nitrate, fumarate, DMSO and oxygen (under micro-aerophilic conditions) as electron acceptors. Sulphide, hydrogen, formate and acetate act as electron donors for respiratory growth (Janssen et al., 2002). Fumarate, maleate and malate support fermentative growth. The optimal growth temperature is 37-40 °C.

1.3 Extracellular electron transfer mechanism

Effective extracellular electron transfer is one of the hallmark physiological features of *Geobacter* species. The capacity to exchange electrons with its extracellular environment defines the unique ecological niche of *Geobacter* species and is an important feature of the many practical applications of this genus (Holmes et al., 2006a)

1.3.1 Microbial Nanowires

One of the most surprising discoveries in the study of extracellular electron transfer in *Geobacter* species has been the finding that *G. sulfurreducens* (Malvankar & Lovley, 2012) and *G. metallireducens* (Tremblay 2012), produce pili that are electrically conductive. Initial indications that pili were important in extracellular electron transfer came from the observation that *G. metallireducens* expressed pili when grown on Fe(III) or Mn(IV) oxides, but not when grown with soluble, chelated Fe(III) as the electron acceptor (Childers et al., 2002). Studies on pili in *G. sulfurreducens* have demonstrated that this organism can produce pilin-like filaments from several different proteins, but the most abundant filaments are those comprising PilA (Klimes et al., 2010).

Deletion of the gene for PilA, the structural pilin protein, inhibited Fe(III) oxide reduction (Reguera et al., 2005a). Conducting atomic force microscopy demonstrated that the pili were conductive across their diameter (Reguera et al., 2005a). The atomic force microscopy revealed that there were other proteins associated with the pili, but they acted as insulators. Therefore, it was proposed that a method for electron transfer to Fe(III) oxide was longrange electron transport along the pilin filaments. Further, although electron exchanging between cytochromes is the accepted method for biological electron transfer over distance, it was suggested that cytochromes did not mediate the electron transport along the pili (Reguera et al., 2005a). This concept was seriously questioned (L. Shi et al., 2007) because there was no known mechanism for electron transfer along protein filaments. However, subsequent studies have provided a mechanism.

The pili of *G. sulfurreducens* possess metallic-like conductivity comparable to synthetic conducting polymers, such as the organic metal polyaniline (Malvankar & Lovley, 2012). When pilin preparations were spotted on a two-electrode system, they formed a network that conducted electrons between the two electrodes. Preparations from a ΔpilA mutant had conductivities comparable to the buffer control. Treating the pilin

preparation to denature any cytochromes that might have remained associated with the pili had no impact on conductivity. Upon cooling from room temperature, the pilin conductivity increased exponentially, a hallmark of quasi-one-dimensional organic metals. The temperature response would not have been observed if electron hopping between cytochromes was responsible for the electron transfer. Indeed, conductivity of the nanofilaments has a temperature dependence similar to that of a metal (Malvankar & Lovley, 2012). Finally, a study suggested that aromatic amino acids required for pili conductivity and long-range extracellular electron transport in *Geobacter sulfurreducens* (Vargas et al., 2013).

The possibility of electron transport along a protein filament without the involvement of cytochromes is a paradigm shift in biology. The metallic-like mechanism for electron transport along the pili of *G. sulfurreducens* under in vivo conditions is fundamentally different than the conductivity proposed for filaments of other microorganism such as *Shewanella oneidensis*, which was only demonstrated in fixed preparations and was reported to be dependent on the presence of cytochromes (Gorby et al., 2006). However, transport of electrons along bacterial nanowires was demonstrated in *Shewanella oneidensis* MR-1(El-Naggar et al., 2010).

1.3.2 Cytochromes and Multi-copper Proteins

One of the most characterizing features of *Geobacter* species is their abundant c-type cytochromes and the large diversity of cytochromes encoded in their genomes (Butler et al., 2010a), *Geobacter* species possess ca. 100 c-type cytochrome genes per genome, with the exception of *G. lovleyi* (Butler et al., 2010a). There are nine families of well-conserved c-type cytochromes, four of which are encoded together and may constitute a quinone: ferricytochrome c oxidoreductase. However, most of the cytochromes are poorly conserved among the genus and some cytochrome families have only been found in a single species of *Geobacter* (Butler et al., 2012). This, coupled with the fact that the function of c-type cytochromes has only been significantly studied in *G. sulfurreducens*, makes it difficult to make broad generalizations about cytochrome function in *Geobacter* species. PpcA is a family of triheme periplasmic c-type cytochromes that is well conserved. These are among the most abundant c-type cytochromes in the genus and were first studied biochemically in the closely related *Desulfuromonas acetoxidans* (Banci et al., 1996) and *G. metallireducens* (Afkar and Fukumori, 1999; Champine et al., 2000) and then with more detailed functional studies in *G. sulfurreducens* (Lloyd et al., 2003).

PpcA purified from *G. sulfurreducens* contained the expected three hemes with a molecular weight of 9.6 kDa and a midpoint potential of 169.5 mV (Lloyd et al., 2003). Although PpcA is related to the earlier studied cytochrome in *D. acetoxidans*, its redox properties are distinct (Pessanha et al., 2006). Purified PpcA reduced Fe(III) and other metals, but its periplasmic location makes direct reduction of Fe(III) unlikely (Lloyd et al., 2003). The heme groups of PpcA are oriented in parallel or perpendicular to each other (Morgado et al., 2010), an arrangement expected to facilitate rapid electron transfer within and between proteins. Deletion of ppcA did not impact fumarate reduction but did impact reduction of the extracellular electron-acceptors Fe(III), AQDS, and U(VI) with acetate as the electron donor. However, with hydrogen as the electron donor, reduction of extracellular electron acceptors in the mutant and wild type were comparable.

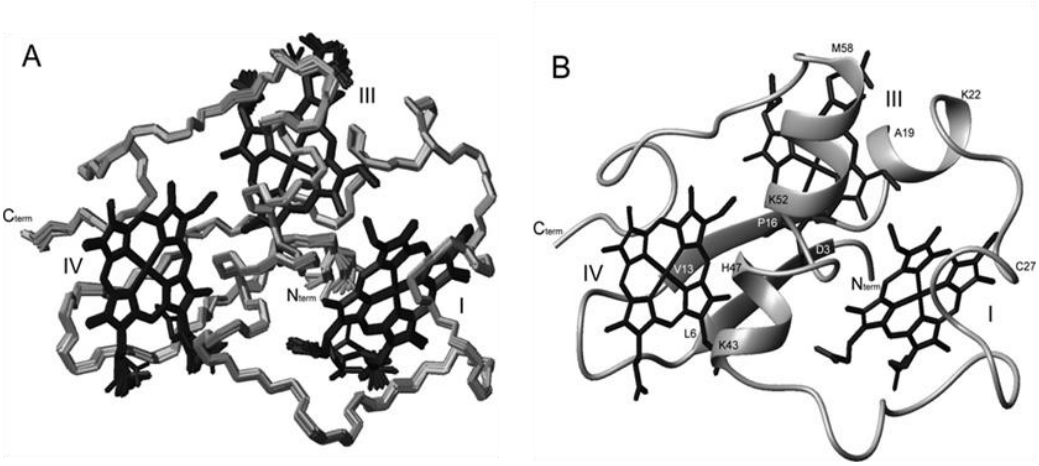


Figure 1.6: solution structure of the c-cyt PpcA of *G. sulfurreducens* (Morgado et al., 2010): (A) Overlay of the 20 lowest energy NMR structures of PpcA at pH 7.1. Superimposition was performed using all of the heavy atoms. The peptide chain and the hemes are coloured grey and black respectively. (B) Ribbon diagram of PpcA structure.

Early studies on *G. sulfurreducens* found significant Fe(III) reductase activity in membrane fractions, which involved cytochromes (Magnuson et al., 2001). One of these cytochromes was purified (Magnuson et al., 2001) and was most likely OmcB (Leang et al., 2003). This cytochrome has a molecular weight of 89 kDa, 12 hemes, and gross midpoint potential of #190 mV with some hemes appearing to have much more negative potentials (Magnuson et al., 2001). The purified protein was capable of reducing Fe(III) oxide and chelated Fe(III). OmcB is embedded in the outer membrane, with a portion of the molecule exposed to the outer surface (Qian et al., 2007). Deleting the gene for OmcB inhibited reduction of Fe(III) citrate and Fe(III) oxide (Leang et al., 2003). Deletion mutants adapted to growth on Fe(III) citrate, but not Fe(III) oxide (Leang & Lovley, 2005). The

presence of multiple RpoS dependent promoters upstream of up-regulated cytochromes in the Fe(III) citrate-adapted mutant suggests that an activated RpoS response permitted *G. sulfurreducens* to compensate for the loss of OmcB (Krushkal et al., 2007). Whereas OmcB is embedded in the outer membrane, several of the *G. sulfurreducens* c-type cytochromes are fully exposed on the outer cell surface.

OmcS is a six-heme c-type cytochrome with a molecular weight of 47 kDa (Qian et al., 2011). Its midpoint redox potential is 212 mV vs. SHE, more negative than that of the periplasmic c-type cytochromes. However, the available evidence suggests that individual hemes span a wide range of potentials. The gene codifying for OmcS is the most upregulated during growth on Fe(III) oxide versus growth on Fe(III) citrate (Holmes et al., 2006a) and this is reflected in the proteome (Ding et al., 2006) and in initial studies that detected omcS transcripts in cells grown on Fe(III) oxide, but not Fe(III) citrate (Mehta et al., 2005). It is also highly expressed under some conditions during growth on electrodes (Holmes et al., 2006b) and in co-cultures of *G. sulfurreducens* and *G. metallireducens* (Summers et al., 2010). Purified OmcS reduced a diversity of potential extracellular electron acceptors for *G. sulfurreducens*, including Fe(III) oxide, U(VI), and humic acids, and also bound Fe(III) oxide (Qian et al., 2011). OmcS is specifically associated with the pili of *G. sulfurreducens* (Leang et al., 2010) and is required for growth on Fe(III) oxide, but not Fe(III) citrate (Mehta et al., 2005).

OmcE is another c-type cytochrome found on the outer cell surface, but its specific localization has yet to be pinpointed. It also has not been purified but is predicted to have a molecular weight of 32 kDa and four hemes (Mehta et al., 2005). Expression patterns of OmcE (Ding et al., 2006; Nevin et al., 2009a), as well as gene deletions studies (Mehta et al., 2005), suggest that OmcE plays a role in extracellular electron transfer in wild-type cells, but cells can adapt to the loss of OmcE.

In contrast to OmcE and OmcS, OmcZ is not required for the reduction of insoluble Fe(III). However, of all *G. sulfurreducens* cytochromes studied to date, only OmcZ is absolutely necessary for high-density current production (Nevin et al., 2009a) . In its mature extracellular form, OmcZ has a molecular weight of 30 kDa, with eight hemes, including an unusual CX14CH motif (Inoue et al., 2010). Its midpoint potential is 220 mV, but as with other multiheme cytochromes individual hemes cover a wide range of potentials. The purified protein can reduce a range of typical soluble extracellular electron acceptors, and Mn(IV) oxides, but only poorly reduced Fe(III) oxide. This corresponds with increased expression of OmcZ during growth on Mn(IV) oxide, but not Fe(III) oxide, versus growth on Fe(III)

citrate (Rotaru et al., 2011)(Holmes et al., 2011b). The poor solubility of OmcZ in water might help maintain it within the extracellular matrix, and is specifically localized at the biofilm–anode interface in high-current density biofilms (Inoue et al., 2010; Inoue et al., 2011). It does not associate with filaments and its expression patterns suggest that its natural function may be to promote the reduction of extracellular soluble electron acceptors. The cytochrome encoded by gene GSU1334 is homologous to OmcZ and a deletion mutant exhibited defects in Fe(III) oxide and U(VI) reduction (E. Shelobolina et al., 2007). However, caution in interpreting such phenotypes is warranted without additional study.

Homologs with four copper-binding sites, two at the N-terminus and two at the C-terminus, are found in all of the *Geobacter* genomes, though the protein size ranges from ca. 800 to 1700 aminoacids (Butler et al., 2010b). Phylogenetically, the omp genes form two distinct clades, the B-type and the C-type, and not all genomes contain both types (Holmes et al., 2008). No homologs were found in the two *Pelobacter* genomes. Various potential roles for OmpB and OmpC have been suggested (Holmes et al., 2008), but purification and characterization of the proteins are required to better evaluate these possibilities. The many other underexplored cytochromes and other putative redox active proteins in *G. sulfurreducens* warrant further study, as do proteins likely to be involved in cytochrome export (Afkar et al., 2005), and the cytochromes in other *Geobacter* species.

Development of genetic systems for *Geobacter* species other than *G. sulfurreducens* can aid in functional analysis, as will the approach of determining which cytochrome functions can be completed in mutants of *G. sulfurreducens* with cytochrome gene sequences from other *Geobacter* species (Yun et al., 2011). A genetic system is available for *G. metallireducens* (Tremblay et al., 2012).

1.3.3 Respiratory chain of two model DMRB

Electron transport chains are a series of biological redox reactions transferring electrons from an electron donor to an electron acceptor. The transfer of electrons is coupled to the translocation of protons across a membrane, producing a proton gradient which supplies the energy to produce ATP. This process is known also as cellular respiration, so the Electron transfer chain is also called Respiratory chain.

Respiratory chains are composed of quinones, cytochromes and Fe-S proteins (Hernandez & Newman, 2001). Indeed, an elevated number of genes coding for *c*-type cytochromes is a typical distinctive characteristic related to the bacteria with a respiratory chain such as DMRB, whose function is to take part in a conductive multi-protein electron transport chain

from the cytoplasm to the cell surface and/or to catalyse terminal electron transfer reactions.

Among DMRB, two organisms have been chosen as models, *Geobacter sulfurreducens* and *Shewanella oneidensis*. Most of the information available about the metabolism and physiology of DMRB are referred to those two species.

In *Shewanella oneidensis* MR-1, as a member of NapC/NirT family of quinol dehydrogenases, inner membrane (IM) *c*-Cyt CymA is capable of oxidizing quinol at IM and reducing the redox proteins, such as *c*-Cyt MtrA, at periplasm (PS). MtrA might also interact with the outer membrane (OM) protein MtrB. Although it is not a *c*-Cyt, MtrB is speculated to facilitate ET across OM to MtrC, an OM *c*-Cyt. Pseudopilus apparatus of T2S, whose formation is regulated by a protein complex in the IM, where only GspF is shown, pushes MtrC and OmcA (another OM *c*-Cyt) from PS through GspD to the surface of bacterial cells where MtrC and OmcA form a functional complex. The cell surface MtrC and OmcA are capable of directly reducing solid Fe(III)/Mn(III, IV) (hydr)oxides.

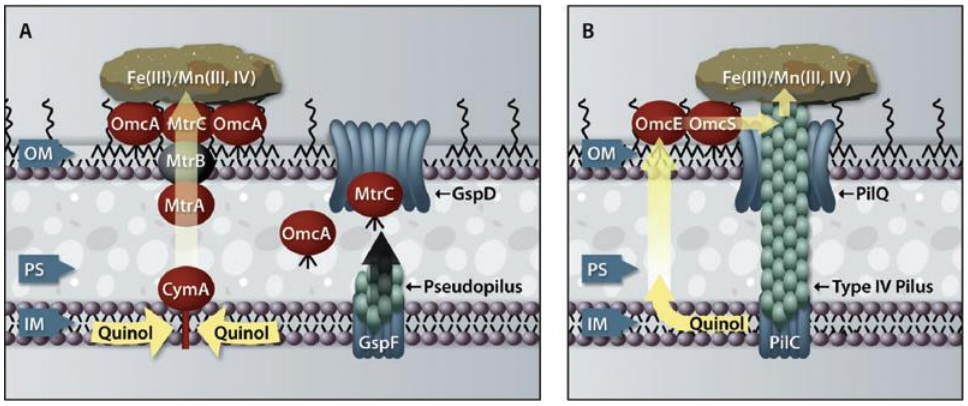


Figure 1.5: Proposed models depicting electron transfer pathways for *S. oneidensis* MR-1 (A) and *G. sulfurreducens* (B) during dissimilatory reduction of solid metal (hydr)oxides (Shi 2007). For simplicity, the quinone-reducing portion of respiratory chain, the peptidoglycan layer and the individual components of the type II secretion system (T2S) and type IV pilus (T4P) biogenesis machine (other than GspD/PilQ, GspF/PilC and pseudo-pilus/pilus apparatus) are omitted from these models. Identified multi-heme *c*-type cytochromes (*c*-Cyts) are in red. Yellow arrows indicate the proposed electron transfer (ET) path. Reproduced from L. Shi et al., (2007).

In *G. sulfurreducens*, OM *c*-Cyts OmcE and OmcS are suggested to transfer electrons to the T4P apparatus, which then transfers electrons directly to solid Fe(III)/Mn(III, IV) (hydr)oxides. The structural components that mediate ET from the IM to OmcE/OmcS in the OM during reduction of solid metal (hydr)oxides have yet to be identified experimentally.

Differently from *G. sulfurreducens*, in *G. metallireducens* the genome hosts a higher number of genes coding for cytochromes. Most studies were conducted using iron as terminal electron acceptor, which is why we will focus this section of the review on the current knowledge about the components of the electron transport chain to ferric iron in *Shewanella* and *Geobacter*. The multitude of phenotypes of different deletion mutants and the catalytic activity of purified enzymes towards different extracellular acceptors is summarized in Table 1.1. Previously published studies on *G. metallireducens* are summarized in the next paragraph.

Table 1.1 Phenotypes of different deletion mutants and catalytic activity of *G. sulfurreducens (K. Richter, Schicklberger, & Gescher, 2012), (K. Richter, Schicklberger, & Gescher, 2012)*towards different extracellular electron acceptors. Reduction rates of the mutants are classified into (-) no phenotype (reduction occurred like in the wild type), (+) mildly affected (reduction rate is slightly impaired compared to the wild type), (++) affected (reduction rate lies roughly between the rate of the wild type and no reduction), and (+++) strongly affected (no or almost no reduction).

Geobacter sulfurreducens		**Electrode**	**Fe(III) oxide**	**Mn(IV) oxide**	**Ferric citrate**	**Humics/ AQDS**	**Cr(VI)**	**U(VI)**
Mutant phenotype	ΔomcB	-	+++		+++	+		
	ΔomcS	++	+++	+++	-	+		
	ΔomcE	-	++	++	-	+		
	ΔomcZ	+++				-		
	ΔomcB			++	-	+++		
	ΔppcA		++			+++		+++
	ΔpilA	+++	+++					++
Catalytic activity of purified enzyme	OmcS		Yes	Yes	Yes	Yes	Yes	Yes
	OmcZ		No	Yes	Yes	Yes	Yes	Yes

1.4 Extracellular electron acceptors

1.4.1 Humic substances

Humic substances are insoluble or soluble organic extracellular electron acceptors. They are not able to diffuse into the outer membrane of Gram-negative bacteria and hence need extended respiratory chain in order to be exploited. Among humic substances, the quinones are the most common redox active moieties. To our knowledge, it was so far not investigated whether there are habitats in which humics serve as the sole anaerobic electron acceptor for microbial anaerobic respiration. Humic acids can serve as electron shuttles that enable electron transfer for instance between a microbial cell and a distantly localized mineral.

1.4.2 Iron

In soil and sediments having neutral pH, reducible iron is solid phase crystalline iron oxides or oxyhydroxides like hematite (Fe_2O_3), goethite (FeOOH), or ferrihydrite (hydrated ferric oxide-oxyhydroxide) that become soluble following the reduction. Several models have been advanced for how *Geobacter* species transfer electrons to insoluble Fe(III) oxides. A wrong calibration procedure of the spectrophotometer in initial studies with *G. metallireducens* (Gorby & Lovley, 1991) resulted in the mistaken suggestion that b-type cytochrome(s) were important in extracellular electron transfer, but subsequent studies demonstrated a role for c-type cytochromes in the reduction of Fe(III) and other metals (D. R. Lovley, 1993). An early model for Fe(III) oxide reduction by *Geobacter sulfurreducens* suggested that it released a low-molecular-weight c-type cytochrome, which acted as an electron shuttle between cells and Fe(III) oxide (Seeliger et al., 1998), but this concept was refuted in the entire scientific community. Evidence consistent with the need for direct contact is the lack of Fe(III) reduction when cells are separated from Fe(III) oxide contained within micro-porous alginate beads (Nevin & Lovley, 2000) or agar (Straub & Schink, 2003). This was observed with *G. metallireducens* (Nevin & Lovley, 2000) as well as *G. sulfurreducens*, *G. bremensis*, and *G. pelophilus* (Straub & Schink, 2003). In contrast, *Shewanella* (Nevin & Lovley, 2002b)(Nevin & Lovley, 2002a) and *Geothrix* species, and Fe(III)-reducing enrichment cultures (Straub & Schink, 2003), produced shuttles that permitted reduction of Fe(III) oxide at a distance. Further, *G. metallireducens* also did not appear to produce chelators that could solubilise Fe(III), whereas *Shewanella* (Nevin & Lovley, 2002b) and *Geothrix* (Nevin & Lovley, 2002a) species did solubilize Fe(III) under similar conditions. Although some of the components that appear to be involved in electron transfer to Fe(III) oxides have been identified, the understanding of how these, and potentially other components, fit together is far from complete. As noted above, OmcS is likely to have an important role in Fe(III) oxide reduction because (1) OmcS expression is highly upregulated

during growth on Fe(III) oxide (Mehta et al., 2005); (2) gene deletion studies indicate that the OmcS is required for Fe(III) oxide reduction (Mehta et al., 2005); (3) OmcS is specially associated with pili (Leang et al., 2010), which, as described above, are electrically conductive and are required for Fe(III) oxide reduction; and (4) purified OmcS can transfer electrons to Fe(III) oxide and may bind Fe(III) (Qian et al., 2011). The simplest explanation for these observations is that electrons that are transported along the pili are transferred to Fe (III) oxide via OmcS. There is no obvious route for electrons to get to OmcS other than the pili and the lack of Fe(III) reduction in the absence of OmcS suggests that electrons cannot be directly transferred from the pili to Fe(III) oxide. There is little information on how electrons are transferred to the pili. This could conceivably take place in the periplasm, or even the inner membrane, but the requirement for OmcB, which is located in the outer membrane, suggests that electron transfer near the outer surface of the cell is more likely. The fact that OmcB is embedded in the outer membrane suggests that it might be difficult for OmcB and pili to associate closely enough for electron transfer between the two. The need to mediate electron transfer from OmcB to the pili at the outer cell surface may explain why other potentially redox-active outer-surface components, such as other c-type cytochromes and the putative multi-copper proteins OmpB and OmpC, are important in Fe(III) oxide reduction.

The role of other outer-surface cytochromes in Fe(III) oxide reduction is not completely understood. OmcE can be an abundant c-type cytochrome under some growth conditions, but cells can eventually overcome deletion of omcE and reduce Fe(III) oxide (Mehta et al., 2005). It has been proposed that OmcZ localized in an extracellular matrix could be important in Fe(III) oxide reduction (Rollefson et al., 2011), but this is not consistent with several observations:(1) OmcZ is not required for Fe(III) oxide reduction (Nevin et al., 2009a), (2) low levels of OmcZ in cells growing on Fe(III) oxide (Holmes et al., 2011b), and (3) purified OmcZ only poorly reduces Fe(III) oxide (Inoue et al., 2010). If OmcB is the conduit for electrons out of the cell and toward pili, then there is a need to clarify which is the electron donor for OmcB. Periplasmic cytochromes are potential sources, ferrying electrons from the inner membrane to the outer membrane. As noted above, a number of periplasmic c-type cytochromes have been identified in *G. sulfurreducens*, but no electron transfer link between these cytochromes and OmcB, or any other electron acceptor, has been documented. Diagrams for how the electrons may flow to Fe(III) oxide from *G. sulfurreducens* are available (Qian et al., 2011), but clearly we are still at the hypothesis stage and more research on electron transfer out of the cell is warranted. Novel strategies for elucidating important components are likely to be helpful. For example, adaptive evolution for improved Fe(III)

oxide reduction in *G. sulfurreducens* provided further evidence for the importance of pili in Fe(III) oxide reduction as well as identifying an additional c-type cytochrome that may be involved (Tremblay et al., 2011). Studies on species other than *G. sulfurreducens* are also warranted to look for commonalities that are general features of electron transfer to Fe(III) oxides in all *Geobacter* species. For example, unique PilA sequences are conserved in *Geobacte*r species (Reguera et al., 2005a) and recent gene deletion studies have demonstrated that PilA is required for Fe(III) oxide reduction in *G. metallireducens* (Tremblay et al., 2011). In contrast, outer-surface cytochromes' sequences are poorly conserved in *Geobacter* species (Butler et al., 2010a), suggesting that there is less specificity in cytochrome requirements. However, there is still an opportunity to look for commonality in mechanisms. For example, if electrons cannot be directly transferred from pili to Fe(III) oxides, then it would be expected that *G. metallireducens*, which does not have an OmcS homolog (Butler et al., 2010a), would possess another cytochrome, which like OmcS, is associated with pili and necessary for Fe(III) oxide reduction. Additional research is also required on the early steps of electron transfer across the inner membrane and to the electron carriers responsible for the terminal steps in electron transfer to Fe(III) and other extracellular electron acceptors. Although possible electron carriers can be identified from genome sequences, experimental studies are required before definitive models can be developed. One of the key features of extracellular electron transfer in *Geobacter* species is the poor energy yields available from this mode of respiration in comparison with the reduction of soluble electron acceptors within the cell (Esteve-Núnez et al, 2004; Mahadevan et al., 2006). This can be attributed, at least in part, to the fact that intracellular reduction of electron acceptors consumes protons along with electrons, but when electrons are transferred out of the cell, this proton sink is lost, requiring export of protons that does not contribute to the development of a proton-motive force across the inner membrane (Mahadevan et al., 2006).

1.4.3 Electrodes

Like Fe(III) oxide, electrodes represent an insoluble, extracellular electron acceptor. Initial studies with *G. sulfurreducens* suggested that it did not produce electron shuttles in order to promote electron transfer to electrodes (Bond & Lovley, 2003) and electrochemical studies supported this conclusion (Busalmen et al., 2008; Marsili et al., 2008; Marsili et al., 2010; H. Richter et al., 2009). This is consistent with the similar concept of direct electron transfer to Fe(III) oxide. However, there are major differences between the electrodes and Fe (III) oxide because electrodes function as stable long-term electron acceptors, whereas once Fe(III) is reduced in one location cells need to find additional sources of Fe(III). The

stability of the electrode as an electron acceptor makes it possible for *Geobacter* to produce thick (>50 mm) biofilms on electrodes (Franks et al., 2009; Nevin et al., 2009a; Reguera et al., 2006), which are not formed during growth on Fe(III) oxide. Thus, the necessity to transfer electrons through a biofilm may require different electron transport strategies and may place different selective pressures on cells. Fashioning one coherent model for electron transfer from *G. sulfurreducens* to electrodes that can accommodate all the data available in the literature is difficult. There is substantial confusion in the literature because models generated from preliminary data are often ruled out as more data becomes available. For example, early studies in our laboratory investigated electron transfer in systems producing relatively low amounts of current in which most of the cells were closely associated with the anode surface. Under those conditions, OmcS was highly expressed and was essential for current production (Holmes et al., 2006a). In contrast, in subsequent studies with systems producing much more current, OmcS was not highly expressed and cells adapted to produce current comparable to that of wild type when OmcS was deleted (Nevin et al., 2009a). Rather, OmcZ was highly expressed in the high-current density biofilms. OmcZ and OmcS do not appear to have equivalent functions, based on their different localization and other factors, and it is generally the case that when OmcS is highly expressed OmcZ expression is low and vice versa. The geometry of the electrode material may also influence gene expression patterns, and presumably electron transfer pathways. Therefore, instead of attempting to develop one universal model for electron transfer to electrodes, most research groups have focused on electron transfer in thick (>50 mm) electrode biofilms, which produce high-current densities, because a major goal is to understand the production of high-current densities in order to further optimize current output. An initial observation in the development of higher current densities was that the increase in current was proportional to the increase in biomass on the anode, suggesting that cells at great distance from the anode were contributing to current production (Reguera et al., 2006). Subsequent studies have confirmed the high metabolic activity of such cells (Franks & Nevin, 2010). The finding that deleting pilA prevented high-current densities led to the hypothesis that networks of pili in the *G. sulfurreducens* biofilms conferred conductivity on the biofilm and a route for electrons released from cells at distance to be transported to the electrode (Reguera et al., 2006). Consistent with this concept, modelling studies indicated that the high current density in microbial fuel cells would be feasible only if *Geobacter* biofilms were assumed to be electrically conductive (Torres et al, 2008). However, other studies suggested that biofilms of bacteria act as insulators (Dheilly et al., 2008). Measurement of the conductance of viable *G. sulfurreducens* biofilms with a novel two-electrode system revealed that the biofilms that had been grown with an

electrode as the electron acceptor had remarkable conductivity, comparable to that of synthetic organic conducting polymers, such as polyaniline and polyacetylene (Malvankar & Lovley, 2012). In contrast, biofilms grown in the same system, but with fumarate as the electron acceptor, had low conductivity. Biofilms of *Escherichia coli* and *Pseudomonas aeruginosa* were not conductive. Evaluation of different strains of *G. sulfurreducens* with different biofilm conductivities demonstrated a strong correlation between the abundance of PilA in the biofilm and conductivity, suggesting that the conductivity was related to the extent of pilin production. The temperature dependence of biofilm conductivity was similar to that of pilin preparations, demonstrating a metallic-like conduction mechanism, which was further confirmed with electrochemical gating studies (Malvankar & Lovley, 2012). These results suggested that the biofilm conductivity was related to the metallic-like conductivity of the pilin network. None of these results support the concept of electron hopping through biofilms via c-type cytochromes. Further, denaturing the c-type cytochromes in the biofilms had no impact on conductance and there was no correlation between conductance and cytochrome content of the biofilms. These results suggest that the novel metallic-like conductivity in *G. sulfurreducens* can be attributed to the surprising metallic-like conductivity of its pilin networks. Consistent with the apparent importance of pili in conduction of electrons through *G. sulfurreducens* biofilms, the gene for PilA is among the most highly upregulated genes in current-producing biofilms (Nevin et al., 2009a). Selective pressure for enhanced current production yielded a strain of *G. sulfurreducens* that produced more pili (Li et al., 2011). Deletion of *pilA* significantly inhibited current production, with only cells near the electrode surface remaining metabolically active (Reguera et al., 2006). Although the pilin constructed of PilA may have a structural role in biofilm formation under some conditions (Reguera et al., 2007), the pilA deletion mutant readily formed thick biofilms on the graphite electrode material if fumarate was provided as an alternative electron acceptor (Nevin et al., 2009a).

The concept of electron transport through *G. sulfurreducens* biofilms via conductive pilin networks contrasts with many studies that have suggested that more traditional electron transfer via cytochromes moves electrons through the biofilms. Biofilms of wild-type *G. sulfurreducens* growing on electrodes are visibly red, due to the cytochrome abundance. Many studies have provided evidence that cytochromes are oxidized and reduced in *G. sulfurreducens* biofilms in electrical contact with electrodes (Fricke et al., 2008; Jain et al., 2012; Marsili et al., 2008; Marsili et al., 2010) but the interpretation that this represents electron transfer through the biofilm by electron hopping via c-type cytochromes in analogy with redox hydrogels (H. Richter et al., 2009) is not consistent with the studies (Malvankar & Lovley, 2012) on biofilm conductance.

The likely explanation for this apparent discrepancy is that the electrochemical analyses only probed the biofilm-electrode interface and not the entire biofilm (Dumas et al., 2008; Franks & Nevin, 2010). The cytochromes at the interface may function as an electrochemical gate, promoting electron transfer to the electrode surface (Dumas et al., 2008). A likely candidate for a cytochrome functioning as an electrochemical gate is the outer-surface c-type cytochrome OmcZ. The omcZ gene is one of the most highly upregulated genes in current-producing cells, and if omcZ is deleted, the cells produce low levels of current (Nevin et al, 2009a). There is much higher resistance for electron transfer to electrodes in cells lacking OmcZ, which was originally interpreted as OmcZ conferring conductivity throughout the biofilm (H. Richter et al., 2009). However, this cannot be correct as the conductance of biofilms of a strain with lower abundance of OmcZ was higher than those of wild type (Malvankar & Lovley, 2012). Further, cells throughout the biofilm express omcZ (Reith, 2011). OmcZ accumulates at the biofilm-electrode interface, consistent with the electrochemical gate hypothesis (Inoue et al., 2011). The reason that OmcZ or other cytochromes might be required to facilitate current production is that a significant energy barrier might exist across the biofilm-electrode interface similar to a semiconductor–metal interface. The wide range of reduction potentials (420 to 60 mV) of the multiple hemes in OmcZ (Inoue et al., 2010) might help overcome this energy barrier in a manner similar to electrochemical gating in molecular electronics.

1.4.4 Chromium and other metals as Extracellular Electron Acceptors

The display of multiple low-potential c-type cytochromes on the outer surface of *Geobacter* species confers the capacity to reduce a wide diversity of soluble electron acceptors at the outer cell surface. Reduction of these electron acceptors may be rather nonspecific. For example, deleting the genes for individual outer-surface cytochromes only partially inhibited the ability of *G. sulfurreducens* to reduce humic substances and anthraquinone-2,6-disulfonate (AQDS). Only when the genes for OmcB, OmcE, OmcS, OmcT, and OmcZ were deleted in the same strain, both humic substance and AQDS reduction did not occur (Voordecker et al., 2010). Although the final product of U(VI) reduction is U(IV), the initial reduction of U(VI) may be a one electron transfer followed by disproportionation of U(V) to U(VI) and U(IV) (Renshaw et al., 2005). Initially it was considered that U(VI) might be reduced in the periplasm (Lloyd et al., 2003), but the accumulation of uranium in the periplasm that was a main line of evidence for periplasmic reduction was later found to be an artefact (E. Shelobolina et al., 2007). Systematic deletion of the genes for the most abundant outer-surface c-type cytochromes in a study comparable to one on reduction of humic substances has indicated that the site of reduction is

the outer surface of the cell. Purified OmcZ (Inoue et al., 2010) and OmcS (Qian et al., 2011) reduce U(VI), and it is likely that many low-potential c-type cytochromes will be capable of U(VI) reduction (D. R. Lovley, 1993). It seems likely that the other metallic ions that *Geobacter* species can reduce may also be reduced in a similar nonspecific manner. In vitro studies with the abundant periplasmic c-type cytochrome of the closely related *Desulfuromonas acetoxidans* demonstrated that these cytochromes could reduce elemental sulphur in vitro (Pereira et al., 1997) and periplasmic reduction of sulphur has been a model.

1.5 Electroactive bacteria

Electroactive bacteria generate energy by coupling the flow of electrons in the membrane to the electrochemical proton gradient. The electrons flow from primary electron donors to terminal electron acceptors through intermediate electron transfer agents, such as outer membrane cytochromes and periplasmic proteins (K. Richter et al., 2012). Because of their unique properties, some DMRB were recently termed among the electroactive bacteria. Extracellular electron transfer in electroactive bacteria is not yet well understood. Electrochemical methods like the one shown in this thesis work can measure and characterize this process.

Production of electrical current from electrodes placed in microbial cultures was observed nearly a century ago (Potter, 1911). Since that observation, the attempts to convert the chemical energy of organic matter directly into electrical energy using devices like microbial fuel cells (MFC) were explored throughout the 20th century (Roller et al., 1984), and many species have been found to be electroactive.

1.6 Bioremediation

Bioremediation is the utilization of organisms or derivatives from organisms to degrade pollutants. The main advantage of bioremediation versus phisico-chemical remediation technologies is the reduced cost. Moreover, bioremediation is often a permanent solution because it allows the complete transformation of the pollutant to its molecular constituents like carbon dioxide and water and the eventual addiction of pollutant would not require any intervention because the bacteria are already in the contaminated place (Allen et al., 2008). In this chapter, we will review briefly the current biological method for Cr detoxification, with particular respect to those involving DMRB.

1.6.1 Chromium

Chromium is widely used heavy metals, which found application in stainless steel and non-ferrous alloys electroplating, dyes and pigment, and tannery industry (Beukes et al., 1999). The most common oxidation states of chromium are Cr(III) and Cr(VI). Cr(III) is essential in animal glucose metabolism (Mertz 1993), while Cr(VI) has no known biological function. While Cr(III) solubility and toxicity are low (Cervantes et al., 2001; Huvinen et al., 2002), Cr(VI) is highly soluble in water and toxic to life. In humans, Cr(VI) is known to induce skin sensitization, breathing problems and stomach disease, and is a suspected carcinogen. Cr(VI) affects expression of fundamental genes involved in sensing heavy metal load (Kimura et al., 2008) and can cause cell apoptosis through DNA fragmentation. The effect of small daily intake of Cr(VI) is not yet clear, however the precautionary principle suggests that we minimize the Cr(VI) in drinking water, while waiting for undisputed scientific evidence. Moreover, the most recent opinion tends to confirm the chromium danger and reject the role of Cr(III) as a micronutrient. Since Cr(VI) is also very toxic to microorganisms and plant, sites contaminated by chromium cannot be used for agricultural and recreational purposes. As all heavy metals, Cr(VI) is not biodegradable and it is a long-term public health concern.

Both European and Irish environmental regulations for Cr(VI) established a threshold of 5-30 μg L^{-1} in groundwater and drinking water. In order to meet these stringent requirements, efficient Cr(VI) remediation techniques are needed. Conventional methods for Cr(VI) remediation include off-site chemical reduction of Cr(VI) to sparingly soluble Cr(III). Although effective, these techniques are limited by high operating cost and toxic sludge generation (Zhou et al., 2008). Also, these methods are inadequate to treat the source of contamination if the groundwater is not easily accessible.

Bioremediation is a complementary strategy for Cr(VI) detoxification in which microorganisms reduce or complex Cr(VI) to less toxic and mobile forms (Alam et al., 2006). Several anaerobic respiratory bacteria can use Cr(VI) as a terminal electron acceptor, and therefore reduce it to Cr(III), which is removed from the groundwater flow (Brim et al., 2006; Mabbett et al. , 2004).

Current remediation treatment involves reduction of Cr(VI) to Cr(III) using chemical reducing agents such as Fe at low pH, followed by adjustment to near-neutral pH and subsequent precipitation of Cr(III). Recent studies, however, have shown that micro-organisms can also reduce Cr(VI) efficiently at circumneutral pH, and could be used to treat Cr(VI)-contaminated water. Chromium (Cr) has three main valence forms, Cr(0), Cr(III), and Cr(VI). Cr(III) occurs naturally in the environment (e.g. in chromite), while Cr(0) and Cr(VI) are industrial by-products. Chromium is an essential alloy in stainless steel and is used in furnaces, bricks, dyes and pigments, chrome plating, chemical catalysts, leather tanning and wood

preserving. Chromium is discharged in the environment from mining, chemical manufacturing, fossil fuels, combustion, waste incineration and steel making.

1.6.2 Limits of current bioremediation methods

Bacterial reduction of metallic ions has been shown to occur for U(VI), Se(VI), Cr(VI), Mo(VI), Se(IV), Hg(II), Ag(I) and others (Bradley & Chapelle, 1998; D. R. Lovley, 1993). A wide range of bacteria has been identified that are capable of carrying out a complete reduction of Cr(VI) to Cr(III) by oxidation–reduction reactions of biotic and abiotic nature. Microbial reduction of Cr(VI) to Cr(III) can be considered as an additional chromate resistance mechanism

which is not usually a plasmid-associated trait (Cervantes et al., 2001). Cr(VI) reduction outside the cell generates Cr(III) which cannot cross cellular membranes. Three Cr(VI) reduction mechanisms have been described (Cervantes and Campos-Garcıa 2007): (i) In aerobic conditions, chromate reduction has been commonly associated with soluble chromate reductases that use NADH or NADPH as cofactors. (ii) Under anaerobiosis, some bacteria, like *Pseudomonas fluorescens* LB300 (Ganguli & Tripathi, 2002; Ramírez-Díaz et al., 2008a; Ramírez-Díaz et al., 2008b), can use Cr(VI) as an electron acceptor in the electron transport chain.

(iii) Reduction of Cr(VI) may also be carried out by chemical reactions associated with compounds such as amino acids, nucleotides, sugars, vitamins, organic acids or glutathione. For instance, ascorbate is capable of reducing Cr(VI), and riboflavin derivatives FAD and FMN are essential coenzymes for chromate-reducing flavoenzymes (Ramírez-Díaz et al., 2008a).

Microorganisms have evolved diverse resistance mechanisms to cope with chromate toxicity. These systems include direct strategies that involve the efflux of toxic chromate ions from the cytoplasm or the transformation of Cr(VI) to innocuous Cr(III) outside the cell. Several probable Cr(VI) membrane transporters have been identified and they have been grouped into a large superfamily, although only two bacterial homologous able to extrude chromate are well characterized. Many bacterial species are reported to reduce Cr(VI) to Cr(III), but the biochemical properties of only a few Cr(VI) reductases have been elucidated. The diverse characteristics of these ancient enzymes and their wide distribution support the hypothesis that reduction of chromate is a secondary role for Cr reductases. Diverse bacterial species seem to display indirect systems of tolerance to Cr. After chromate exposure, these bacteria show a varied regulatory network that involves the expression of genes for several different metabolic processes as a Cr stress defensive strategy. These include genes for sulphur or iron homeostasis and ROS detoxification. These indirect systems of tolerance to Cr include mechanisms focused to maintain the integrity of the cells by

protecting them from oxidative stress or to repair the damages caused by Cr derivatives.

1.6.3 Current molecular biology and genetic approach to improve bioremediation

Microbial catalysts have enormous catabolic potential for remediating wastes because of their huge number of pathways and plasticity; however, the interactions between bacteria and pollutants are often complex and suitable remediation is not often available. Moreover, many anthropogenic compounds lack good biological catalysts, and apparently good biocatalysts fail to transform pollutants when transferred to (D. R. Lovley & Phillips, 1994) the environment (J. Ramos et al., 2011). Hence, the field remains a fertile area for the application of new biotechnological methods to facilitate bioremediation such as metabolic engineering, proteomics, reverse genetics, transcriptomics, metabolomics and genome-scale metabolic modelling. In addition, follow-on studies are important for determining why pollutants persist. Metabolic engineering involves redirecting the cell's metabolism to achieve a particular goal using recombinant engineering (Pieper & Reineke, 2000).

1.6.3.1 Genetic methods

Current bioremediation processes use native microorganisms, environmental isolates from contaminated soil and sediments, and strains selected through conventional sub-culturing procedures. Although existing microorganisms are effective for bioremediation, numerous laboratory studies demonstrated the usefulness of genetic engineering methods for the creation of strains with a) higher toxicity resistances, b) aggregated degradation pathways, and c) faster bioremediation rates. These genetic strain improvement methods mimic natural processes of gene flow and DNA evolution that occur in subsurface biofilms (Coppi et al., 2001)(Massoudieh et al., 2007).

1.6.3.2 Increased resistance to toxicity

The low resistance of microorganisms used in bioremediation to the toxicity of soil contaminants limits the field application of bioremediation. Heavy metals, novel chemicals, solvents and pesticides are of particular concern since microorganisms may not have developed metabolic pathways to degrade these pollutants. Several works report methods and strategies to improve microbial resistance. The most common strategies are based on genetic engineering. For example, *Stenotrophomonas* sp. strain YC-1, a native soil bacterium that produces methyl parathion hydrolase was genetically engineered by incorporating *Pseudomonas syringae* genes possessing a broader substrate range for organophosphates (OPs), allowing the strain to degrade a mixture of six OP pesticides (0.2 mM each) completely within 5 h. The broader substrate specificity in combination with the rapid degradation rate made the engineered strain a promising candidate for in situ remediation of OP-contaminated sites (Yang et al., 2006). In another study, an engineered *Deinococcus radiodurans* expressing toluene dioxygenase was able to oxidize toluene, chlorobenzene, 3,4-dichloro-1-butene, and indole, and was also tolerant to the solvent effects of toluene and trichloroethylene at levels exceeding those of many radioactive waste sites in the presence of radionuclide irradiation (Lange et al., 1998; T. Zhang et al., 2010). Also, eight DNA-shuffled toluene *ortho***-**monooxygenase genes expressed by engineered *Escherichia coli* showed an improved degradation capability against chlorinated compounds (Rui et al., 2004). Toxicity resistance is a very complex character, and may be the result of several site-specific genetic modifications. Genome shuffling generates a large amount of mutants, among which the ones that present the desired characteristics can be isolated with a well-defined screening method.

1.6.3.3 Aggregated degradation pathways

Existing degradation pathways from different microorganisms can be combined in a single strain through genetic engineering methods. The insertion of foreign degradation cluster(s) in the chromosome or in plasmids (Lajoie et al., 1993) result in stable strains, even in field conditions. The catabolic genes can be stably maintained also in groundwater aquifer material without specific selective pressure for the introduced genotypes (Paul et al., 2005). The resulting strain can degrade completely a target pollutant, thereby simplifying the bioremediation treatment in the laboratory and in the field. For example, complete mineralization of polychlorinated biphenyl (PCB) was achieved through the stable incorporation of *Burkholderia xenovorans* LB400 genes into the chlorobenzoate degrading bacterium *Cupriavidus necator* JMP134-X3 through the mini-TN5 transposon system and biparental mating (Bro et al., 2006). The genetically

modified strain was added to non-sterile, PCB contaminated soil and a strong increase in PCB mineralization was observed. Genes encoding the 2,4-dinitrotoluene (DNT) degradation pathway enzymes were taken from another Burkholderia strain and incorporated into the genome of the psychrotolerant rhizobacterium *Pseudomonas fluorescens*. The engineered strain was stable and capable of growing on 2,4-DNT as sole nitrogen source, even at low temperatures (Martin et al., 2003¸Monti et al., 2005). In another study, the genes encoding an alcohol dehydrogenase and an esterase from *Pseudomonas fluorescens* were cloned into a vector and functionally expressed in *E. coli*, demonstrating that three recombinant enzymes can be applied in alkane biodegradation (Kirschner et al., 2007).

1.6.3.4 Accelerated bioremediation rate

A quite different approach was adopted to increase metal reduction rate in the dissimilatory metal reducing bacterium (DMRB) *Geobacter sulfurreducens*. Following *in silico* model prediction, the authors engineered the strain in order to increase ATP consumption. This was achieved through the creation of ATP consuming futile cycles and through the reduction of internal ATP reserves. Genome-wide analysis of gene transcript levels showed that the modified strain produced lower amount of biomass per equivalent of electron transfer and had a higher rate of respiration than the control strain. Respiration rates in DMRB are strictly correlated with metal reduction rates, therefore the engineered strain may increase metal bioimmobilization rates (Ro et al., 2006)(Izallalen et al., 2008).

As with any other application of genetically engineered microorganism, risks and ethical concern may arise from the open-field application and should be discussed within the current regulatory regime (Snow et al., 2005). Restrictions on the use of genetically modified microorganisms can be avoided by other using genetic modification methods instead, such as genome shuffling. In fact, an organism treated by genome shuffling may be not considered a GMO (Roller et al., 1984) (Ahmed, 2003).

Genome shuffling is a powerful tool to improve bacterial phenotypes producing highly recombined and evolved progeny in a short time. In brief, a large number of mutants, generated through random mutagenesis are screened for improved phenotype characteristics (e.g., higher degradation rate of a pollutant) and recombined by multiple rounds of protoplast fusion. Genome shuffling saves work and time with respect to conventional mutagenesis methods but, it has been little applied to bioremediation. Nonetheless, Dai and Copley used genome shuffling to improve the degradation rate of PCP by *Sphingobium chlorophenolicum* (Dai & Copley, 2004). After three rounds of shuffling, several faster and more PCP tolerant strains were obtained.

G. metallireducens use the same respiratory chain for Fe(III) and Cr(VI) bioreduction. In the case of Cr(VI), the reduction to Cr(III) serves as protection against the toxicity of Cr(VI). Genome shuffling will enhance the Cr(VI) tolerance and Cr(VI) bioremediation rate of *G. metallireducens.*

1.6.4 Engineering improvement

The limiting factors in bioremediation process design are the bioavailability of the pollutant to degrading microorganisms and its solubility. This is particularly important for polyaromatic hydrocarbons (PAH) and PCBs. The addition of chemical surfactants improves PAH bioavailability (Rodriguez & Bishop, 2008) and increases degradation rates. The addition of Tween 90, a non-ionic surfactant, increases bioremediation rates of chlorinated pesticide contaminated soil in methanogenic granular sludge (H. U. Kim et al., 2008), (Baczynski & Pleissner, 2009). In another example, coconut fatty alcohol sulphate (CFAS) was added to soil in order to increase bioremediation rate of organophosphate insecticide (Bobiles et al., 2009). However, the addition of synthetic surfactants or surfactants derived from agricultural products increases costs, particularly for inaccessible soils and groundwater, and may raise environmental concern. A possible solution to this problem is the use of environmentally friendly, microbially produced surfactants, which can be generated in situ during the bioremediation treatment, thereby reducing costs and simplifying the bioremediation treatment. Many microorganisms produce biosurfactants when they grow on water-immiscible substrates. The most common biosurfactants are glycolipids, where the carbohydrates are attached to a long-chain aliphatic acid. However, other more complex surfactants, such as lipopeptides, lipoproteins, and heteropolysaccharides, were reported (Sauer, 2001)(Desai & Banat, 1997). Also, bio-emulsifiers, such as glycolipids and polymeric surfactant (e.g., lipoprotein and lipopeptides) increase bioremediation rate of hydrocarbon polluted soils, because they increase the displacement of hydrocarbons from soil particles (Al Hasin et al., 2009; Calvo et al., 2009). The most studied biosurfactants are the rhamnolipids, produced by *Pseudomonas aeruginosa*. They are probably the oldest biosurfactants identified (Kuyper et al., 2005)(Y. Zhang & Miller, 1992) and one of the first that enter industrial production (Kuyper et al., 2005; LaGrega et al., 2011)(Mukherjee & Bordoloi, 2011). Many other microbially produced surfactants have been identified and it is expected that they will contribute to faster oil product biodegradation (W. Wei et al., 2001a)(Okoh & Trejo-Hernandez, 2006). While crude oil is a common substrate for many aerobic and anaerobic microorganisms, it is difficult to degrade it in a cost-effective manner when spread over a large surface, as in the case of the recent (April, 2010) oil spill in the offshore oil rig off Louisiana coast (USA). Microbially produced biosurfactants will enable faster and more sustainable bioremediation treatment after these rare but catastrophic events occur.

1.6.5 Chromium bioremediation

Cr(VI) bioremediation occurs through the biosorption of Cr(VI) in the biofilm EPS or the bioreduction of the toxic form Cr(VI) to the less toxic form Cr(III). In the first case, biofilms need to be periodically removed when saturated in Cr(VI). This strategy is therefore less fit for *in situ* bioremediation but more efficient for *ex situ* processes (Gadd, 2010). Bioreduction to Cr(III) provides a long-term solution to Cr(VI) contamination. Although there is no known strain capable of growing with Cr(VI) as sole electron acceptor, numerous facultative and strict anaerobic microorganisms can reduce Cr(VI) to Cr(III) via their co-metabolism. Dissimilatory metal reducing bacteria (DMRB) predominate in natural environments as biofilms and have shown marked Cr(VI) bioreduction capability (Bond & Lovley, 2003)(Gadd, 2010). For example, *Cellulomonas,* a biofilm-forming, fermentative subsurface isolate can reduce Cr(VI) directly or through Fe(III) reduction (Viamajala et al., 2007). A well-known methanogenic strain, *Methylococcus capsulatus* was found to have Cr(VI) reductase activity. Cr(III) was accumulated both on cell surfaces and in the growth medium (Al Hasin et al., 2009). In another laboratory study, Chai et al. (2009) demonstrated the Cr(VI) bioreduction capability of a native soil microbial consortium recovered from a contaminated subsurface in close proximity to a steel alloy factory. A novel (facultative/anaerobic) microorganism tolerant to high Cr(VI) concentrations, *Pannonibacter phragmitetus* was isolated from the soil and characterized (Borsodi et al., 2003). Other experiments with soil microcosms contaminated with Cr(VI) showed the presence of Cr(VI) tolerant *Bacillus* and *Enterobacteria* (P. Molokwane & Nkhalambayausi-Chirwa, 2009; P. E. Molokwane et al., 2008).

Cr(III) is usually immobilized in extracellular aggregates and in the EPS . This may be also due to the anionic nature of many EPS compounds, which assist in the metal immobilization process and determine the morphology of metal precipitates in the biofilms (Pieper & Reineke, 2000)(Gadd, 2010). There is evidence that Cr(VI) reduction occurs not only in close proximity to the cells, but also at large distances, indicating indirect biochemical reduction. It is not yet clear if these effects are related to biofilm growth mode. Energy calculations suggest that biofilms should have higher Cr(VI) reduction power, since they can immobilize precious Cr(VI) reductases, rather than dispersing them into the environment. The chemical form of Cr(III) in biofilms is little known. Similar to what happens for other metals, the formation of Cr(III)-phosphate complexion compounds has been observed.

However, due to the short time span of chromium contamination, most Cr(VI)-reducing bacteria such as *Geobacter metallireducens* have not evolved efficient metabolic pathway for Cr(VI) reduction and they exhibit low

tolerance to Cr(VI) concentration usually encountered in contaminated environments. We believe that accelerating evolution *G. metallireducens,* may improve their Cr(VI)-tolerance and Cr(VI)-reduction capabilities. More efficient Cr(VI) reducers may be then used for field Cr(VI) bioremediation. The common use in industries of chromium, its high solubility and the subsequent contamination concerns have led to the development of remediation techniques to cope with the pollution. In the past researchers affirmed that trace quantities are required for some metabolic activities, e.g. glucose and lipid metabolism. Today it is accepted that chromium is highly toxic, and is considered a priority pollutant in many countries. In the environment two oxidation states are predominant: Cr(VI) is the most toxic and mobile form commonly encountered, while Cr(III) is less soluble and less toxic. Indeed, Cr(III) is considered less mutagenic than Cr(VI) (Cervantes et al., 2001)

Cr(VI) reduction in the field has been also attempted. In order to avoid the periodic injection of electron donor in the subsurface, Faybishenko et al (2008) used a slow hydrogen releasing compound to provide constant electron donor concentration in a Cr(VI) contaminated groundwater (Faybishenko et al., 2008). Hydrogen served as electron donor and bacteria removed Cr(VI) below the background concentration.

1.6.6 *Geobacter* bioremediation of metals

The ability of *Geobacter* to reduce soluble ions of metals to less soluble forms shows promise as a bioremediation tool. Metals may be removed from water in this manner in reactors, or stimulating the activity of *Geobacter* species for in situ immobilization is an option. In some instances, *Geobacter* species might naturally attenuate the movement of metals via reduction. Uranium has been the contaminant metal of greatest focus because the rapid kinetics of bacterial U(VI) reduction and low solubility of U(IV) make this process an attractive option for removing uranium from groundwater below drinking water standards (Williams et al., 2011), and references therein). The rather nonspecific nature in which *Geobacter* species reduce U(VI) (see above) and the fact that even in uranium-contaminated environments U(VI) is likely to be a minor electron acceptor (Finneran et al.,2002) make it difficult to definitely determine if *Geobacter* species are the agents for U(VI) reduction in studies in which dissimilatory metal reduction has been stimulated to promote uranium bioremediation. However, the consistent pattern of effective U(VI) removal being associated with increased growth and activity of *Geobacter* species at least at some sites (Williams et al., 2011), and references therein) suggests that *Geobacter* species play a role. Stimulating the activity of *Geobacter* species may also remove a variety of other toxic metals that *Geobacter* species have the potential to reduce in pure culture, but the reduction of these

contaminants may be indirect in subsurface environments, because as noted above in Section 5, these electron acceptors can also be reduced by Fe(II) that *Geobacter* species generate during Fe(III) oxide reduction.

Although the commonly considered approach to stimulating the activity of *Geobacter* species for bioremediation of uranium and related contaminants is to add organic electron donors, a more effective approach might be to provide *Geobacter* species electrons with electrodes (Gregory & Lovley, 2005). Long-term stimulation of anaerobic respiration has several potential negative impacts (Williams et al., 2011). These include (1) release of trace metals and arsenic that were associated with Fe(III) oxides into the groundwater (Burkhardt, 2010), (2) deterioration of the groundwater quality from accumulations of dissolved Fe(II) or sulphide, and (3) aquifer plugging due to biomass or mineral accumulations (Williams et al., 2011). Further, reductive immobilization of uranium in this manner leaves the uranium contamination in the subsurface. Therefore, a better alternative may be to feed *Geobacter* species electrons with electrodes (Gregory & Lovley, 2005). Maintenance of the electron addition to the subsurface with electrodes is much simpler than complex pumping strategies for the controlled introduction of organic electron donors and the electrode strategy is sustainable, easily powered with solar panels. Further, this strategy specifically provides electrons for the reduction of the soluble contaminant of interest and the U(IV) produced precipitates on electrodes. It would be a simple matter to periodically remove the electrodes, extract the U(IV) under aerobic conditions in bicarbonate (Phillips et al., 1995), and return the electrodes to the subsurface. This approach would alleviate all the negative side effects of adding the organic electron donors listed above as well as remove the uranium from the subsurface.

1.7 Genome shuffling

Geobacter metallireducens is an important model organism for many novel aspects of extracellular electron exchange and the anaerobic degradation of aromatic compounds, but studies of its physiology have been limited by a lack of techniques for gene deletion and replacement. Therefore, a genetic system was developed for *G. metallireducens* by making a number of modifications in the previously described approach for homologous recombination in *Geobacter sulfurreducens.* Only in 2012 a genetic system for G. metallireducens has been developed (Tremblay et al., 2012).

Genome shuffling is a powerful technique for engineering of microbial strains for desirable properties. It is a tool to accelerate phenotypic improvement (Y. Zhang et al., 2002). It consist of 3 main steps: 1) mutagenize an initial strain by a physico-chemical agent such as nitrosoguanidine (NTG) or UV radiation to generate genetic variability; 2) to

enhance genetic variability by the exchange of genetic material among the cells through the recombination promoted by protoplast fusion, allowing bacterial cells to take those evolutive advantages reserved to the higher eukaryotes. 3) Finally, the individuals showing an improved phenotype are selected among the combinatorial library.

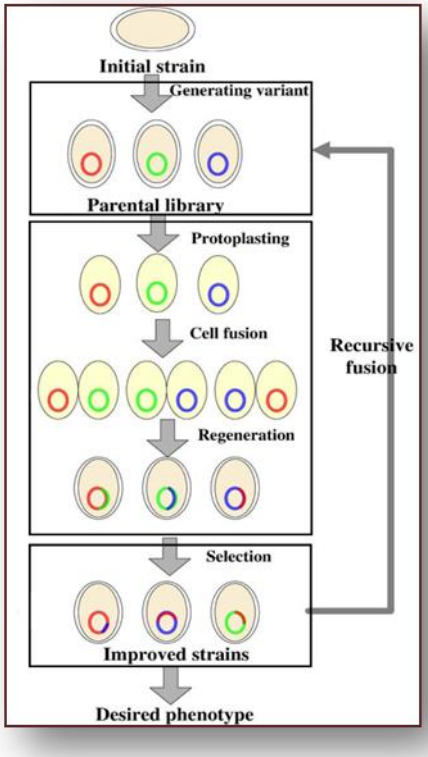


Figure 1.9: Genome shuffling cycle: every cycle consist of: 1) random mutagenesis of the initial strain by NTG; 2) recombination of the mutants genomes promoted by protoplast fusion; 3) selection of the individual having an improved phenotype. Immagine reproduced from (Gong, Zheng, Wu, Chen, & Zhao, 2009)

The rationale of genome shuffling is based on the evolutive success of the millenary selective breeding based on biparental mating, which has led to the successful improvement of plants and animals to meet human needs. Genome shuffling mimics and accelerates evolutionary processes at a DNA molecular level, and allows the breeding and improvement of individual genes and sub-genomic DNA fragments, combining the advantage of multi-parental crossing allowed by DNA shuffling with the recombination of entire genomes normally associated with conventional breeding. When applied to a population of phenotypically selected bacteria, many of these new strains show marked improvements in the selected phenotype thanks to the evolution-directed strategy. Asexual evolution is the sequential process of accumulating punctual mutations. Selection of the test results in the capture of only a single mutant. Previous works report that genome shuffling accelerates the velocity of the improved mutant obtainment of 10-

20 times (Y. Zhang et al., 2002). Asexual evolution is slow, as individuals within a population evolve alone as opposed to sharing information and evolving as a group. Genetic diversity is lost and deleterious mutations that are difficult to lose accumulate. Parental breeding allows the information within a population to be shared. Mating within a selected population consolidates genetic information by providing a mechanism for the combination of useful mutations and the loss of deleterious mutations. Sexual evolution thus produces populations containing individuals that have a far greater fitness than their parents.

Although fine-tuning the relative levels of gene expression in a specific pathway may lead to improved phenotypes, the majority of applications require more global techniques which can alter the entire cellular milieu in a more global fashion. Many recent efforts focused on the development of global approaches for phenotypic improvement, such as artificial transcription factor engineering, global transcription machinery engineering, ribosome engineering, and genome shuffling. These methods are combinatorial in nature, that is, they are based on generating genetic (and hence, phenotypic) diversity in a population followed by screening and selection for improved phenotypes (Santos & Stephanopoulos, 2008). While many of these approaches are still nascent, they are particularly powerful in that they can be used to sample phenotypic space more effectively and are more amenable to phenotypic transfer. The technology of genome shuffling is one of the typical contributions to the tools of combinatorial engineering. This method was firstly presented by Stemmer group in 2002 (Y. Zhang et al., 2002). They have ever focused on strain improvement through DNA shuffling and directed evolution. The technology of DNA shuffling is a method for in vitro homologous recombination of pools of selected mutant genes by random fragmentation and polymerase chain reaction (PCR) reassembly (Crameri et al., 1998; Stemmer, 1994). Directed molecular evolution of several genes and pathways has been successfully accomplished by DNA shuffling (Christians et al., 1999; Coco et al., 2001; Crameri et al., 1997; Ness et al., 1999). As a similar strategy for strain improvement, the technology of genome shuffling was presented. Genome shuffling was described as a process that combines the advantage of multi-parental crossing allowed by DNA shuffling with the recombination of entire genomes normally associated with conventional breeding. It was regarded as the application of combinatorial methods for phenotypic improvement and was praised as a major milestone in strain-improvement technology and metabolic engineering (Stephanopoulos, 2002). At present, genome shuffling has been successfully applied as an effective whole-cell engineering approach for the rapid improvement of industrially important microbial phenotypes (Table 1.2).

Table 1.2 Published papers on enhancement of strain tolerances

Microorganism	Results	Ref.
Lactobacillus	Shuffled strains produced three-fold more lactic acid than the wild type at pH 4.0.	Patn aik et al. (2002)
Lactobacillus rhamnosus	After three rounds of genome shuffling, four strains that could grow at pH 3.6 were obtained.	Wang et al. (2007)
Lactobacillus rhamnosus	The glucose consumption of the best performing strain from the second round of genome shuffling was 62.2% higher than that of the wild type.	Yu et al. (2007, 2008)
Streptomyces pristinaespiralis	A 100 mg L^{-1} pristinamycin resistant recombinant was obtained after four rounds of protoplast fusion.	Xu et al. (2008)
Candida krusei	A mutant, which can grow on the plates containing 0.85% acetic acid, was isolated and selected after four rounds of genome shuffling.	Wei et al. (2008)
Saccharomyces cerevisiae	After three rounds of genome shuffling, the improved strain was obtained, which can maintaining high-cell viability up to 55 °C and also can tolerate 25% (v/v) ethanol stress.	Shi et al. (2009)
Saccharomyces cerevisiae	Two round of genome shuffling improved acetic acid tolerance.	Zheng et al. (2010)
Bacillus amyloliquefaciens	Two rounds of genome shuffling, a strain produces 10.3-fold surfactin than wild type.	Zhao et al. (2012)

Strain improvement has focused also on metabolic engineering. This strategy has emerged as the discipline that utilizes modern genetic tools for the targeted genetic manipulation of microbes (Bailey, 1991). The tools of metabolic engineering have remarkably enabled targeting of necessary genetic changes to express a desired phenotype. The literature was exhaustive with examples demonstrating the efficacy and efficiency of these approaches in improving phenotypes of industrial strains. For example, the strain of E. coli has been engineered for the production of lycopene (Alper et al., 2005), amino acids (J. H. Park & Lee, 2008) and alcohols (Atsumi et al., 2010) through metabolic engineering method. The improvement of *Saccharomyces cerevisiae* for the production of ethanol was obtained by the application of metabolic engineering method (Alper et al., 2005; Bro et al., 2006; Nissen et al., 2000). The applications of metabolic engineering for complex phenotypes have been spurred on by more-comprehensive

genetic tools and increased cellular knowledge of microorganisms. For example, the mevalonate pathway has been incorporated in *E. coli* and *S. cerevisiae* to produce amorphadiene and artemisinic acid, respectively, which are precursors of the antimalarial drug artemisin (Majander et al., 2005; Martin et al., 2003; Ro et al., 2006)

Although remarkable accomplishments have been achieved by the use of metabolic engineering, the application of this approach is limited in the absence of detailed knowledge about genotype–phenotype relationships. Thus the engineering of strains by metabolic engineering approach is usually confined to well-characterized hosts, such as *E. coli, S. cerevisiae* and *B. subtilis*. Thanks to various experimental and computational tools, metabolic engineering is now moving towards global-scale strategy. Systems metabolic engineering and system biotechnology have been applied to improve the performance of microbial strains in bioprocesses (Durot et al., 2009; H. U. Kim et al., 2008; J. H. Park et al., 2008). The general strategy of systems metabolic engineering for strain improvement relies on the integration of upstream and downstream bioprocesses. In the application of systems metabolic engineering, strains were improved by performing metabolic engineering within a systems biology framework, in which entire cellular networks are optimized and fermentation and downstream processes are considered at early stages. Thus, regulatory, metabolic and other cellular networks are engineered in an integrated manner. Systems-level engineering of microorganisms can be achieved by integrating high-throughput analysis and predictive computational modelling or simulation. The information obtained from the results of genomic, transcriptomics, proteomic, metabolomics and fluxomics studies, and those predicted by computational modelling and simulation, are considered together within the global context of the metabolic system. Based on the global cellular information, the strains can be engineered for desired properties. These systems approaches to cell design were called systems biotechnology. The application of systems metabolic engineering and systems biotechnology has recently led to some remarkable achievements for the overproduction of value added products. The whole genome engineering approaches have advanced the field of constructing phenotypes at a faster pace compared with traditional tools. Inspired by natural evolution, the technique termed “evolutionary engineering” has been coined to describe the process of iterative cycles of variation and selection of improved phenotypes (Sauer, 2001). Evolutionary engineering follows nature's ‘engineering’ principle by variation and selection. In this approach, genetic diversity was created by mutagenesis and recombination, and then continuous evolution of large populations was processed under selection pressure over many generations relying on the cell's inherent capacity to introduce adaptive mutations. Due to its distinct advantages over the rational approach, evolutionary engineering has found its place in the

metabolic engineer's toolbox for strain development. This approach has been applied successfully to a number of industrial microorganisms and processes. Xylose utilization is of commercial interest for efficient conversion of abundant plant material to ethanol. However, the most important ethanol-producing organism, *S. cerevisiae*, is incapable of xylose utilization. Using evolutionary engineering approach, Sauer have developed a selection procedure for the evolution of *S. cerevisiae* strains that are capable of anaerobic growth on xylose alone (Sonderegger & Sauer, 2003). In similar effort the improvement of mixed-sugar utilization by a xylose-fermenting *S. cerevisiae* strain was achieved using the strategy of evolutionary engineering (Kuyper et al., 2005). Although rational method and global technique have been successfully applied in strain improvement respectively, the two strategies should be combined in engineering the complex phenotypes (Santos & Stephanopoulos, 2008). Recently, combinatorial approaches become an attracting strategy for optimizing cellular phenotype, including techniques to fine-tune pathway expression, to generate randomized knockout and overexpression libraries, as well as more global approaches to develop complex and multi-genic properties (Santos & Stephanopoulos, 2008). It is now broadly accepted that the optimized pathways require the balanced expression of several enzymes. So many new approaches, such as synthetic promoter libraries and engineering post-transcriptional control, have been developed for fine tuning expression to obtain an optimum balance between pathway expression and cell viability. Keasling and his colleague invented a method for tuning the expression of multiple genes within operons by generating libraries of tuneable intergenic regions (TIGRs), recombining various post-transcriptional control elements and screening for the desired relative expression levels (Pfleger et al., 2006). This technology is useful for optimizing the expression of multiple genes in synthetic operons, both in prokaryotes and eukaryotes. It is an excellent example of the potential of combinatorial searches for pathway optimization.

Moreover, classical methods for improvement of microbial strains have relied upon either mutagenesis followed by selection for improved properties, or manipulation of specific genes known to play an important role in the desired phenotype. The first strategy is time-consuming, requiring many generations of mutation and selection to allow accumulation of multiple beneficial mutations in a single strain. The efficacy of the second strategy is limited by the ability to predict which mutations will improve a particular phenotype. Thus, it is not possible to take advantage of mutations in genes that are not obviously related to the phenotype of interest but may nevertheless improve microbial fitness or performance under a particular set of conditions. In contrast to them, genome shuffling has the advantage of simultaneous genetic changes at different positions throughout the entire genome by recursive protoplast fusion or crossing without the necessity for

network information (Petri & Schmidt-Dannert, 2004). However, genome shuffling has limits in practice to control the yield of metabolic by-products.

Currently, genome shuffling has not been applied to increase metal toxicity resistance. Due to metal resistance and metal reducing capability are very complex characters depending on a great number of genes related and not fully understood, genome shuffling, as a black box tool involving random mutations and their mix can overcome the problem related to insufficient knowledge and comprehension of the molecular and physiologic mechanisms. The technique can also overcome other problems such as time and costs of the conventional engineering methods.

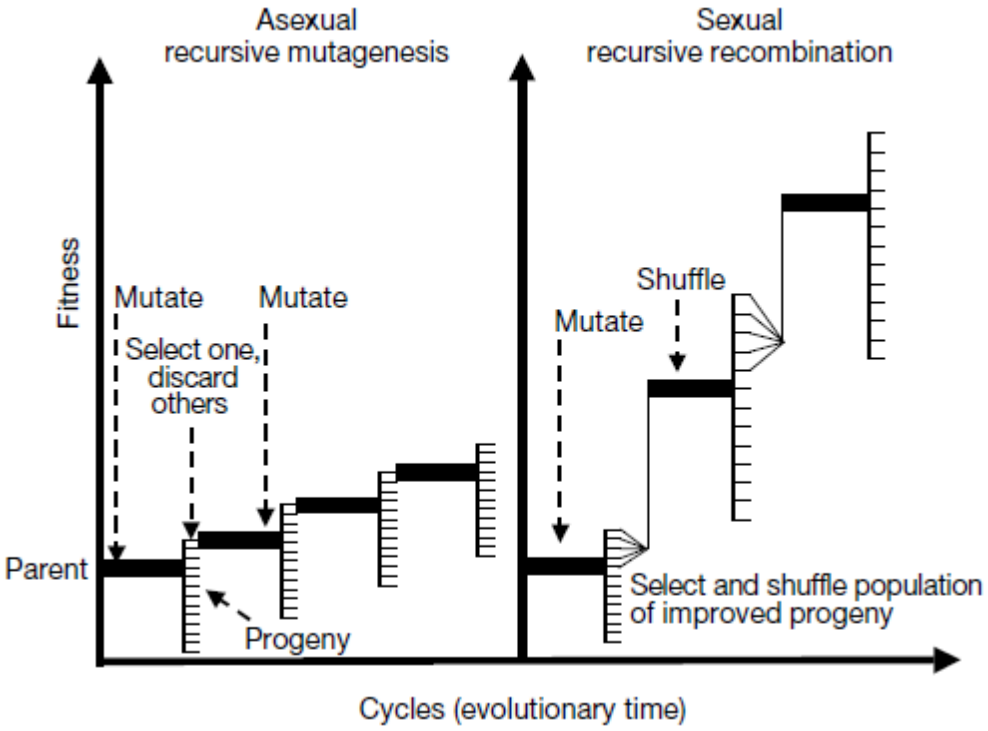


Figure 1.10 Asexual versus sexual evolution. Reproduced from (Y. Zhang et al., 2002)

However, only a few work have been published on Gram negative and only one on anaerobic species, because of the difficulty about the engineering because the outer membrane makes protoplast fusion more difficult and the manipulation of anaerobic bacteria. Dai demonstrated that the efficiency of recombination in fused (Dai et al., 2005) *E. coli* protoplasts is lower than that achieved using Gram-positive organisms, but it is nevertheless high enough for practical applications.

The great number of genome shuffling present in literature show haw this technique has been applied to overcome phenotypical character difficult to be improved, especially for industrial purposes. Whole genome engineering

approach already used successfully to improve stress tolerance and product yield in microorganisms (Bajwa et al., 2010), For example, genome shuffling has been applied to improve production of tylosin by *Streptomyces fradiae* (Y. Zhang et al., 2002), acid tolerance in *Lactobacillus* (Patnaik et al., 2002), and degradation of pentachlorophenol in *Sphingobium chlorophenolicum* (Dai & Copley, 2004). Other reports have described successful use of protoplast fusion (without the initial mutagenesis step) to combine metabolic capabilities of two different organisms. For example, protoplasts of *Acinetobacter* sp. A3 and *Pseudomonas putida* DP99 have been fused to generate strains with enhanced abilities to degrade hydrocarbons (Hanson et al., 1996), and protoplasts of *Kluyveromyces* sp. Y-85 and (W. Wei et al., 2001b) *Saccharomyces cerevisiae* E-15 have been fused to generate strains with an enhanced ability to produce sorbitol under fermentation conditions (Hanson & Desai, 1996).

1.8 Electrochemistry: a tool to investigate EET in viable DMRB

Bioelectrochemistry is a sub-discipline of electrochemistry (EC) where biological material is the object of the study (D. Chen et al., 2007).

BEC is the key to study electron transfer pathway across the membranes and its mechanisms in pure proteins, extract, and viable biomass. BEC is particularly important for DMRB and EAB. Electrochemistry is an interdisciplinary truly pushes the field forward and accelerates the understanding and engineering of these biofilms. The core discipline amongst all of the research efforts in this field is electrochemistry. This scientific discipline can itself be regarded as classically interdisciplinary. It is settled between (physical) chemistry and physics; however, its tools and methods are widely used in different fields spanning from materials science via synthetic chemistry to botany (C. Zhang et al., 2008)Collecting electrons from bacterial metabolism has been investigated as a potential sustainable energy source, while electricity can be used to enhance fermentations of reduced organic chemicals (McKinlay & Zeikus, 2004).

The vast majority of published works on extracellular electron transfer in electroactive bacteria is based on *in vitro* observations of protein fraction or purified proteins (Magnuson et al., 2001). However, in the last years electrochemical techniques have been focused as a powerful tool to characterize viable electroactive bacteria. Since they have shown very complex and branched extracellular electron transfer pathways, in vitro results must be validated also *in vivo*. Electrochemical analyses, such as those used in protein film voltammetry, do not damage the electroactive biofilm but unveil the mechanistic details of extracellular electron transfer *in vivo*.

1.8.1 Electrochemical Cells

Electrochemical (EC) cells have are devices used to study the electron transfer properties of DMRB such as *G. metallireducens*. EC are three electrodes systems. Reference electrode, working electrode and counter electrode are incorporated into the system in conjunction with electronic control hardware, the potentiostat, which acts by controlling the electric potential at the electrode of interest to determine the effect of applied voltage on microbial extracellular electron transfer. Potential at the site of oxidation is kept constant through the Potentiostat detecting changes in resistance across the electrochemical cell and adjusting the current flow at the site of reduction in response. The act of establishing a constant voltage at the electrode at which the reaction of interest occurs is defined as poising and is sometimes performed in electrochemical devices in order to study the mechanisms by which microbes transfer electrons to solid surfaces. In

this type of arrangement, the electrode at which microbes perform oxidation to generate electrons is referred to as the working electrode, due to the fact that it is the location of the redox reactions of primary interest. Likewise, the electrode that electrons generated by microbial metabolism flow to is designated the counter electrode, or auxiliary electrode in accordance with its supporting role. This 3-electrode design may therefore be distinguished from a 2-electrode microbial fuel cell in which the electric potential difference between the anode and the cathode is established primarily by the microbes colonizing the anode (Logan & Regan, 2006).

The reference electrode itself is a half cell with a known electric potential that functions as an orientation point when establishing a potential at another electrode. A porous junction separates the electrolyte within the reference electrode from the rest of the MFC. The oversaturated reference electrolyte generates a highly stable voltage at a piece of metal wire such as silver or mercury. The wire passes the signal from the electrolyte to the electrode's cable or connector and on to the electrical device being used to control the MFC. Several types of reference electrodes exist; the most common used in aqueous solutions are listed in Table 1.3.

Table 1.3: Common reference electrodes.

Name (abbreviation)	Potential vs. SHE (V)	Reaction
Standard hydrogen electrode (SHE)	0.000	$H_2 \Leftrightarrow 2e^- + 2H^+$
Saturated calomel electrode (SCE)	+0.242	$Hg_2Cl_2 + 2e^- \Leftrightarrow 2Hg^+ + 2Cl^-$
Silver-silver chloride electrode (sat.)	0.197	$AgCl + e^- \Leftrightarrow Ag^+ + Cl^-$

The electric potential of the reference electrode is determined by the Nernst equation

$$E = E^0 - \frac{RT}{nF} ln \frac{c_R^0}{c_O^0} \qquad \text{(Eq. 1)}$$

Where E = potential of the reference electrode and

E^0 = standard cell potential

The potential of the selected reference electrode is used continually by the Potentiostat as a constant standard to maintain a constant potential across an electrochemical cell (Compton & Banks, 2007). The presence of a reference electrode in an electrochemical setup also allows for voltammetry experiments to be run on an analyte at the working electrode.

1.8.2. Chronoamperometry (CA)

CA is an electrochemical technique where the current passing through the working electrode is measured as function of the time. Chronoamperometry is generically used in those applications where the redox processes depend on the diffusion occurring from the region placed at *d* distance from the electrode surface. For this reason the reaction solution should not be stirred. Nevertheless, in electrochemical cell where viable and live biofilm are grown the stirring is necessary to not limit the biofilm growth.

1.8.3 Cyclic voltammetry

Voltammetry is a powerful electroanalytical tool used for characterizing half-cell reactions. All voltammetry involves changing the voltage at a working electrode versus a reference while measuring the effect of the voltage shift on current production (Armstrong et al., 2000). In this situation the working electrode may be defined as the electrode at which the reaction of interest occurs. The characteristics of the resulting current vs. potential plot depend on several factors including the rate of the electron transfer reaction(s), the chemical reactivity of the electroactive species, and the voltage scan rate. The most basic form of voltammetry is linear sweep voltammetry (LSV). LSV is the measurement of the change in current production at the working electrode as voltage is swept from an initial potential to a final potential in either the positive or negative direction (Compton & Banks, 2007). If the sweep is in the negative direction, an oxidized species present within the electron double layer of the working electrode will become reduced resulting in the flow of electrons out of the working electrode. If LSV is run from a positive potential to a negative potential the species will be reduced, resulting in the start of an electron flow out of the working electrode. In either case the magnitude of electrical current flow increases as the sweep proceeds until a maximum is reached when the potential at the working electrode equals the redox potential of the species. As the sweep continues past the redox potential of the species and the current flow into or out of the working electrode decreases as the system regains equilibrium (Armstrong et al., 2000). The current response to the oxidation or reduction of a species results in a peak in the current vs. potential plot (S. M. Cohen & Halper, 2002). At the end of an oxidation or reduction peak, the potential at the working electrode is considered positive or negative enough that all reactants reaching the surface are immediately oxidized or reduced (Faraoni et al., 1990),(Y. Kim & Amemiya, 2008). This peak current is therefore not dependent on diffusion of species to the electrode surface. Sigmoidal curves are another voltammetry feature indicating electrochemical activity at the working electrode. A sigmoidal curve is an increase or decrease in current flow during a voltage sweep that is not followed by a subsequent drop or rise, as with a peak (Rodgers &

Amemiya, 2007). The presence of such a curve denotes that in addition to simply being oxidized and reduced at the electrode surface, the analyte is also capable of catalysing a sustainable electron flow into or out of the electrode. For this reason sigmoidal curves are also referred to as catalytic waves. The voltage at the inflection point of the wave is approximately equal to the reduction potential of the half-cell reaction driving the current flow.

Cyclic voltammetry is a widely used voltammetry technique in which the voltage is held at an initial potential then scanned in the positive or negative direction until arriving at a final potential. The direction of the sweep is then reversed and the potential is returned to the initial value. A useful characteristic of cyclic voltammetry is that an analyte can be shown to be capable of being reversibly oxidized and reduced if forward and the reverse potential sweeps both exhibit a wave or peak with midpoints no more than 60 mV in difference. If a redox system remains in equilibrium throughout the potential scan, the electrochemical reaction is said to be reversible (Armstrong et al., 2000). Peaks generated by a reversibly oxidized and reduced analyte (n=1) are separated by approximately 60 mV on a cyclic voltammogram (Léger et al., 2003).Voltammetry is an electrochemical method studying how the current change as function of an applied potential. The resulting curve I = f(E) is called voltammogram. The potential can be varied step by step or continuously while the relative current value is measured. The shape of the voltammogram depends on the speed of potential variation and on whether the solution is stirred or quiescent (mass transfer). In our experiments, the solution is not stirred to prevent noise, while the potential of the working electrode hosting the electroactive biofilm changes and the resulting current is measured.

Voltammetry requires at least two electrodes: working electrode, which makes contact with the electroactive biofilm, applies the potential and allows the transfer of charge to and from the biofilm. A second electrode acts as the other half of the cell. This second electrode must have a known potential with which to gauge the potential of the working electrode, furthermore it must balance the charge added or removed by the working electrode. The reference electrode is poised at a known potential. Its only role is to act as reference in measuring and controlling the working electrodes potential and at no point does it pass any current. The auxiliary electrode passes all the current needed to balance the current observed at the working electrode. To achieve this current, the auxiliary will often swing to extreme potentials at the edges of the solvent window, where it oxidizes or reduces the solvent or supporting electrolyte. These electrodes, the working, reference, and auxiliary make up the modern three electrode system. There are many systems which have more electrodes, but their design principles are generally the same as the three electrode system. For example, the rotating ring-disk electrode has two distinct and separate

working electrodes, a disk and a ring, which can be used to scan or hold potentials independently of each other. Both of these electrodes are balanced by a single reference and auxiliary combination for an overall four electrode design. More complicated experiments may add working electrodes as required and at times reference or auxiliary electrodes. In practice it can be very important to have a working electrode with known dimensions and surface characteristics. As a result, it is common to clean and polish working electrodes regularly. The auxiliary electrode can be almost anything as long as it doesn't react with the bulk of the analyte solution and conducts well. The reference is the most complex of the three electrodes, there are a variety of standards used and its worth investigating elsewhere. In most voltammetry experiments, a bulk electrolyte (also known as a supporting electrolyte) is used to minimize solution resistance. It is possible to run an experiment without a bulk electrolyte, but the added resistance greatly reduces the accuracy of the results. With room temperature ionic liquids, the solvent can act as the electrolyte.

To perform cyclic voltammetry (CV) experiments on electroactive microbial biofilms, the following is needed for a three-electrode set-up: a working electrode (WE), a reference electrode (RE), and a counter electrode (CE). Using this set-up, a current–potential polarization curve can be recorded using a potentiostat for 1) controlling the voltage between the WE and the RE and 2) for measuring the current low between the WE and CE.

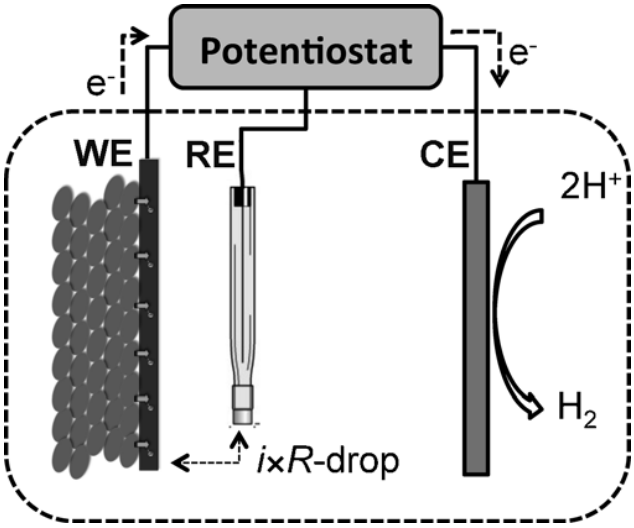


Figure 1.11: A potentiostatic three-electrode experiment on an anodic biofilm that is housed in a single-chamber electrochemical cell; here the counter reaction at the CE is the reductive hydrogen evolution from protons. Reproduced by permission from (Harnisch & Freguia, 2012).

This principle is shown in Figure 1-12 for the example of a bio-anode, which also indicates the voltage drop between the reference electrode and the working electrode (determined by i*R, where R is the resistance owing

to the electrolyte between the WE and the RE, known in this case as uncompensated resistance). This resistance causes a drop in potential in the solution and in the biofilm during current flow owing to Ohm's law. This drop increases with solution resistivity and with the distance between the WE and the reference point. Therefore, it is always advisable to place the RE outside the path of ion migration between the WE and the CE.

Note: many sections of chapter 1.6 are extracted from a book chapter (Pastorella et al., 2012)*. The chapter has been written by the same author of the present work and all permissions have been obtained.*

2. Materials and methods

2.1 Solutions and buffers

2.1.1 Buffers

TBE buffer, 1L: TRIS 1,21 g, EDTA 0,292 g, pH=8

2.1.2 Media composition

2.1.2.1 Media for cultivation of Geobacter

NB iron oxide (per liter): 0.38 g KCl, 0.2 g NH_4Cl, 0.6 g NaH2PO4·H2O, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g $NaHCO_3$, 10 mL DL Minerals, ferrihydrite 50-100mM. The medium was split to 10 ml anaerobic tubes, flushed by N_2/CO_2 80/20 v/v for 10 min and autoclaved 15 min at 121°c 1,5 atm.

NBFC (per liter): 4,64 Fumarate; 19.0 g KCl; 10g NH_4Cl; 3,45 g NaH_2PO_4; 0.8 g $CaCl_2 \cdot 2H_2O$; 1.5 g of NTA; 0.1 g $MnCl_2 \cdot 4H_2O$; 0.5 g $FeSO_4 \cdot 7H_2O$; 0.17 g $CoCl_2 \cdot 6H_2O$:; 0.10 g $ZnCl_2$; 0.03 g $CuSO_4 \cdot 5H_2O$; 0.005 g $AlK(SO_4)_2 \cdot 12H_2O$; 0.005 g H_3BO_3; 0.09 g Na_2MoO_4 , 0.04 $NiCl_2$; 0.02 g $Na_2WO_4 \cdot H_2O$; 0.10 Na_2SeO_4 ; 4 g g$SO_4 \cdot 7H_2O$; 0.002 g biotin; 0.002 g folic acid, 0.01 g pyridoxine HCl; 0.005 g riboflavin; 0.005 g thiamine; 0.005 g nicotinic acid; 0.005 g pantothenic acid; 0.0001 g cobalamin; 0.005 g p-aminobenzoic acid; 0.005 g lipoic acid; 1,66 g acetate; 2 g $NaHCO_3$.

NBFA (per liter): 13.7 g Fe(III) citrate; 19.0 g KCl; 10g NH_4Cl; 3,45 g NaH_2PO_4; 0.8 g $CaCl_2 \cdot 2H_2O$; 1.5 g of NTA; 0.1 g $MnCl_2 \cdot 4H_2O$; 0.5 g $FeSO_4 \cdot 7H_2O$; 0.17 g $CoCl_2 \cdot 6H_2O$:; 0.10 g $ZnCl_2$; 0.03 g $CuSO_4 \cdot 5H_2O$; 0.005 g $AlK(SO_4)_2 \cdot 12H_2O$; 0.005 g H_3BO_3; 0.09 g Na_2MoO_4 , 0.04 $NiCl_2$; 0.02 g $Na_2WO_4 \cdot H_2O$; 0.10 Na_2SeO_4 ; 4 g g$SO_4 \cdot 7H_2O$; 0.002 g biotin; 0.002 g folic acid, 0.01 g pyridoxine HCl; 0.005 g riboflavin; 0.005 g thiamine; 0.005 g nicotinic acid; 0.005 g pantothenic acid; 0.0001 g cobalamin; 0.005 g p-aminobenzoic acid; 0.005 g lipoic acid; 1,66 g acetate; 2 g $NaHCO_3$.

NBA (per liter): 2,3376 g NaCl; 19.0 g KCl; 10g NH_4Cl; 3,45 g NaH_2PO_4; 0.8 g $CaCl_2 \cdot 2H_2O$; 1.5 g of NTA; 0.1 g $MnCl_2 \cdot 4H_2O$; 0.5 g $FeSO_4 \cdot 7H_2O$; 0.17 g $CoCl_2 \cdot 6H_2O$:; 0.10 g $ZnCl_2$; 0.03 g $CuSO_4 \cdot 5H_2O$; 0.005 g $AlK(SO_4)_2 \cdot 12H_2O$; 0.005 g H_3BO_3; 0.09 g Na_2MoO_4 , 0.04 $NiCl_2$; 0.02 g $Na_2WO_4 \cdot H_2O$; 0.10 Na_2SeO_4 ; 4 g g$SO_4 \cdot 7H_2O$; 0.002 g biotin; 0.002 g folic acid, 0.01 g pyridoxine HCl; 0.005 g riboflavin; 0.005 g thiamine; 0.005 g nicotinic acid; 0.005 g pantothenic acid; 0.0001 g cobalamin; 0.005 g p-aminobenzoic acid; 0.005 g lipoic acid; 1,66 g acetate; 2 g $NaHCO_3$.

2.1.2.2 Media and procedures for cultivation of Shewanella

The culture was grown aerobically for 24 hours (h) at 30 ºC in Luria-Bertani medium (LB). Subsequently, the culture was centrifuged at 13,400 rpm for 20 min, and the LB medium was replaced with 10 ml of defined medium (DM) containing per litre: NaHCO3 2.5 g, CaCl2·2H2O 0.08 g, NH4Cl 1.0 g, MgCl2·6H2O 0.2 g, NaCl 10 g, HEPES 7.2 g. Vitamins mixture (1 ml) and trace metal solution (10 ml) were added to the DM as previously described, and 15 mM lactate was added to the medium as electron donor [4]. The cells were grown aerobically in DM at 30 ºC for 2 days, under shaking condition at 150 rpm. Following centrifugation for 20 min at 13,400 rpm, the pellet was washed three times with DM medium, to remove soluble redox mediators from the inoculum.

2.2 Strains

Geobacter metallireducens (DSMZ 7210) and *G. sulfurreducens* (DSMZ 12127) and *Shewanella loihica* PV- 4 (DSM 17748) strains were purchased from the German microbial bank DSMZ and maintained in NB Iron Oxide. The media are described in section 2.2.

To ensure the culture maintenance -80°c stock cultures were prepared transferring 1ml of actively growing culture to a cryotube containing 0.5 ml of 50 % DMSO.

2.3 Anaerobic handling of *Geobacter*

G. metallireducens and *G. sulfurreducens* were grown following strict anaerobic handling procedures (Speers, Cologgi, & Reguera, 2009). In brief, N_2/CO_2 (Air Products, Ireland) and N_2 (BOC, Ireland) of commercial purity were passed through a copper hot scrubber to remove traces of oxygen. Unless diversely specified, all the transfers of cultures were performed with syringes and needles that had been flushed with oxygen-free N_2 or were performed under a stream of oxygen-free gas in an anaerobic chamber. The media were stored in Balch-type tubes and vials, which are more stable than Hungate-type tubes and result in lower oxygen intrusion. Balch-type tubes and vials are closed with a thick butyl rubber stopper which is held in place by a tear-off aluminium crimp seal. The media and every stock solution needed were purged with N_2:CO_2 80:20, then the stoppers are inserted as the tubes and vials are withdrawn from gassing needles. Media were then sterilized (20 min, 1atm) prior to storage (up to two months) and use. Sterile injection syringes and needles were flushed repeatedly with anaerobic N_2 then used to transfer *G. metallireducens* or *G. sulfurreducens* to a fresh medium tube.

Genome shuffling, strain selection, and solid medium experiments were carried out in a vinyl anaerobic chamber (CoyLab, USA). The anaerobic chamber provides an anaerobic environment for all the operations that

cannot be performed in anaerobic tubes. The chamber is filled by a N_2-CO_2-H_2 75-20-5% v/v atmosphere. A palladium catalyst removes O_2 traces (below 1 ppm) that react with H_2 to form water. It should be noted that *G. metallireducens* cannot use H_2 as an electron donor (Jain et al., 2012).

2.4 Bacterial growth and media

2.4.1 NB Iron Oxide

NB Iron Oxide was prepared according to the literature (D. R. Lovley et al., 1987). NB Iron oxide is a basal medium containing acetate as sole electron donor, basal salts, minerals and amorphic ferric oxide (or ferrihydrite) as solid electron acceptor. In this medium, both *G. metallireducens* and *G. sulfurreducens* maintain the metal reducing phenotype. The ferrihydrite was prepared by the neutralization of iron chloride and four washes by distilled water to remove the chloride. All the cultures used were first grown on NB Iron Oxide and then transferred to NBFC (*G. metallireducens*) or NBFA (*G. sulfurreducens*).

2.4.2 NBFC

NBFC is a basal medium where acetate serves as electron donor and Fe-citrate as a soluble electron acceptor. Both *G. metallireducens* and *G. sulfurreducens* can be cultivated on NBFC. However, in the present study only the first was sub-cultured on this medium. NBFC was used also for the genome shuffling procedure to maintain the salt and osmotic strengths.

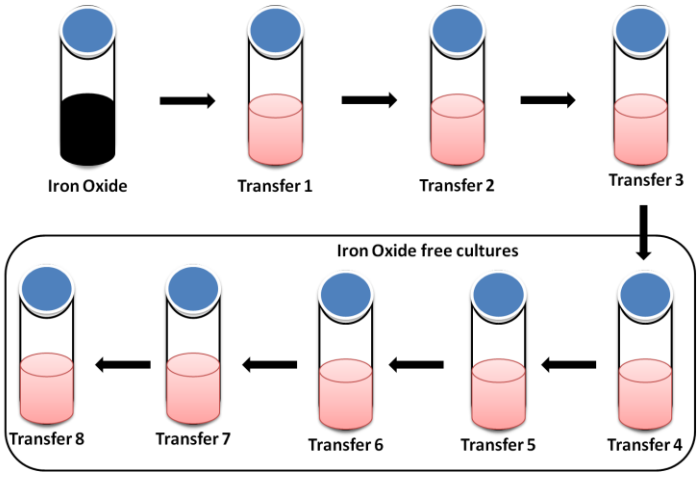


Figure 2.1: *G. metallireducens* and *G. sulfurreducens* were cultured on NB Iron oxide to maintain the metal reducing phenotype. At least three 0.1 to 10 ml transfer (dilution factor: 10^6) were necessary to assume that iron oxide was depleted: in NB iron oxide the solid metal concentration was 50-100 mM. After three transfers, its concentration was 50-100 10^{-9} M. After 8-10 transfers the metal-reducer phenotype can be lost and a new transfer-line can be started.

2.4.3 NBFA

NBFA is a basal medium where acetate serves as electron donor and fumarate as soluble electron acceptor. This medium is used to subculture *G. sulfurreducens* to prevent the iron oxide entering in the electrochemical cells.

2.4.4 NBA

NBA is a basal medium containing acetate and missing electron acceptors used to grow *G. metallireducens* and *G. sulfurreducens* in electrochemical cells. The lack of electron acceptor is crucial for the biofilm formation on the electrode surface.

2.4.5 Growth curve

A growth curve is an empirical model that gives information about the evolution of the biomass during a certain time. The biomass was evaluated by spectrophotometric analysis of a *G. metallireducens* culture repeated every 3 h. Two identical cultures were used in order to avoid working at night time, transferring 0.1 ml of a stationary phase *G. metallireducens* culture into a 100 ml NBFC bottle. The first culture was transferred 12 h before the second and 3 ml of culture were taken every 3 h. Due to the medium changing colour during the growth, 1 ml of culture was centrifuged at 2000g 5 min and the supernatant was used to zero the spectrophotometer (Varian 50, Australia). The other 2 ml were put in two 1.5 ml tubes, vortexed and the optical densities were analysed at 600 nm.

2.5 Genome shuffling

Genome shuffling (GS) consists of cycles of: random mutagenesis, protoplast fusion and selection of the improved phenotype strains. Another selection step was added before the mutagenesis, in order to find the limit of the tolerance. In the following, I will provide a detailed description of each step, with particular regards to the specific steps needed for selection of metal-resistant strains.

2.5.1 Mutagenesis

G. metallireducens was grown in NBFC from wild type strain sub-cultured and maintained in NB iron oxide because planktonic growth is needed for the genome shuffling procedure. Due to the unstable metal-reducing phenotype of *G. metallireducens*, all the steps for a genome shuffling round were completed no more than 10 subcultures on NBFC (Figure 2.19). It has been shown that the metal reducing phenotype decreases significantly after approximately 10 subcultures in NBFC.

A late exponential phase culture from NBFC medium was transferred in fresh NBFC medium spiked with the mutagenic agent NTG (40 g L^{-1}) and incubated at 30°c for 6 days. The obtained mutants were then selected for

the Cr(VI) resistance. By this selection step, we removed those strains not having an improved phenotype from the pool participating at the recombination, increasing the efficiency of genome shuffling. The most resistant strains were able to resist up to 2.5 mM Cr.

2.5.2 Protoplast fusion

The selected mutants were collected by centrifugation at 5000g for 10 min and treated with 25 ml L^{-1} lysozyme in half volume of medium to degrade the cell wall. After an incubation at 30°C for 30 min, the wall debris were removed by a 10 min at 10000g, 4°C centrifugation and the supernatant was placed in a new tube. 0.5 mL of PEG6000 was added and the solution was incubated at 30°C for 90 min with gentle shaking in order to promote protoplast fusion and genetic recombination. The reaction was stopped by placing the solution in ice. Fused cells were collected by centrifugation at 10000g, 4°C for 40 min, the supernatant was discarded and the pellet was re-suspended in 1 volume NBFC + 50mM NAG to stimulate regrowth.

2.5.3 Selection

G. metallireducens genome shuffled mutants were then selected by 3mM Cr. This chromium concentration is higher than the one the wild type strain can tolerate eliminating all the cell that have not took part on genetic recombination or took part on it but the recombination has not improved the chromium resistance mechanism.

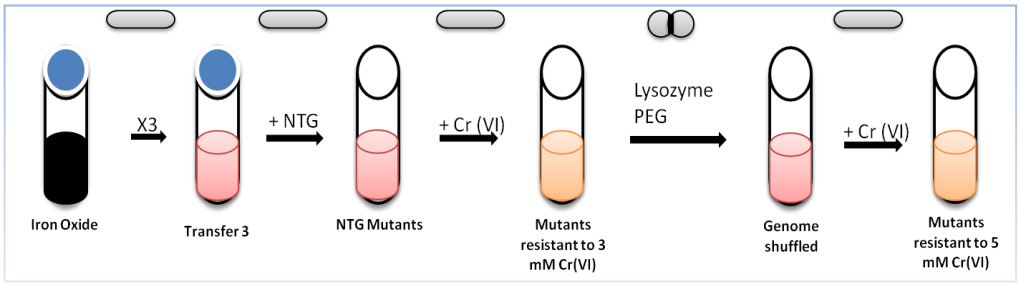


Figure 2.2: Diagram of the genome shuffling process.

2.6 Electrochemical cells assembly

2.6.1 Electrodes preparation.

A graphite electrode was machine cut into 2 by 1 by 0.1 cm electrodes. Freshly cut glassy graphite electrodes were polished using P400 sandpaper. The electrodes were polished by sonication to remove the residual graphite powder, soaked overnight in 1 M HCl to remove metals and other contaminants, washed twice with deionized water to remove organic substances, and then stored in deionized water. After each

experiment, electrode surfaces were cleaned with an additional 1 N NaOH treatment (to remove biomass), and the entire surface was refreshed through sandpaper polishing and cleaning as described above to remove immobilized electron transfer agents. These working electrodes were attached to 0.1 mm Pt wires via miniature nylon screws inserted into heat-pulled 3 mm glass capillary tubes (Fisher scientific, Ireland) and soldered inside the capillary to copper wires. ITO electrode was cut from a commercial ITO-covered plastic film (Sigma Aldrich), sterilized by Et-OH for 20 min and UV light for 30 min. These working electrodes were directly attached via a crocodile clip that ensured electrical contact throughout the experiment. Counter electrodes consisted of a 0.125 mm diameter Pt wire (Sigma-Aldrich, Ireland) that was also inserted into a 3 mm glass capillary and soldered to a copper wire. The resistance of each electrode assembly was measured, and electrodes with a total resistance of higher than 0.5 were discarded. Reference electrodes were connected to bioreactors via a salt bridge assembled from a 3 mm glass capillary and a 3 mm Vycor frit (Bioscience, Ireland).

2.6.2 Electrochemical cell

Electrochemical cells were jacket cells (Bioscience, Ireland) closed by a custom made Teflon lid. Electrode capillaries were inserted through ports in the custom made Teflon lid which was sealed with an O-ring gasket. This lid fit onto a 20 ml conical electrochemical cell (Bioscience, Ireland), which had been previously washed in 8 M HNO_3 . After the addition of a small magnetic stir bar, the cell was autoclaved for 20 min. Following autoclaving, the salt bridge was filled with 0.1 M Na_2SO_4 in 1% agar. A saturated reference electrode (Fisher Scientific, Ireland) was placed at the top of this agar layer and covered in additional 1 M Na_2SO_4 to ensure electrical contact. The reactors were connected to a water bath to maintain cells at 30°C. To maintain the strict anaerobic conditions required by bacteria, all reactors were operated under a constant flow of sterile humidified N_2:CO_2 (80:20 [vol/vol]), which had been passed over a heated copper column to remove trace oxygen. Each reactor was located above an independent magnetic stirring unit. Autoclaved bioreactors flushed free of oxygen, filled with sterile and anaerobic NBA medium, and incubated at 30°C were analysed before each experiment to verify anaerobicity and the absence of redox-active species. Electrochemical cells showing residual peaks in differential pulse voltammetry (DPV), anodic current in cyclic voltammetry (CV), or baseline noise were discarded as having possible electrode cleanliness or connection noise issues. These autoclaved, verified bioreactors were then used for growth of cultures.

A typical bioreactor was inoculated with 40% (v/v) of early stationary phase *G. metallireducens or G. sulfurreducens* culture. After inoculation, a potential step of 0.2 V versus the standard hydrogen electrode (SHE) was

applied and the reactors were incubated. CA and CV were used to analyse the biofilms formed at graphite or ITO electrodes. The parameters for the techniques were chosen as it follows. CA: E = 0.0V vs. SCE; CV: equilibrium time 5 s; scan rate1mV/s, E_i =−0.8 V vs. SCE, E_f = 0.0V vs. SCE. Scan rate analysis was performed on the biofilm depleted of acetate, with scan rate 1 mV/s.

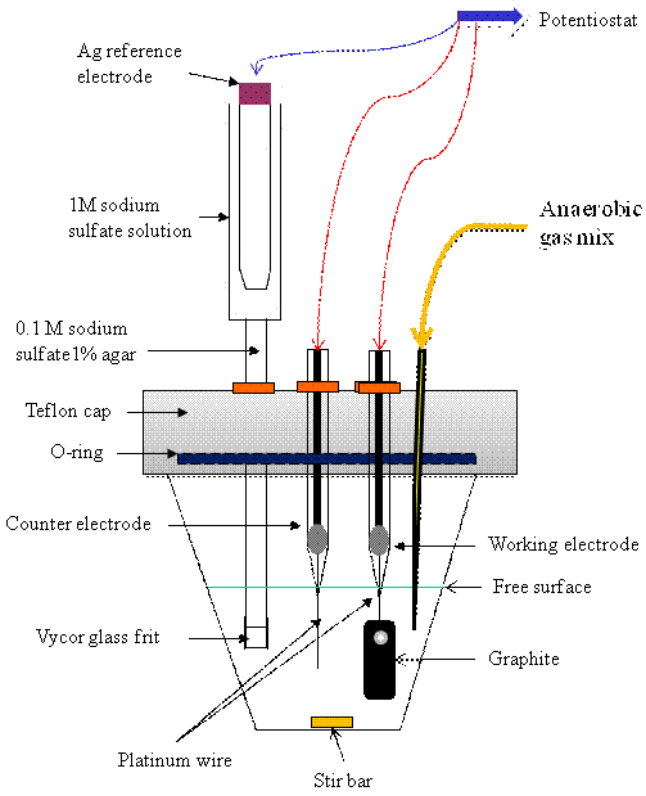


Figure 2.3: Scheme of the electrochemical cell used in the *G. metallireducens* characterization. Modified from (Marsili et al., 2008).

2.6.3 *Shewanella* growth on graphite electrode

The washed *S. loihica* PV-4 cell suspension was adjusted to O.D=520 nm, then purged for 0.5 h with purified N_2, and finally 5 ml of this suspension was added to the electrochemical cell filled with 5 ml of DM medium. Lactate was added to a final concentration of 15 mM. After 24 h, the spent growth medium was replaced with fresh DM medium, to promote the electroactive biofilm growth. Following the first medium change (MC), 15 mM lactate was injected twice at about 48 h and 72 h, to maintain non-limiting electron donor concentration in the EC.

2.6.4 *Geobacter* growth on graphite electrode

G. metallireducens and *G. sulfurreducens* were previously cultivated in NBFC or NBFA, respectively. 4 ml of an actively growing culture were

transferred using anaerobic technique to the electrochemical cell filled with 6 ml of NBA medium containing acetate 20 mM. Every 24 h, the spent growth medium was replaced with fresh NBA medium, to promote EAB growth.

2.6.3 Spectroelectrochemical cell

Spectroelectrochemical cell was a 5 ml cuvette closed by a custom made Teflon lid. Working electrode was an ITO covered plastic film (Sigma Aldrich, Ireland). Counter and reference electrodes, medium, anaerobic gas and parameter for techniques are described in 2.4.2.

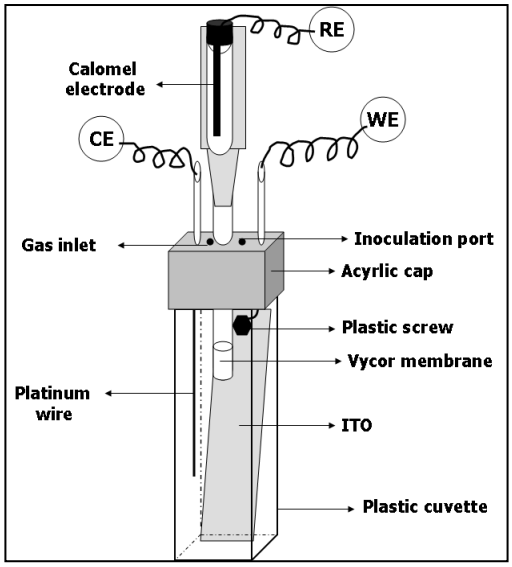


Figure 2.4: Scheme of the custom made "minicell" used for spectroelectrochemical analysis. Figure reproduced from Jain et al., (2011).

2.7. Electrochemical instrumentation.

A 5 channel potentiostat (VMP, BioLogic, France) was connected to the three-electrode cells described above (Figure 2.3). Software from the same producer (EC-Lab v10.19) was used to run simultaneous multi-method electrochemistry routines, which include CA, CV, DPV. All measurements, with the exception of CA, were performed in succession without stirring enabled.

2.7.1 Cyclic Voltammetry

CV was carried out at 0.001 V s^{-1} scan rate from -0.8 to 0.0 V versus standard hydrogen electrode (SHE) while the headspace was continuously flushed with N_2/CO_2 80/20.

2.7.2 Differential Pulse Voltammetry (DPV)

DPV was carried out at 0.005 V/s scan rate from -0.8 to 0.0 V versus standard calomelane electrode (SCE) while the headspace was continuously flushed with N_2/CO_2 80/20 v/v.

2.8 Microscopy

2.8.1 Fluorescence spectroscopy

Fluorescence spectroscopy of the spent medium collected from the EC containing graphite anode was performed using a LS-50B luminescence spectrometer (Perkin Elmer, UK). Before analysis, the spent medium was centrifuged at 13,400 rpm for 0.33 h and filter-sterilized via 0.22 µm filter (Millipore, USA). The fluorescence excitation spectra (200 – 400 nm) at 430 nm emission wavelength and emission spectra (350 – 600 nm) at 360 nm excitation wavelength were recorded. The excitation and emission slit widths were 2.5 nm with photomultiplier tube (PMT) voltage of 600V.

2.8.2 Confocal microscopy

S. loihica PV- 4 biofilms grown at graphite electrode was collected after 96 h of the EC operation. The samples were removed from the EC in an anaerobic chamber (Coy Laboratory, USA), followed by staining for 0.5 h in 1 mg ml-1 acridine orange. After rinsing to eliminate excess dye, the samples were fixed to a glass slide. The confocal images were captured with a laser scanning microscope (Zeiss LSM 510, USA), using argon laser 488 nm as excitation source. The objective was a PLAN apochromatic 63 x oil immersion, with numerical aperture 1.40. Fluorescence was recorded with a low pass filter at 505 nm. A series of images were taken along the biofilm thickness (Z axis) at regular intervals (0.5 µm).

2.8.3 Scanning electron microscopy (SEM)

S. loihica PV- 4 biofilm coated graphite electrodes were removed from the EC after 96 h of operation in the laminar air flow. The biofilm sample was fixed with 2% glutaraldehyde in filtered (0.22 µm) phosphate buffer saline (PBS) for 2 hours and dehydrated using ethanol gradient (beginning with 20 %, 40 %, 60 %, 80 % and ending with 100 % ethanol). The samples were then air-dried, sputter coated with gold using a sputter coater, and then the samples were observed with Zeiss SEM.

3. Results

3.1 Optimization of bacterial growth

G. metallireducens, G sulfurreducens and *S. loihica* PV-4 were grown using media available in literature, as described in Chapter 2. *G. metallireducens* was also grown on plates according to the first version of genome shuffling procedure. Agar plates were prepared by adding 1.5 % agar to NBFC medium. In NBFC-agar Fe(III) reduction was clearly visible as colourless zone (Figure 3.1) earlier than visible colonies (Figure 3.2). Moreover, cysteine was necessary added to promote growth on solid medium.

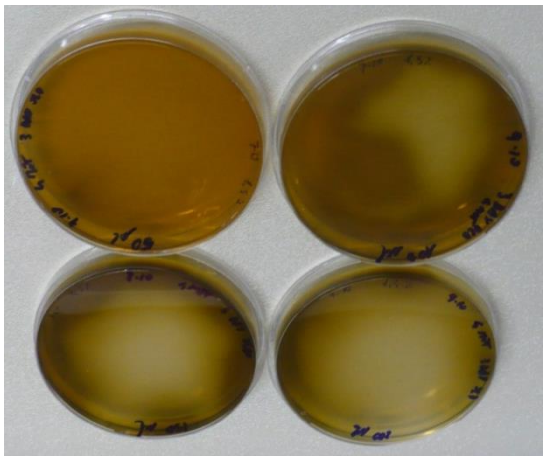


Figure 3.1 *Geobacter metallireducens* growing on NBFC-agar at different times top-left) 1h; top-right) 24h; bottom-left) 48h; bottom-right) 72h. *G. metallireducens* uses Fe(III) as final electron acceptor of its respiratory chain. The clear parts of the agar show the reduction of Fe(III).

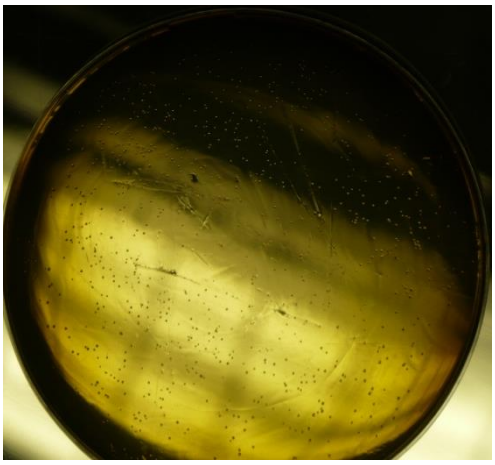


Figure 3.2 *G. metallireducens* on NBFC-agar. The colonies are visible 6 days after the spreading.

3.1.2 Preliminar characterization of graphite as electrodic surface

Most of the electrochemical experiments in this work employ commercial graphite electrodes, polished with sandpaper (400 particles per inch, P400). *G. metallireducens, G. sulfurreducens* and *S. loihica* PV-4 formed a thin biofilm when grown on graphite surface in potentiostat-controlled electrochemical cell and in lack of soluble electron acceptors. Figure 3.3 show SEM picture of (A) bare graphite, (B) and *S. loihica* PV-4 biofilm on graphite. (C) Confocal microscopy picture of the *S. loihica* PV-4 biofilm on graphite electrode collected after 96 h of cultivation at 0.2 V vs. Ag/AgCl. The roughness was a primary factor determining the electroactivity of the biofilm.

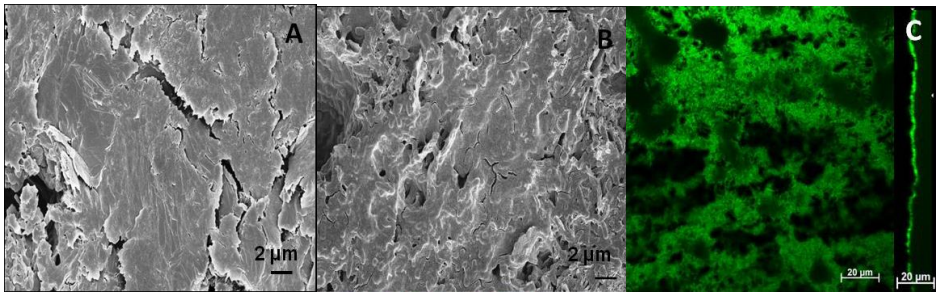


Figure 3.3 (A) SEM picture of bare graphite, (B) and *S. loihica* PV-4 biofilm on graphite. (C) Confocal microscopy picture of the *S. loihica* PV- 4 biofilm on graphite electrode collected after 96 h of cultivation at 0.2 V vs. Ag/AgCl.

3.2 Electrochemical characterization of *G. metallireducens*

G. metallireducens form a thin biofilm within 24 h. This electroactive biofilm was characterized by CA, CV, DPV. In the following sub-sections, we report the characterization of *G. metallireducens* wild type (Section 3.2.4). Additionally, the spectroelectrochemical characterisation of G. *metallireducens* EAB is included in Section 3.2.7.

3.2.1 Chronoamperometry

G. metallireducens grown in Fe(III)-citrate was inoculated at 50% v/v, thus the final concentration of Fe(III)-citrate was 20 mM. In the electrochemical cell poised at oxidative potential, Fe(III) citrated serves both as electron acceptor and redox mediator. Therefore, the current output in the first day was mostly due to Ferric citrate. The current output declined to a stable value when the red/ox forms of Ferric citrate reached an equilibrium concentration. It then started growing again, as the cells grow, thus oxidizing acetate and transferring the corresponding electrons to the electrode. Following two media changes, Fe citrate was mostly depleted and the residual cells were forced to form a biofilm to respire the electrode.

As the EAB grow, current increased at a rate of approximately 5.7 μA h^{-1}, corresponding to a generation time of approximately 7 h. This is compatible with the generation time observed in the growth curve in ferric citrate medium (Figure 3.4).

In all experiments described, the potential step used was 0.24 V vs. SHE. This potential was chosen based on literature (Bond and Lovley, 2003) about other bacteria and our preliminary electrochemical experiments. As recently demonstrated, the attachment phase is most rapid when electron donor-limited cells (compared to mid-log-phase cells) were used as the inoculum, (Marsili 2008). This phase was followed by a growth phase, characterized by an exponential increase in current, which doubled at a rate typically observed for *G. metallireducens* reducing Fe(III)-citrate.

3.2.2 Cyclic voltammetry

CV can assess the respiratory rate, which is relevant for metal reduction. Several tests were required to find the best condition (i.e. the operative potentials) to grow a good electroactive biofilm. Cyclic voltammetry in a 3-electrode electrochemical cell can detect catalytic properties of intact bacterial biofilms in real-time. CV revealed a sigmoidal anodic current profile characteristic of catalytic activity (Fig. 3.4). This positive current can be observed above a potential of approximately 0,2V reflecting a flow of electrons from bacteria to the working electrode. First derivative analysis of voltammetry results (Figure 3.4-C, inset) allowed estimation of the potential at which the rate of increase of the catalytic wave reached a maximum [similar to what is typically termed E_{CAT} in purified protein studied (Armstrong, 2005)]. This potential (0.15 V - 0.01V) was consistent across all experiments. The steepness of the wave was also stable across multiple experiments and treatments, as determined by the width of the derivative peak at half-maximal height (Anderson et al., 2000).

3.2.3 Differential pulse Voltammetry:

Although CV is the most informative method used to investigate catalytic substrate oxidation by adsorbed enzymes, it has a low detection limit, and subtraction of the ohmic (capacitive) current is necessary to reveal small features. Furthermore, when electron transfer between adsorbed enzymes and electrodes is slow, as is expected for complex electron transfer chains studied in stationary electrodes, CV requires substantial time, and proper derivative analysis requires post-processing of data. In comparison, pulse methods have the potential to reveal characteristic peaks while cancelling out capacitive current, even at higher scan rates, and are often used as complementary techniques to CV.

Preliminary experiments with *G. sulfurreducens* biofilms indicated that DPV could also be used to monitor biofilms non-destructively, across a range of scan rates (up to 50 mV s^{-1}) and pulse heights (up to 100 mV). The

parameters chosen (see Materials and Methods) represent a compromise between the time of analysis and sensitivity. When performed on mature biofilms, DPV always revealed a broad peak, which increased in height with the age of the biofilm. These voltammograms were highly reproducible, with the peak centred at −0.105 ± 0.005 V versus SHE.

3.2.4 *G. metallireducens* at graphite electrode

I used a thin graphite electrode as described in the Materials and methods section. I have characterized two different types of biofilms: biofilm growing on the electrode surface by the application of an adequate potential (0.2 V), and a biofilm naturally formed in a tube containing the culture and no potential applied.

Figure 3.6 shows the electrochemical analysis of a *G. metallireducens* electroactive biofilm. After the first 30 hours, anodic current increases steadily for more than 250 hours. A 2000 µA current production was reached after 210 hours. The first part of the graph is relative to the bacterial attachment onto the electrode surface and the current does not increase. This is due to the presence of Fe(III)-citrate at 20 mM, which serve as electron acceptor and redox shuttles. Between 24 and 70h a rapid growth is observed, while later the current increases with a minor rate until the maximal value is reached. The inflection at 170h indicates the addiction of 20mM acetate, which was depleted by the following medium change at 205h.The decreasing slope with time of the anodic current vs. time is due to the accumulation of a thick, poorly conductive biofilm: this envelope limits the diffusion of nutrient to cell in proximity of the electrode surface.

Cyclic voltammetry shows (Figure 3.4) clear sigmoidal shaped voltammetric curves since the 125th hour. The increase of the current value at the inversion and the shift of the sigmoidal peak toward negative values mean that a fully and thin electroactive *G. metallireducens* biofilm developed on the surface of the working electrode, according to the Differential Pulse Voltammetry (Figure 3.4), which show at t=0 only a major peak around -0.1V vs. SCE that disappears during the biofilm development. In contrast, three peaks at -0.53, 0.38 and 0.30 develop as the biofilm grows.

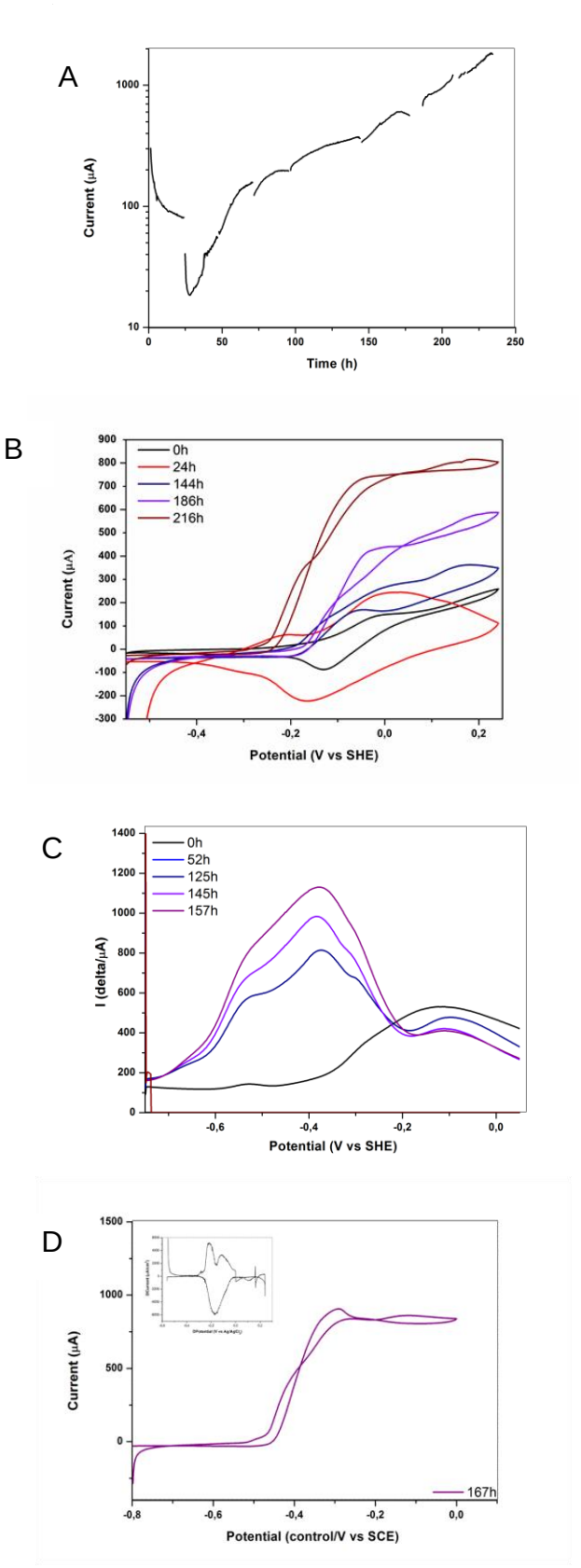


Figure 3.4 Electrochemical characterization of *G. metallireducens* wild type biofilm. (A) Chronoamperometry. A 2000 μA current production was reached after 210 hours. A full medium change was performed every 24h. (B) Cyclic Voltammetry. The production of a clear sigmoidal shaped curve since 144th hour, the increase of the current value and the shift of the

sigmoidal peak toward negative valuesindicate the development of a *G. metallireducens* electroactive biofilm on the surface of the working electrode. (C) Cyclic Voltammetry with inset of its first derivative. *G. metallireducens* was grown on electroactive biofilm. The first derivative amplifies the curve shape making the oxidation and the reduction curves more evident; (D) Differential Pulse Voltammetry of *G. metallireducens* growing as electroactive biofilm The increasing with time of three peaks (-0,54; -0,38; -0,29 V) indicates the formation of a viable and growing electroactive biofilm

3.3 Genome shuffling of *G. metallireducens*.

NTG is a mutagenic agent used to induce single point mutations in bacterial genomes. This kind of mutation is random, thus it is not possible to anticipate which cellular function will be altered. It is likely that alterations of DNA in bacteria slow their growth also in optimal condition, sometimes they lead to cell death. As reported in the literature, in genome shuffling experiments one of the most common and effective mutagenic agents is NTG and the adequate concentration required in this kind of approach is about 40-50 mg L^{-1}. Our genome shuffling protocol involves growing bacteria in basic medium (NBFC) enriched with the mutagenic agent NTG at 40 mg L^{-1}. During the incubation time, at 30°C in anaerobic conditions and darkness, bacteria with non-lethal mutations survived and grew. Most of these experiments were unsuccessful. Considering the regular vitality of stored cultures concurrently to such negative results, the most likely reason of non-growth is death of bacteria due to the presence of NTG or severe damage to the cell membrane.

To verify if cultures of *G. metallireducens* were differently affected by several NTG concentrations a vitality assay was carried out using several NTG concentrations: 20, 30, 40, and 50 mg L^{-1}. To simulate the exact conditions required from the procedure, the solution was enriched with Cr(VI) to final concentration of 1mM. Since all samples grew normally after one week, 40 mg L^{-1} was choosen as working concentration.

3.3.1 Test of different lysozyme action times in liquid culture of *G. metallireducens*.

To induce fusion of two or more cells, it is necessary that such cells are devoid of cell walls, a multifunctional structure separating the contents of the cell from the outer membrane. Cell walls provide protection for cells against mechanical damage and allow cells to survive in a medium of lower osmotic potential than that of its protoplasm. Genome Shuffling concerns contact and fusion between cells of the same species (or not) and sharing of DNA. To allow blending of genetic material of respective bacteria, it is necessary to remove barriers. The agent most commonly used for this purpose is lysozyme. Bacteria from which cell wall has been removed are weak and protracted exposition to lysozyme action could be lethal. Conversely, an overly soft lysozyme operation may not remove totally the wall, preventing protoplasts fusion and genome shuffling effect.

In order to identify the time required to obtain enough removal of the wall components, several samples were examined after different times of incubation with lysozyme: 30 minutes, 45 minutes and 60 minutes. Lysozyme activity was observed using an optical microscope, looking for loss of shape of bacterial cells due to the loss of the cell wall. The first

sample, following 30 minutes of exposition, shows a large amount of rod-shaped cells and just a few cells of a round-shape, indicating loss of wall. After 45 minutes, only a small number of rod-shape cells were observed. The last sample, 1 hour after the beginning of the experiment, was totally made of round-shape bacteria ready to be employed in the protoplast fusion. Then, tests on how different polyethylene glycol (PEG) action times in *G. metallireducens* liquid culture were performed. Void of their external protection, bacteria have expose the cellular membrane made of lipid components. To induce the fusion of such membranes it is sufficient to submerge cells in a soap that allows aggregation of bacteria to form large fused protoplasts. The agent suitable for the task is PEG 6000.

As well as for lysozyme, PEG requires time to perform its function. The incubation period should be enough to promote encounter between wall-less bacteria alive in the solution. Contact between them, supported by gentle shaking, makes possible their fusion. Subsequently, bacteria should be collected and inoculated in NBFC enriched with NAG, an essential component indispensable to correct wall restoring. Centrifugation was used to pellet the cells and remove the PEG: centrifugal force allows the formation of a compact pellet of bacteria on the bottom of tubes, thus removal of PEG solution is easier and cells are ready to be re-suspended in usual medium. Also in this step, time plays a fundamental role. How long do protoplasts need to be submerged with PEG to clash and fuse? This represents the first issue to address. In the followed experiment samples were examined after several periods of incubation with PEG at 50% concentration (as reported in protoplast fusion protocol): 30 minutes, 1 hour, 2 hours and 3 hours.

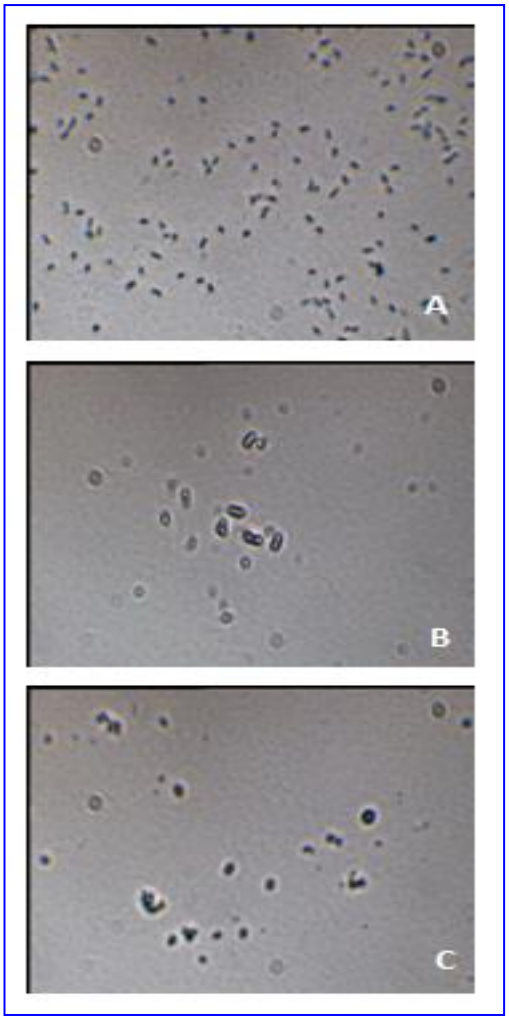


Figure 3.5: Images from optical microscope of *G. metallireducens* cells treated by PEG: A - untreated sample; B - after 30 minutes; C – after 3 hours. Fused cells formation was optimal after 30 min.

Evaluation of results was made by observation under the optical microscope searching protoplasts for aggregates. As shown in Figure 3.5, untreated protoplasts have a round shape and appear widespread uniformly in the sample (section A). After 30 minutes of PEG treatment, most of the protoplasts are part of 2 or 3-cell aggregates. With a higher resolution it could be possible to observe these cells sharing their cellular membrane (section B).

The amount of protoplasts forming aggregates is largely increased 3 hour afer the beginning of the experiment. As shown in Figure 3.5 C, at this stage of treatment, fusion could involve two or more cells, also up to 6 cells together.

For physiological reasons, the probability of survival of 5/6 cells fused together and sharing 5 or 6 different genomes is unlikely. Thus, in the genome shuffling procedure the optimum action time allow 2-cell aggregates. The sample examined up after 1 hour of treatment (section B) shows a good amount of this kind of aggregates. Leaving cells for a longer

time, 1.5 hours should improve PEG- induced fusion of protoplasts in the solution.

3.3.2 Test of different PEG concentrations to improve protoplast fusion

Adding PEG to a solution makes it more viscous and incubating protoplasts in such a solution with gently shaking allows cells to swim through it and for fusion to occur between some of them that collide. An initial concentration of PEG 6000 solution is 50%. To find the right final concentration to induce fusion, samples were examined from several culture/PEG solutions: ¼ PEG, ½ PEG and ¾ PEG. Evaluation of results was made by observation under the optical microscope searching protoplast aggregates fused by cellular membrane. Half culture and half PEG produced a good compromise between enough percentage of fused protoplasts and right viscosity of medium. Only ¼ of PEG does not allow fusion of membranes but, conversely, ¾ of PEG gives rise to a solution too viscous to work. However, applying the finding to the genome shuffling procedure, bacteria likely not survive or are not present in the solution after the treatment. The reason could be the low protoplast vitality after PEG fusion, but the easiest explanation is a too slow centrifugation that leaves protoplast in the supernatant. Thus cells are lost and with that the results are negative. Viscosity of PEG solution seems to affect the sedimentation of fused protoplasts during centrifugation, thus, a higher speed of rotation could help this process and improve the pellet recovery. At the same time, decreasing the percentage of PEG 6000 added to the protoplast solution could support this attempt. In executing the genome shuffling protocol modified with the improved velocity of centrifugation (20000g), 2 different percentages of PEG were added for the protoplast fusion step: 40% and 50%. In 4 days bacteria were grown. The higher speed allows separation between bacteria and the viscous medium required for protoplast fusion. Bacteria generate the pellet on the bottom of tubes as oval brown patches. Following removal of the supernatant and resuspension of the pellet in NBFC enriched with NAG, bacteria can regenerate their wall and grow as usual. The result was manifested at all PEG concentrations meaning that the percentage is not influencing the Genome Shuffling procedure.

3.3.3 Test for protoplasts vitality after PEG fusion.

As mentioned before, big aggregates of 4-6 cells are not able to survive and replicate themselves. The reason why that happens is the complexity of sharing different genetic materials and coexistence of many cellular mechanisms (replication, duplication, energy machinery, etc.). However, 2-cell aggregates are weak and fragile, because of strong treatment with lysozyme and PEG-mediated fusion. Thus, it is not well known if *Geobacter metallireducens* bacteria survive and how many of them. If there are not live

cells, genome shuffling cannot be realized. PEG-mediated fusion is likely the most important step in the genome shuffling protocol. Furthermore, they are in a highly viscous solution and recovering them by centrifugation is not a reliable procedure. Several samples were examined, centrifuged and re-suspended, for several periods of incubation with PEG: 1 hour, 1 hour and a half, 2 hours, 2 hours and a half, 3 hours.

To verify if the fused protoplast vitality is affected by growth medium after the treatment, two different media were used to re-suspend the pellet after PEG treatment and centrifugation; GPB and the usual NBFC. Observing colour of cultures after resuspension could be used to determine if bacteria are alive or not. If media are coloured brown, as pure NBFC is, there is not bacterial replication occurring. Conversely, if media are coloured a light yellow, bacteria are alive, have replicated themselves and reduced the Fe(III) in the solution. Even after about 2 weeks, samples were not still grown either in NBFC or GPB. Analysing the result, the absence of growth suggests new findings are needed to improve the protocol.

Performing the first cycle of Genome Shuffling the experiment was unsuccessful. After two weeks, bacteria had not grown in NBFC medium following the complete treatment. That upholds results previously described. Absence of growth suggests further research is required to determine the limiting step that prevents bacterial growth or causes loss of cells.

3.4 Characterization of *G. metallireducens* genome shuffling mutants.

3.4.1 Chromium resistance test

The mutant strains obtained by genome shuffling were selected based on the chromium tolerance. The mutants showed a doubled capability with respect to the wild type.

G. metallireducens processed by genome shuffling were firstly analysed as community. The first analysis was the determination of the maximal tolerance to Cr(VI). This analysis gives a first and important quantification of the genome shuffling success as it selects those strains able to tolerate a Cr(VI) concentration higher than the concentration that the wild type strain can tolerate.

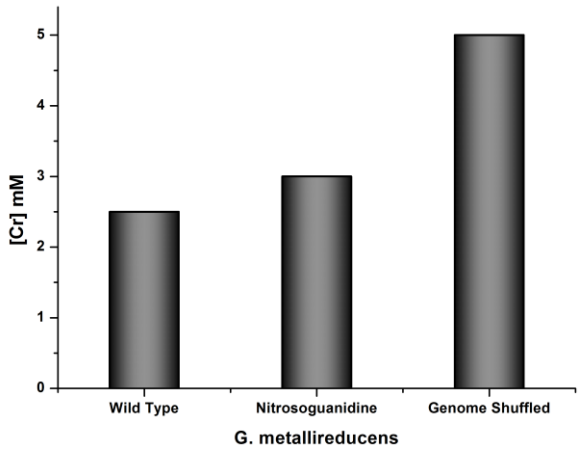


Figure 3.6: Cr(VI) resistance test of *G. metallireducens*: the wild type strain was able to tolerate 2.5mM Cr. When the wild type strain was treated with 40 mg L^{-1} NTG, random mutagenesis and selection provided mutants able to tolerate 3mM Cr. The recombination of the genomes of the mutants by genome shuffling and the following selection provided strains with further improved resistance phenotype.

Chromium toxicity tests showed that the wild type strain can tolerate a maximum concentration of 2,5 mM Cr(VI) (Figure 3.6). The mutagenic action of NTG was confirmed by the increasing of the Cr(VI) tolerance of the WT strain treated by 40 mg L^{-1} NTG and auxotrophic mutant production (data not shown). The genome shuffled population can tolerate and grow at 5mM Cr(VI). A double increase of metal tolerance can be considered an important result because it was obtained by only one round of genome shuffling.

The strains mix contains all the individuals generated by the application of the genome shuffling protocol. We chose to characterize this mixture containing numerous mutants to verify if mutants with higher electroactivity could be observed without further selection and purification in rapid

electrochemical experiments (Section 3.5). Those experiments were carried on in absence of Cr(VI), as the bacterial tolerance of the metal was already tested. Moreover, Cr(VI) could give an electrochemical signal at low potentials.

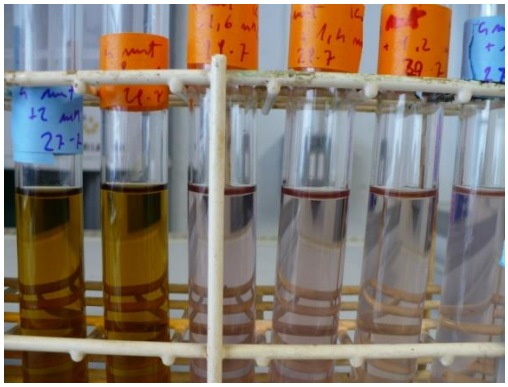


Figure 3.7 *G. metallireducens* Cr(VI) resistance test. Cr(VI) was added to 10ml NBFC. Cultures grown on the highest Cr concentration was transferred to NBFC agar containing higher Cr concentration in order to select the most tolerating colonies.

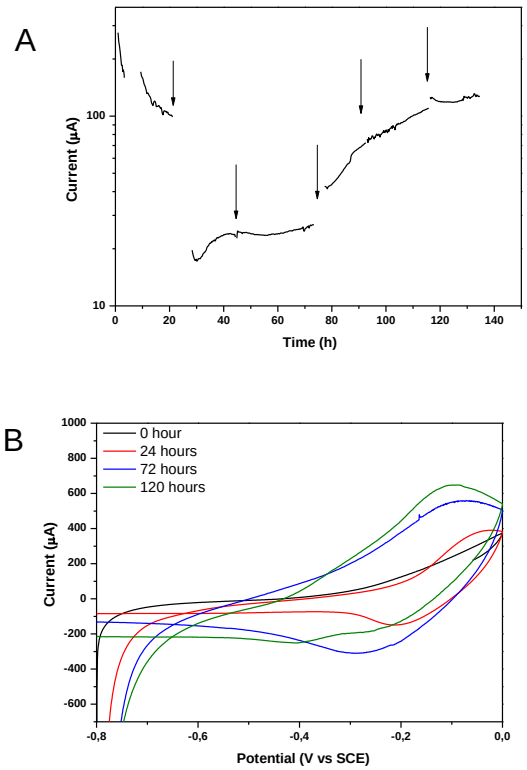
3.5 Electrochemical characterization of improved *Geobacter metallireducens* produced by genome shuffling

Following genome shuffling procedure, we selected the best performing mutants with respect to Cr(VI) tolerance and grow them as electroactive biofilms as previously shown for *G. metallireducens* WT. For the first series of experiments, we used the whole mix of cells produced by genome shuffling. This mixture contains numerous mutants, which likely exhibit a wide spectrum of electroactivity. The scope of these experiments was to monitor the progress of genome shuffling protocol and to verify if mutants with higher electroactivity can be observed without further selection and purification in rapid electrochemical experiments. Those experiments were carried on in absence of Cr(VI), as the bacterial tolerance of the metal was already tested. Moreover, Cr(VI) could give an electrochemical signal at low potentials.

Figure 3.8 shows the result of a typical electrochemical experiment. As for the other Chronoamperometry *G. metallireducens* experiments, the current produced during the first 24h (CA, panel A) was mostly due to the electron shuttle behaviour of Fe(III)-citrate. Following the first medium change (24h), the planktonic cells and the iron citrate were removed from the cell and the current increased steadily starting from 8 µA. The current

then increases slowly with time, reaching approximately a stable value of 120 µA after 140 h. This is a much smaller current (4-5 fold) than that observed in *G. metallireducens* WT experiments. The result suggests that the mutant mix has overall low electrochemical activity, likely because most Cr(VI)-tolerant mutants are not electroactive. The CV support this observation, as it shows a broad, convoluted peak at high potential (>-0.3 V), which correspond to most of the current delivered in the CA. The typical electrochemical features of *G. metallireducens* WT were not observed. However, some similarity with Geobacteraceae was observed. In fact, the current did not change significantly after medium change, indicating that DET remained the dominant EET mechanism. A thin biofilm with the typical orange-red colour was observed at the end of the experiments, indicating that the mutant mix contained some strains capable of forming electroactive biofilm. Because of its much higher sensitivity, DPV shows some small features at low potential that are compatible with *G. metallireducens WT*. However, most of the EET occur at high potential, as the main peak is centred at -50 mV. The first derivative of the CV at 90h did not show the well-defined features of *G. metallireducens* CV.

The comparison of low scan-rate CVs for G. *metallireducens* WT and the GS mutant mix is shown in Figure 3.8 for the same biofilm age (48h).



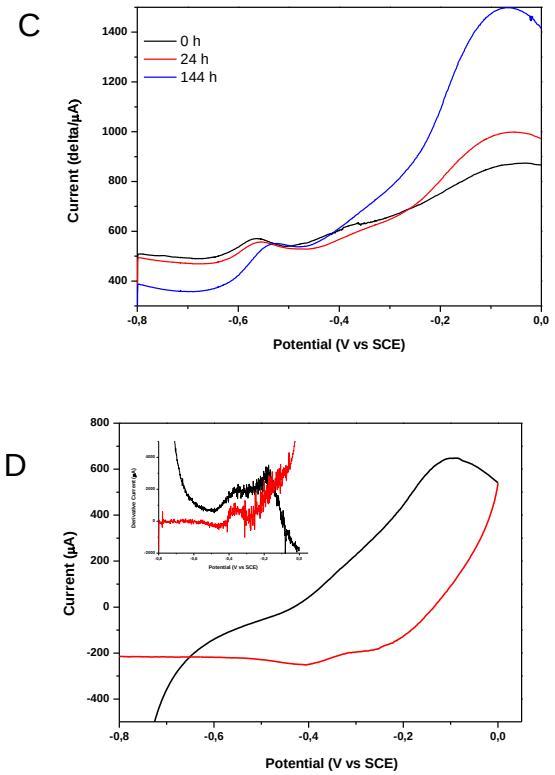


Figure 3.8 Electrochemical characterization of *G. metallireducens* strains genome shuffling mutant mix. (A) CA of genome shuffling mutants mix. 120µA were produced after 140h. Medium change, CV and DPV are evidenced by the arrows (approx every 24 h); (B) CV of *G. metallireducens* genome shuffling mutant mix. The voltammogram does not show the typical sigmoidal curve characteristic of the turnover electron transfer, however the increase of both ohmic and faradaic current with time indicate the development of a biofilm on the electrode; (C) DPV of *G. metallireducens* genome shuffling mutant mix. A large, convoluted peak centered at 0.07 V indicated sluggish extracellular electron transfer at high potential; (D) First derivative of CV after 120 h of growth (I = 90 µA). The electroactive features of the WT are nearly hidden by the high potential peaks of the other strains.

The difference between the two CAs and CVs, as the WT shows a well-defined sigmoidal curve. The poor electroactivity of the mutant mix is likely due to its heterogeneous nature, as less electroactive bacteria (but Cr(VI)-tolerant) limited the current output.

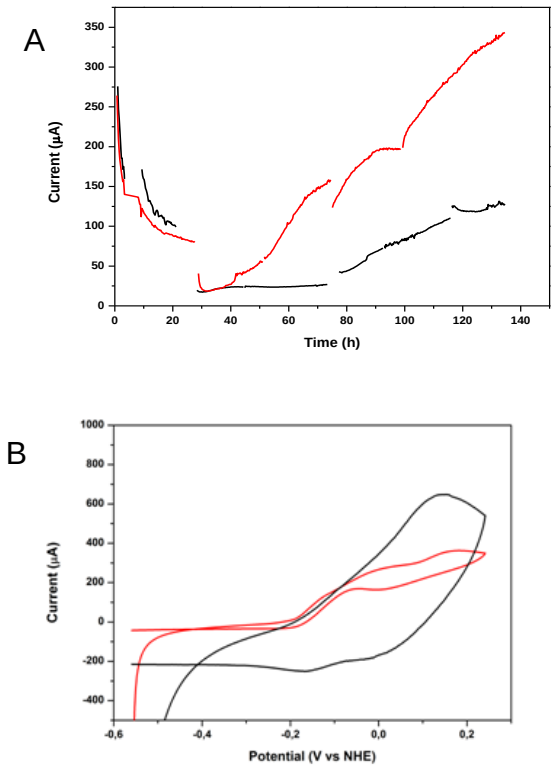


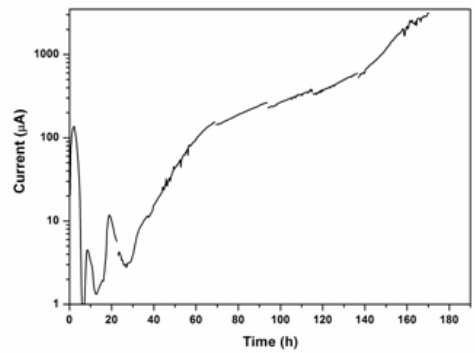
Figure 3.9: Comparison of CAs and CVs of *G. metallireducens* wild type and genome shuffling mutant mix. Red trace: *G. metallireducens* wild type; black trace: *G. metallireducens* genome shuffling mutant mix. (A) In the CA, both inocula behave similarly in the first 24h, due to the presence of the redox mediator ferric citrate. Following the first medium change, the current raised faster in both experiments, although at different rates. After 140 h, the wild type produced 3 time more current than genome shuffling mutant mix. (B) CV after 48 h. The current produced was approximately 90 µA for both inocula. The thickness of the mutant mix curve indicates the presence of a poorly conductive bio-interface. This is likely due to the predominance of not electrochemically active mutants in the mix.

3.5.1 Single mutant selection and characterization

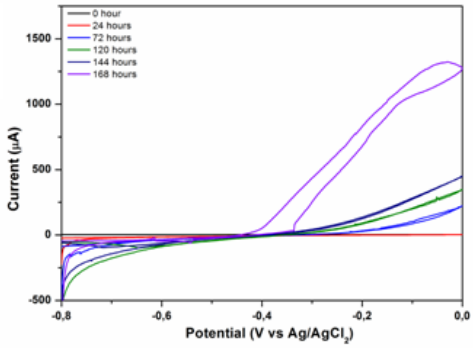
The mutant mix was spread onto solid NBFC medium to isolate single colonies. Twenty-nine mutants were picked up and transferred again in the same medium to ensure the purity of cultures. In both cases, the colonies were picked up immediately after they appeared. The selected strains were then transferred to NB Iron oxide medium in order to maintain the extracellular metal-reducing phenotype. No Cr(VI) was added in order to avoid stress and speed up the microbial growth. Moreover, mutant at this stage of genome shuffling were already selected about Cr(VI) resistance. Among the mutants isolated from solid medium, we chose those that were able to reduce Fe(III) to Fe(II) and form magnetite. Magnetite appeared earlier than for the parent strain (3 weeks vs. 4 weeks, data not shown). The ten best performing mutants (those that form magnetite earlier) were characterized in electrochemical cells poised at oxidative potential, as previously described for the parent strain. Of these 10 mutants, 9 showed

the same electroactivity of the parent strain (data not shown). The mutant M23 produced two-fold the current observed in the wild type and six-fold than current observed in the genome shuffling mutant mix.

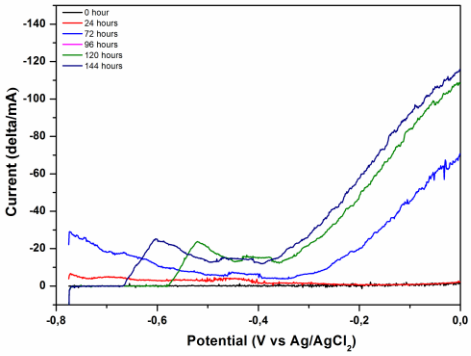
A



B



C



D

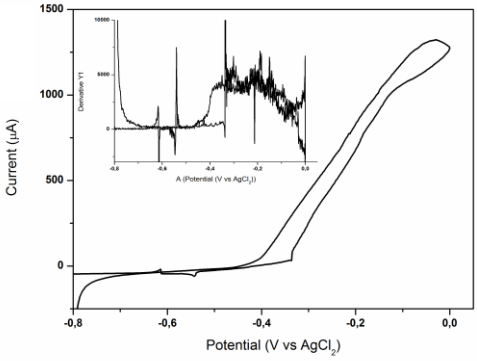


Figure 3.10: Electrochemical characterization of *G. metallireducens M23*. (A) CA shows that 2900 µA were produced after 168h. CV and DPV were performed every 24 h immediately before medium change; (B) CV of M23 *G. metallireducens* biofilms. No sigmoidal curve was observed in the range of potential test, suggesting a different EET mechanism with respect to the WT. The increase of current with time indicates the development of a *G. metallireducens* electroactive biofilm on the working electrode; (C) Differential Pulse Voltammetry of *G. metallireducens* M23. Most of the electroactivity occurs at high potential (>-0.3 V), differently form what observed with *G. metallireducens* WT. increasing of current at 0V indicates a difference with respect to the other strain tested.; (D) CV of *G. metallireducens* M23 biofilm after 168h and first derivative (inset). The derivative did not show any recognizable feature in the range of potential tested.

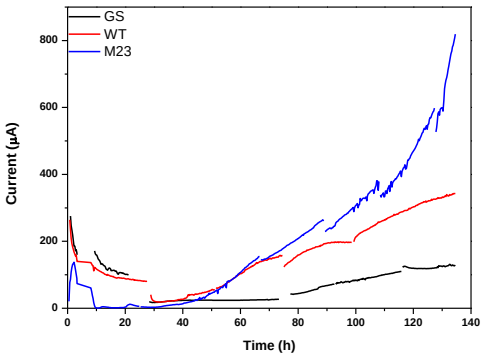
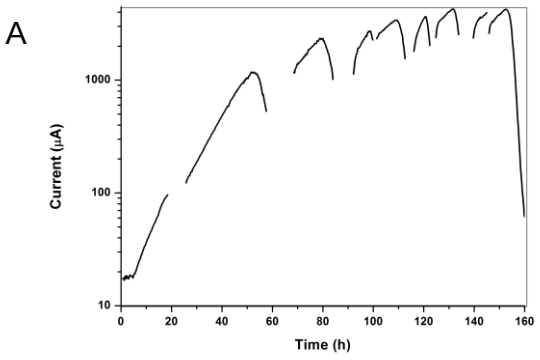


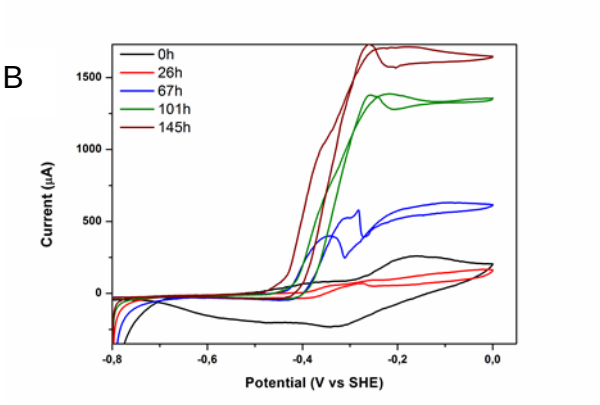
Figure 3.11: comparison of CAs and CVs of *G. metallireducens* biofilms grown on a graphite electrode poised at 0.2 V. Red line: *G. metallireducens* WT; black: *G. metallireducens GS; Blue: G. metallireducens M23.* A: Chronoamperometry of *G. metallireducens* wild type, genome shuffling mutant mix, and M23 mutant. The current output in the first 24h is similar due to the mediating action of ferric citrate.

Figure 3.11 summarizes the main results of this work. The CA of WT, mutant mix and M23 are compared. Following the first medium change, the WT and M23 increase the current production much faster than the genome shuffled population. After 72 h, M23 current output grew faster than both WT and GS. It is probable that the lower current of GS with respect to WT was due to poor electroactive contribution of not well-selected cells downstream of the GS procedure, which select for overall Cr(VI) tolerance. However, the additional selection step returns mutants in which the Cr(VI) tolerance is coupled with high electroactivity. It should be noted that the performance of the laboratory strain of *G. metallireducens* did not change significantly during the whole work (approx. three years). Thus, the application of GS procedure to *G. metallireducens* effectively improved the Cr(VI)-tolerance. The addition of a second screening based on EET enabled selection of those strains that are both Cr(VI)-tolerant and have improved electroactivity. These strains can be used as starting points for another round of GS or for other genetic improvement methods.

3.2.5 Naturally formed *Geobacter metallireducens* biofilm

We have compared the electroactive biofilms with biofilms grown on a electrode not connected to any potentiostat for 4 days (Figure 3.12). When the electrode was poised at 0.24 V vs. SHE, the anodic current developed immediately reaching 100µA in 24h – as we use the growth medium without Fe(III)-citrate and the electrode was the sole electron acceptor. The plateau current was 4000µA, approximately twice that obtained when forming the biofilm at a posed electrode (Figure 3.12). The numerous peaks in the graph are due to the very high current and biomass compared to the volume of the bioreactor: the consumption of nutrient was too fast and in the night the current decreased. The comparison of the CVs confirms that biofilm formed when no potential is applied and is more electroactive than the one formed in presence of potential. In fact, the sigmoidal curve in the CV is higher and better shaped. Additionally, the biofilm formed on the electrode with potential applied shows sigmoidal shape only after 144 h, while the biofilm formed on the electrode surface when no potential was applied shows sigmoidal shape after 26 h. It is also interesting to look at the CV curve relative to the biofilm developed for 5 days with no potential: the curve is tighter than the curve relative to planktonic cells (0 h) but the absence of sigmoidal shape reveals that the biofilm is not yet fully electroactive. Moreover, this type of biofilm can reach the electroactivity faster than planktonic cells and can reach higher current values (1.6 vs. 0.8mA).





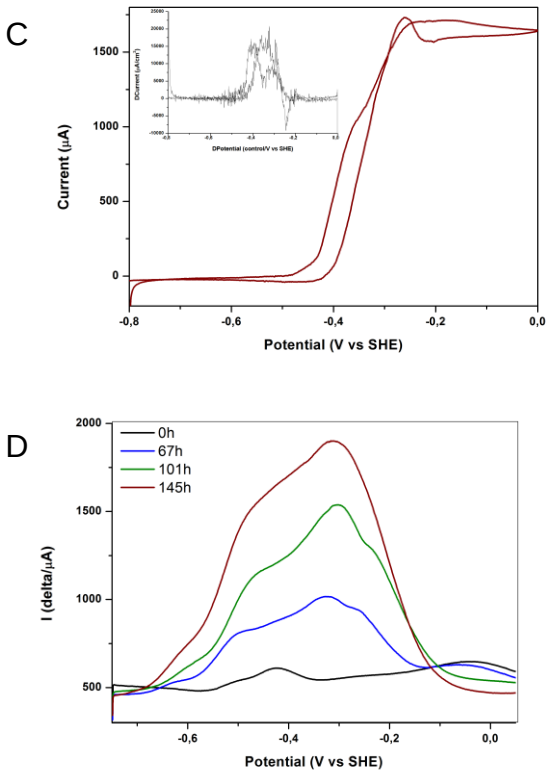


Figure 3.12: Electrochemical analysis of *G. metallireducens* biofilms grown on non-poised electrode. After 5 days the graphite electrode was poised at 0.2V vs SHE. (A) Chronoamperometry of a *G. metallireducens* biofilm formed without any potential. A 4000 µA current production was reached after 210 h. A full medium change was performed every 24 h; (B) CV The shape of CVs is not linear meaning that the not all the cells of biofilm were electroactive; (C) Cyclic Voltammetry with inset of its first derivative. The CV showed in the picture was run at 167 h after the experiment start, after a full medium change. The first derivative amplifies the curve shape making the oxidation and the reduction curves more evident; (D) The DPV peaks at -0.54, -0.38, and -0.29 V increase with time, as a viable and electroactive biofilm is formed.

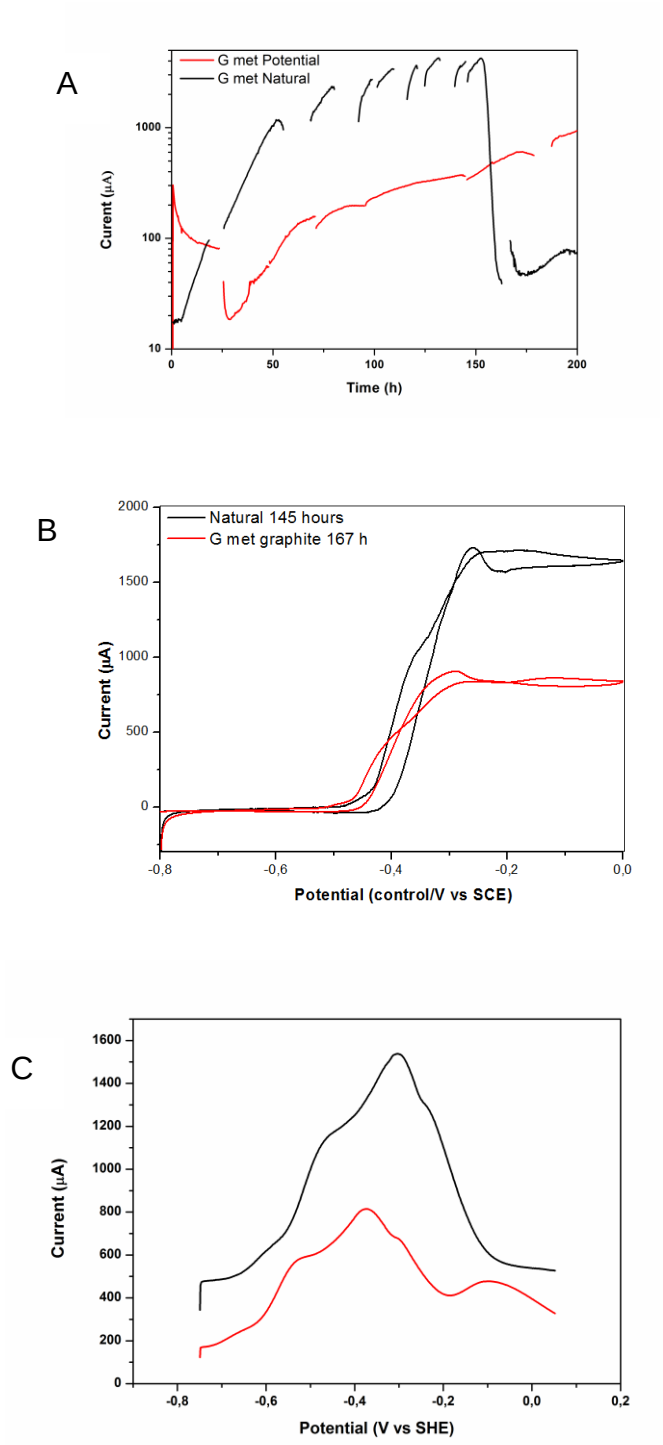
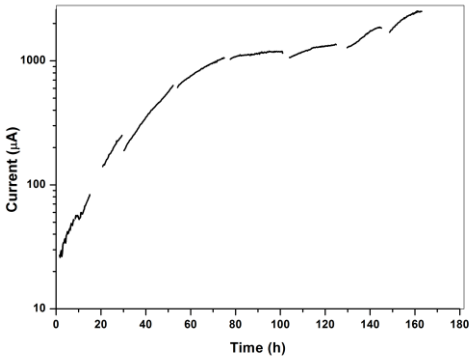


Figure 3.13: comparison of CAs CVs of G. *metallireducens* biofilms grown on a graphite electrode posed at 0.2 V or on a graphite electrode applying no potential. A: comparison of CAs of G. *metallireducens* biofilms grown on a graphite electrode poised at 0.2 V or on a graphite electrode applying no potential; B: comparison of CVs of G. *metallireducens* biofilms. A: biofilm grown on a graphite electrode posed at 0.2 V; B: biofilm formed on a graphite electrode applying no potential. C: comparison of DPVs of G. *metallireducens* biofilms. Red: biofilm grown on a graphite electrode poised at 0.2 V; Black: biofilm formed on a graphite electrode applying no potential.

3.2.7 Electrochemical characterization of *G. sulfurreducens* at graphite electrode.

To better understand and characterize *G. metallireducens*, a comparison with the model G*. sulfurreducens* was necessary. A typical *G. sulfurreducens* experiment is shown in Figure 3.9. Following inoculation of suspended *G. sulfurreducens* cells, we observed a rapidly increasing current, which is the result of catalytic oxidation of acetate in the *G. sulfurreducens* biofilm growing at the electrode (Figure 3.14). The maximum current density was 150 μA cm^{-2} after 36 h, similarly to *G. metallireducens*. After approximately 48 h, the electron donor (acetate) was completely consumed, and the current dropped to near-zero. Following replacement of spent medium with fresh growth medium containing 20 mM of acetate, the current density resumed in 2.5 h to about 90% of the maximum observed at 36 h. Previously, our work (Marsili 2010) demonstrated that such rapid increase in current density exceeds reported growth rates for *G. sulfurreducens*, therefore is not caused by biofilm growth. After this short recovery period, the current density increased with a rate of 13 μA cm^{-2} h^{-1}, which corresponds to a doubling time of approximately 10–11 h, compatible with the reported growth rates for *G. sulfurreducens*. Figure 3.15 compares CAs and CVs of *G. metallireducens* and *G. sulfurreducens* biofilms grown on a graphite electrode posed at 0.2 V vs. AgCl. *G. sulfurreducens* grows faster and produces higher current. Moreover, it is not affected by the initial lag like *G. metallireducens.* CVs has a better defined sigmoidal shape than those performed on *G. sulfurreducens* biofilms (Figure 3.15).

A



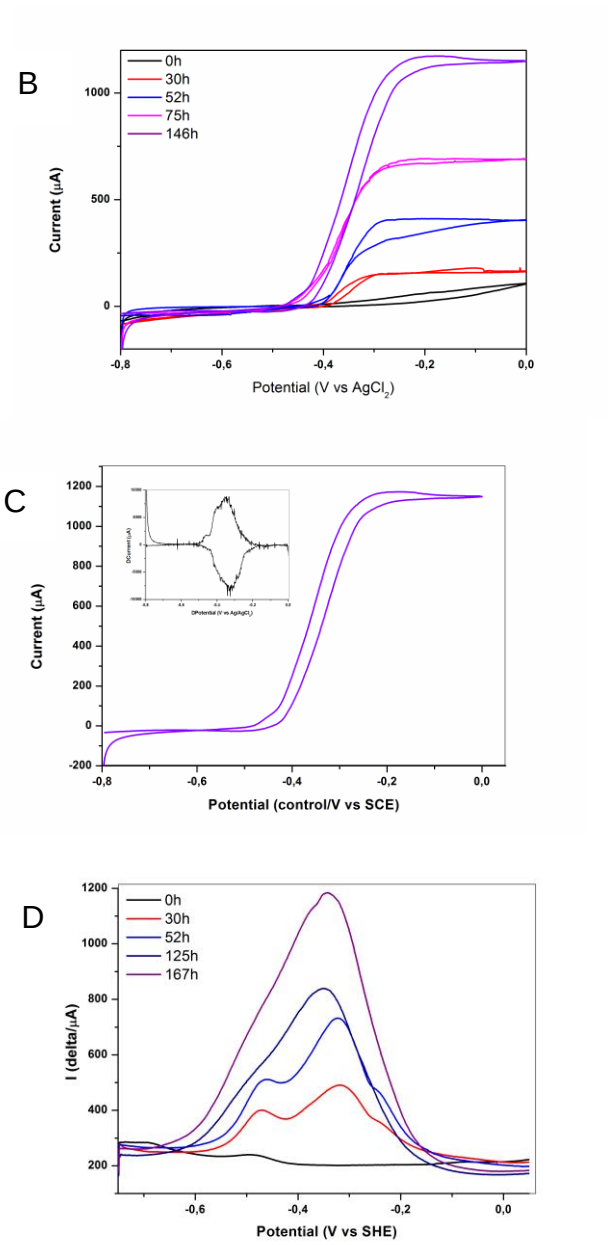


Figure 3.14: Electrochemical analysis of *G. sulfurreducens* grown as biofilm on the surface of a graphite electrode posed at 0,2V vs SHE. The total volume of spent medium was replaced every 24h by fresh medium in order to avoid to stop the biofilm growth A: Chronoamperometry of *G. sulfurreducens* growing as electroactive biofilm A 2500µA current production was reached after 170 hours. B: Cyclic Voltammetry of *G. sulfurreducens* growing as electroactive biofilm The production of a clear sigmoidal shaped curve since 30th hour, the increase of the current value and the shift of the sigmoidal peak toward negative values mean the development of a *G. metallireducens* electroactive biofilm on the surface of the working electrode. C: Cyclic Voltammetry with inset of its first derivative. The CV showed in the picture was run at 146h after the experiment start, after a full medium change. The first derivative amplifies the curve shape making the oxidation and the reduction curves more evident. D: Differential Pulse Voltammetry of *G. sulfurreducens* growing as electroactive biofilm The increasing by the time of three peaks (-0,54; -0,38; -0,29 V) means the formation of a growing electroactive biofilm

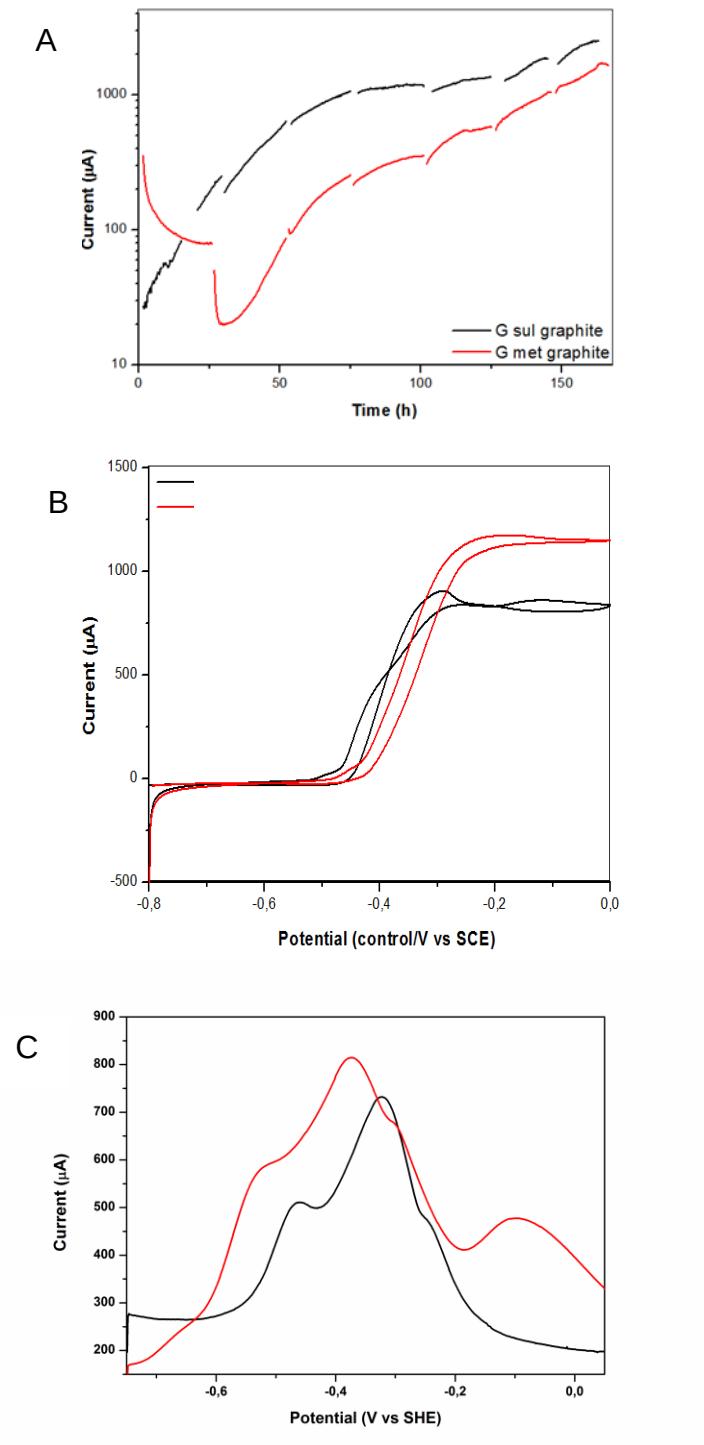


Figure 3.15: Comparison of CAs CVs *of G. metallireducens* and *G. sulfurreducens* biofilms grown on a graphite electrode posed at 0.2 V. A: Comparison *Geobacter sulfurreducens* and *Geobacter metallireducens* growing as electroactive biofilm. *G. sulfurreducens* produced approximately 2500µA current after 170 hours. *G. metallireducens* produced approximately 2000 µA. However, the growth rate is very similar as the curves have a similar slope; B: comparison of the CVs of *G. metallireducens* and *G. sulfurreducens* ran at the 150th h. C: comparison of the DPVs of *G. metallireducens* and *G. sulfurreducens* ran at the 52th h. *G. metallireducens* shows a better electroactivity, as it starts transferring electrons at a lower potential

3.2.8 Electrochemical characterization of *S. loihica* PV-4 at graphite electrode.

As part of the PhD work, cultivation and characterization of electron transfer mechanisms in *Shewanella loihica* PV- 4 viable biofilms formed at graphite electrodes in potentiostat-controlled electrochemical cells poised at oxidative potentials (0.2 V vs. Ag/AgCl) was performed.

3.2.8.1 Chronoamperometry

Figure 3.16 shows the CA of *S. loihica PV- 4* grown as biofilm on graphite electrode. CA, and its first derivative shows that both direct electron transfer (DET) mediated electron transfer (MET) mechanism contributes to the overall anodic (oxidation) current. . A current density of 5±1.2 μA cm^{-2} was immediately observed after inoculation of *S. loihica* PV-4 cell suspension. The anodic (oxidation) current grew steadily at a rate of 3 μA cm^{-2} h^{-1}, and then reached a maximum of 45 ± 12 μA cm^{-2} within 24 h. The experiment was performed using 5 independent replicates. The anodic (oxidation) current shows catalytic oxidation of the lactate and simultaneous reduction of the graphite electrode. After first MC, the chronoamperometry shows a 60 ± 10 % drop in the original current (A). This current pattern shows a contribution to the current generation by suspended *S. loihica* PV-4 cells and/or by soluble electron transfer agents. After first MC, current increases from 23 ± 10 μA cm^{-2} (at 26h) to around 56±15 μA cm^{-2} at 40 h and decreased thereafter but recovered quickly after lactate injection (15 mM) at 48 h. The current increased quickly to 76 ± 14 μA cm^{-2} and 90 ±18 μA cm^{-2} within 6 h of lactate addition (15 mM) at 48 h and 72 h, respectively (A), indicating that lactate was limiting in the EC. Subsequent lactate addition (15 mM) did not result in any further increase.

The maximum anodic current density recorded on graphite was 90 μA cm^{-2}. Fluorescence emission spectra shows increased concentration of quinone derivatives and riboflavin in the cell-free supernatant as the biofilm grows. Differential pulse voltammetry (DPV) showed accumulation of riboflavin at the graphite interface, with the increase in incubation period. This is the first study to observe a gradual shift from DET to MET mechanism in viable *S. loihica* PV- 4 biofilms.

3.2.8.2 Cyclic voltammetry and first order derivatives

The cyclic voltammograms of *S. loihica* PV- 4 biofilm on graphite collected after MC shows two overlapping catalytic waves, one onset at -0.6 V, centered at -0.44 V vs. Ag/AgCl, and the second onset at -0.2V, centered at -0.07 V, indicating two simultaneous catalytic electron transfer processes

at the graphite interface (A). Immediately after first MC (at 24 h after inoculation) the derivative of the CV shows that the electrons are transferred mostly via RC (I) directly to the electrode and RC (III) plays a minor role in the mediated electron transfer (B).Interestingly, first derivative at 48 h shows comparable peaks from both DET and MET at RC(I) and RC (III), respectively. However, with the further increase in the incubation period (at 72 h and 96 h) the electrons are transferred preferentially by RC (III) via MET mechanism, which was evident from the Fig. 3.17 (B). This suggests that with the increase in the incubation period the redox mediators (flavins) produced by *S. loihica* PV- 4 biofilm cells accumulate at the interface and are subsequently used to mediated electrons at graphite electrode.

3.2.8.3 Differential pulse voltammetry

DPV of the *S. loihica* PV- 4 biofilm formed at graphite electrode confirms the above results and shows the accumulation of flavins represented by the increase in the peak height at RC (III) with the increase in the incubation period (Fig. 3.18). Most of Shewanella sp are found to secrete redox-mediators such as flavins and quinones that mediated electron transfer, and an increase in the DPV peak height may represent the accumulation of redox-active mediator at the interface, while a decrease in peak height represents the loss of these compounds. A direct correlation between increases in flavin peak height in DPV with incubation period was observed (data not shown), as reported earlier for *S. oneidensis* MR-1 [Marsili 2008]. DPV shows the similar pattern as observed in the first derivatives of the CVs, i.e. the peak height at RC(I) decreases relative to the increase in the peak height at RC (III) with the biofilm growth. At 48h DPV shows comparable peak intensity at RC (I) and RC (II).

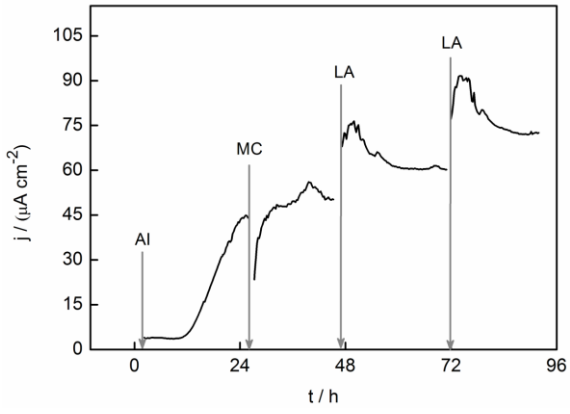


Figure 3.16: Chronoamperometry of *S. loihica* PV- 4 (AI) after inoculation at graphite electrode poised at oxidative potential (0.2 V vs. Ag/AgCl), (MC) medium change after 24 h with the fresh DM medium was followed by (LA) 15 mM lactate addition at 48h and 72h, respectively.

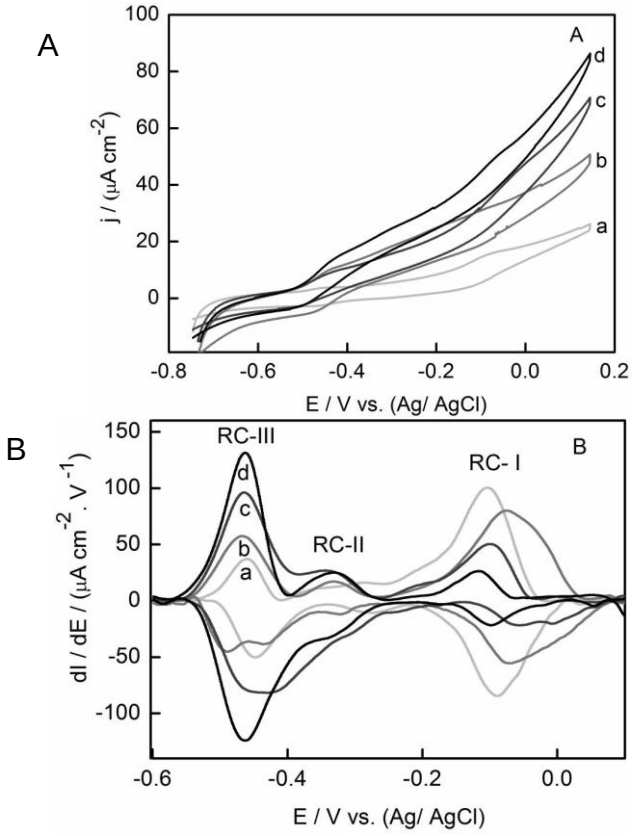


Figure 3.17 (A) Cyclic voltammograms at scan rate = 1 mV s-1 {obtained at (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h after MC}, and (B) first order derivatives of corresponding CVs

{obtained at (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h after MC} of *S. loihica* PV- 4 biofilms formed at graphite electrode. (B) The major redox centers in first order derivatives of CVs were identified as RC- I = -0.07 V, RC- II = -0.35 V, and RC- III = -0.44 V vs. Ag/AgCl.

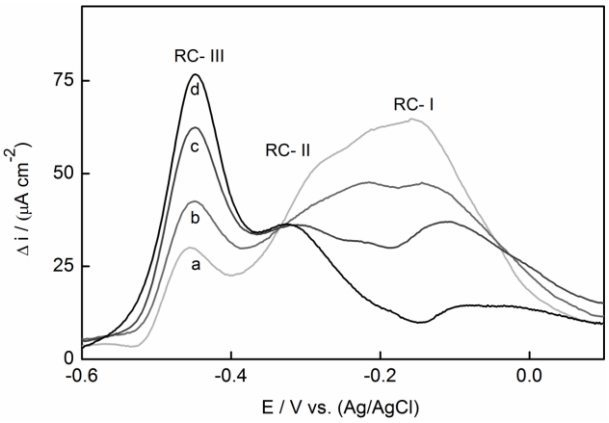


Figure 3.18 DPV of *S. loihica* PV- 4 biofilms associated with graphite electrode, collected at regular time intervals (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h after MC. The major redox centers were identified as RC- I = -0.07 V, RC- II = -0.35 V, and RC- III = -0.44 V vs. Ag/AgCl.

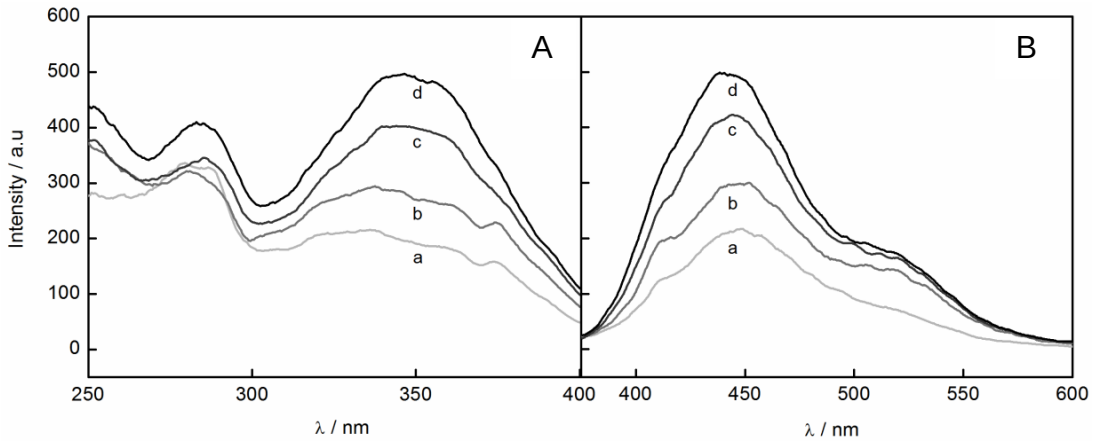


Figure 3.19(A) Fluorescence excitation and (B) emission spectra of the cell free supernatant {collected at (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h after MC} from *S. loihica* PV- 4 biofilms associated with graphite.

3.2.9 Spectroelectrochemical analysis

Electron transfer mechanisms in *Geobacter metallireducens* viable biofilms formed at graphite electrodes were investigated in potentiostat-controlled electrochemical cells poised at oxidative potentials (0.2 V vs. Ag/AgCl) (see materials and methods) (Figure 2.4). The *G. metallireducens* biofilms formed at ITO electrode exhibited an intense reddish-orange color, which indicates the high concentration of Omc, as previously reported for *G. sulfurreducens* (Seeliger et al., 1998) cell suspension. The electronic absorption spectra of the biofilms shows two absorption peaks for Soret band at 409 nm and 419 nm (Figures 3.20, 3.21) that indicate the oxidized and reduced form of cyt c552, respectively.

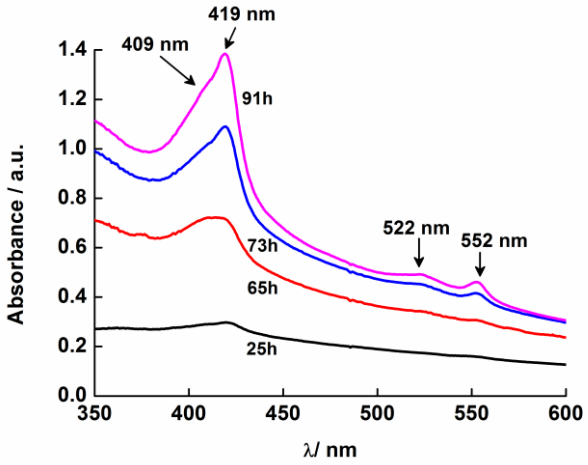


Figure 3.20 Electronic absorption spectra at different stages of *G. metallireducens* biofilm growth on ITO poised at positive potential (0.2 V vs. Pt wire). A clear increase in the peak intensity at 419 nm, 522nm and 552 nm wavelength is seen with biofilm growth on ITO.

The weak absorption bands at 522 nm and 552 nm are characteristic of the reduced form of heme groups in cyt c552 (Figure 3.21). Upon step-wise decrease of the electrode potential from 0.0 to −0.6 V vs. SCE, the absorbance of the peaks at 419, 522, and 552 nm increased (Figure 3.21), suggesting reduction of theoxidized cyt c552. Thus, it appears that *G. metallireducens* biofilms under turnover conditions contain reduced as well as oxidized cyt c552, as reported in *G. sulfurreducens* (Jain et al., 2011).

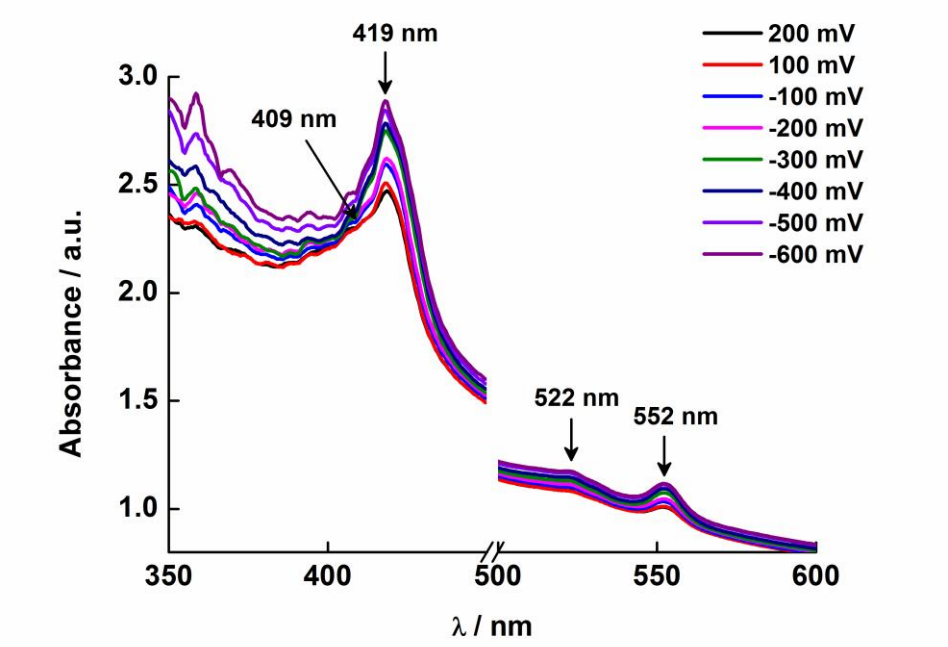


Figure 3.21 Electronic absorption spectra of the *G. metallireducens* biofilm under turnover condition, poised at different electrode potentials.

Figure 3.22 shows absorption spectra of *G. metallireducens* biofilm under non-turnover condition. Biofilm maintained for 24 h in absence of acetate at 0.0 V vs. SCE shows two absorption peaks at 409 nm and 528 nm. These absorption peaks indicate the presence of the sole oxidized form of cyt c552 in the biofilm. The biofilm cyt c552 spectra obtained under nonturnover condition are very similar to the spectra reported earlier for the oxidized state of isolated and purified Omc from *G. sulfurreducens* (Inoue et al., 2010). Upon stepping the electrode potential from 0.0 to −0.6 V, there was a red-shift (419 nm) of the Soret band, indicating reduction of heme groups in cyt c552.

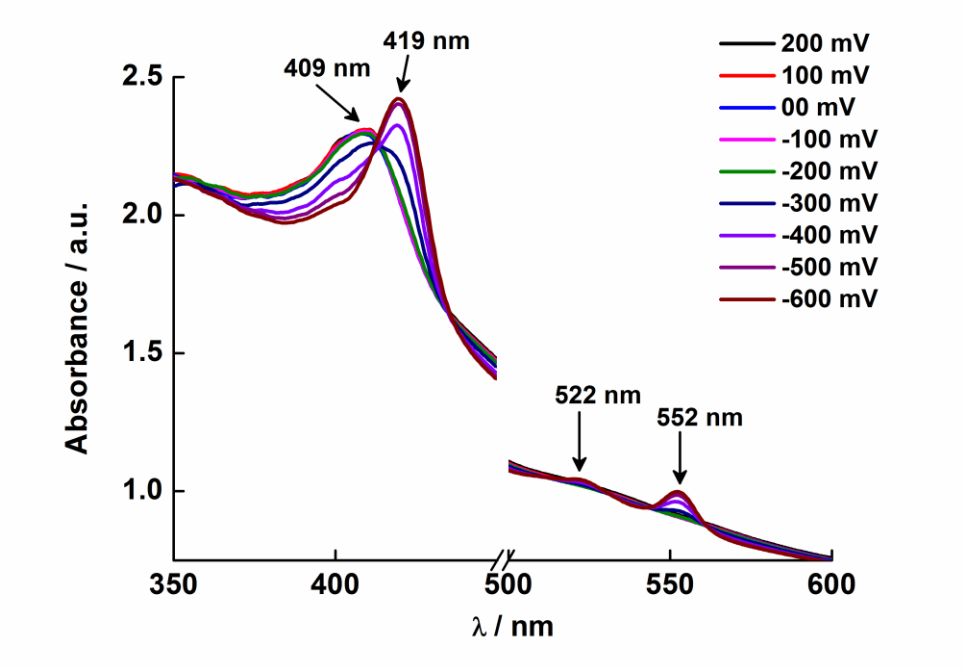


Figure 3.22 Electronic absorption spectra of *G. metallireducens* biofilm under non-turnover condition (c, d). Under non-turnover conditions, the Soret absorption band show Soret peak 419 nm, and weak absorption bands appear at 522 (β band) and 552 nm (α band). Following step-wise decrease of electrode potential from 200 mV to -600 mV vs. Pt electrode, the red shift (419 nm) of Soret absorption band and the gradual increase of the peak intensity at 522 nm and 552 nm were more pronounced than under turnover conditions.

Figure 3.22 shows spectroelectrochemistry analysis of *G. metallireducens* biofilm under non-turnover conditions The background-subtracted absorbance of the biofilm at 552 nm under non-turnover conditions was plotted (Figure 3.23) as a function of applied potential according to Nernst equation:

$$\text{Log } [Ox]/[Red] = (E_{app} - E_{mid}) \cdot (nF/RT) \qquad \text{(Eq. 2)}$$

where [Ox] and [Red] represent the concentrations of the oxidized and reduced species, at a given applied potential (Eapp) and Emid is the midpoint potential of the redox couple determined by the CV.

The first derivative of the curve showed a maximum at E = −0.42 V (E_{mid}) vs. SCE that correspond to an n1/2= 7.5. Since the biofilm cyt c552 has non-Nernstian response, the “n” value cannot be interpreted as the number of electrons involved in the redox process.

However, n = 0.5 describes the level of cooperativity between different heme entities within the cyt c552. Our results indicate a high level of cooperativity within the heme groups of the biofilm cyt c552. Such non-Nernstian behavior is reported for proteins with more than one heme-binding site that display interactions between heme centers that influence

their redox activity. These results suggest electron hopping between non-equivalent hemes in multiheme cyt c552of *G. metallireducens* biofilm.

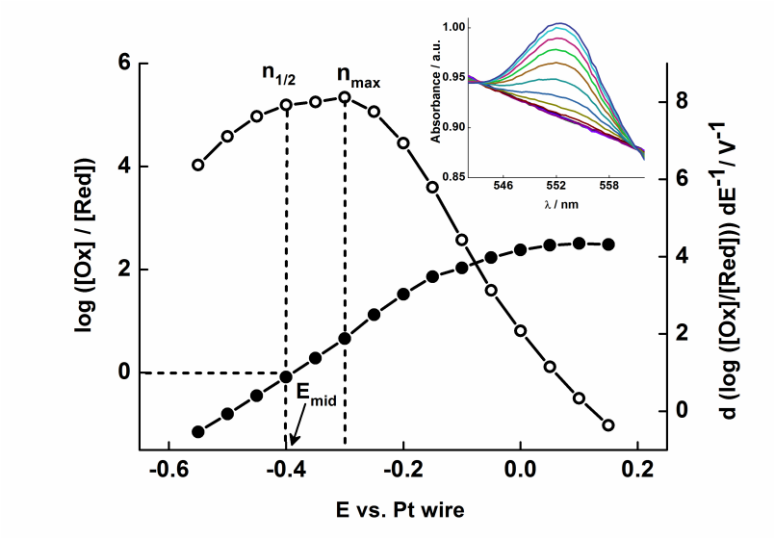


Figure 3.23 Nernst plot of log ([Ox]/[Red])of the biofilm c Cyts (empty circles),and the first derivative of Nernst plot(full circles),as a function of electrode potential. Inset shows the increase in the peak intensity of α band with the increase in electrode potential, which was used for the Nernst plot.

4. Discussion

Dissimilatory metal reducing bacteria (DMRB) can reduce insoluble electron acceptor, such as metals and electrodes, through extracellular electron transfer (EET). Although *G. metallireducens* was the first isolated DMRB (Lovley et al, 1987), most research focused on *G. sulfurreducens*, as it grows rapidly on defined medium and its genetic system is well-established (Coppi et al., 2001). Previous research on *G. sulfurreducens* showed that extracellular reduction occurred in the environment through both direct contact and nanowire-mediated electron transfer (Reguera et al., 2006) Other DMRB, such as *Shewanella* sp., can also use self-produced redox molecules to shuttle electrons form the cell to inorganic electron acceptors (Marsili et al., 2008; von Canstein et al., 2008)

DMRB can reduce metals in soil, sediments, and groundwater, thus they have been employed for bioremediation both in laboratory and on the field. Bio-reduction occurs via extracellular electron transfer in the extracellular space or in the periplasmic membrane, where reduced metals accumulate. Metals like Cr(VI) and U(VI) that are highly mobile and pose a threat to drinking water supply and agricultural production are bio-reduced to Cr(III) and U(IV), which are poorly soluble in water at circumneutral pH and remain immobilized in the soil/sediment matrix.

In this study, we focused on the bio-reduction process of Cr(VI) to Cr(III), which is crucial to Cr(VI) detoxification in subsurface environments and groundwater. Numerous DMRBs, including *Shewanella oneidensis* (Viamajala et al., 2002)and *Enterobacter cloacae* (Wang et al., 1989) can reduce Cr(VI) to Cr(III) under controlled laboratory conditions. However, those species are less promising for improved bioremediation applications. In fact, *E. cloacae* are Enterobacteria, thus they are ubiquitous in soils and sediments. Both *S. oneidensis* and *G. metallireducens* are well-spread in soil and groundwater bacteria. However, *S. oneidensis* is more sensitive to Cr(VI), which can slow down or even stop its growth rate(Viamajala et al., 2004). Indeed, *S. oneidensis* can tolerate 200 µM Cr(VI) (Belchik et al., 2011) while *G. metallireducens* can tolerate 2.5mM (this thesis work)

Geobacter metallireducens is a DMRB capable of extracellular Cr(VI) reduction. Initial experiments (D. R. Lovley, 1993) showed high metal-reduction rate, but the molecular mechanisms of detoxification have not been yet studied in details. DMRBs can detoxify heavy metals by both direct (by transferring electron through electron transfer chain) and indirect bioreduction via Fe or Mn reduction, which in turn reduce Cr(VI). However, the toxicity of Cr(VI) to *G. metallireducens* as well other bacteria limits its application to field bioremediation.

Environmental tolerance is a particularly complex and poorly understood phenotype. These relevant traits include tolerance to products, substrates, by product and the process environmental factors such as temperature, pH and solvent. Many of the tolerance phenotypes like metal toxicity resistance are polygenic, thereby they involve distributed genes in the genome. Therefore, it is very laborious to produce the phenotype–genotype correlation for each desired phenotype and its interaction with each other. Thus, rational metabolic engineering of these tolerance phenotypes becomes a daunting task. On the contrary, the whole genome engineering approaches, such as evolutionary engineering methods like genome shuffling, show more advantages in improving stress tolerance in poorly characterized microorganisms.

A genome shuffling approach has recently been used to increase acid and glucose tolerance in *Lactobacillus* (Gangadharan et al., 2008; Patnaik et al., 2002; Wang et al., 2007; Yu et al., 2008), improve acetic acid tolerance in *Candida krusei*(P. Wei et al., 2008), enhance pristinamycin tolerance in *Streptomyces pristinaespiralis* (Xu et al., 2008), improve thermo-tolerance and ethanol tolerance in *S. cerevisiae* (D. Shi et al., 2009).

Tolerance to environmental stress, such as heavy and toxic metal or low pH, high temperature and organic solvent is a desirable phenotype for industrial and bioremediation strains. In the production of lactic acid, development of low-pH-tolerance strain may decrease the cost of downstream processing. It also reduces potential contamination (van Maris et al., 2004). Organisms capable of surviving under extreme conditions have great application in biological reserve. Most of the reported organic solvent tolerant bacteria are strains of Pseudomonas species, such as *Pseudomonas putida, P. aeruginosa* and *P. fluorescens* (Ramos et al., 1995; Weber et al., 1993).

Several Gram-positive bacteria such as strains of *Bacillus, Rhodococcus*, and *Arthrobacter* also have been reported from natural habitats (Paje & Neilan, 1997; Sardessai & Bhosle, 2002). Lately, new strains based on these organic solvent tolerant bacteria have been constructed by metabolic engineering methods. The bacterial tolerance to hexavalent chromium can be achieved by various biotechnological techniques. Among these is the improvement of microorganism strains for desirable characteristics such as different growth rate and the ability to grow at higher chromium concentration. Whole genome shuffling combines the advantages of dissimilar parents by allowing parental DNA shuffling and hence is believed to improve the characteristics of strains controlled by multiple genes (Y. Zhang et al., 2002). Recursive genomic recombination within a population

of fusant microorganisms can generate strains efficiently with an amplified desirable phenotype.

Although genome shuffling originated from protoplast fusion, it is a different method compared with protoplast fusion. The conventional protoplast fusion refers to the process of fusion between two cells with different genetic traits and obtaining a stable recombinant with the combination of the genetic traits of both parents. In the process of protoplast fusion, recombination results from only two parents per generation. In contrast, genome shuffling allows for recombination between multiple parents at each generation and several rounds of recursive genome fusion were carried out resulting in the final improved strain involve the genetic trait from multiple initial strains. This would highly increase the genetic diversity of “complex progeny”, and remarkably enhance the opportunity for obtaining the high performance strain. So genome shuffling is a new and novel technology which differs from protoplast fusion.

Compared with classical strain-improvement strategies and rational methods for strain engineering, genome shuffling offers more advantages. Firstly, the efficiency of genome shuffling for phenotypic improvement is higher than classical strain-improvement methods, such as mutagenesis and protoplast fusion. In random mutagenic breeding, a single strain was used as the start strain, during each cycle a population of improved mutants was identified from which the single best performer is taken forward. Evolution of microorganisms is slow in mutagenesis for individuals within a population evolve alone as opposed to sharing information and evolving as a group (Gong et al., 2009).

Although classical mutagenic breeding has succeeded in generating many industrial strains, it is a time-consuming and laborious process. These shortcomings of asexual procedure can be overcome in sexual evolution which allows the information within a population to be shared. Mating within a selected population amplifies the genetic diversity of the population by creating new mutant combinations. Although protoplast fusion addresses sexual evolution, it allows for recombination between only two parents per generation. The emergence of genome shuffling technology accelerates the process of strain improvement by recursive protoplast fusion between multi-parent strains. More hybrid strains can be obtained than protoplast fusion because more parents were involved. The application of recursive protoplast fusion ensures the sharing of information within the population which has positive phenotype. So the desired strains which involved the positive trait of multiple parents can be obtained in a rather short period.

Two rounds of genome shuffling were sufficient to achieve results that had previously required 20 years of classical strain improvement (Y. Zhang et al., 2002). In a word, amplifying the genetic diversity of the population in the offspring may be the key advantage of genome shuffling when compared with the classical mutagenic breeding.

Secondly, the technique of genome shuffling is not limited to the microbe which has clear genetic background. Although gene recombination technology allows the recombination between multiple parents, it addresses DNA fragments not whole-microbial genomes. However, cellular phenotype is a manifestation of global gene expression levels, metabolic demand, resource availability, and cellular stresses. The profile of an ideal cell depends on the expression of a large number of genes that are rather poorly understood, mostly unknown, and broadly distributed throughout the genome. So it is difficult to improve the strain by direct genetic manipulation through the control of specific gene. However, genome shuffling is a whole genome-wide engineering strategy and can be performed on the tested microbes without knowing its genetic background.

Unlike the genetic engineering methods for improvement of microbial strains, genome shuffling causes simultaneous changes broadly distributed throughout the genome based on genome plasticity, without the need to know the genome sequence data or network information. Moreover, compared with other molecular breeding techniques, genome shuffling is more convenient and easy to be popularized. The application of genome shuffling does not require any expensive facility. The cost for the genome shuffling is not high and the charge for a round of genome shuffling is equivalent to a cycle of protoplast fusion. In addition, the technique is relatively easy to manipulate and can be generalized in most laboratories. Additionally, strains engineered by genome shuffling, a technique based on protoplast fusion, are not considered to be “genetically modified”, and therefore avoid public distaste reserved for genetically modified organisms (GMOs) (Ahmed, 2003).

Genetic manipulations of G. *metallireducens* might increase tolerance to Cr(VI). Since metal toxicity resistance is a complex phenotype that results from various genic loci, site-specific genetic engineering may be less effective, tedious, and time consuming. Multiple random mutagenesis methods may be applied to increase Cr(VI) resistance, as they result in recombination of multiple changes across the genome. To our best knowledge, random mutagenesis has been not applied to *G. metallireducens* and neither genome shuffling has been applied to improve metal resistance to DMRBs. Additionally, only one genome shuffling work on anaerobic bacteria has been published.

In this study, we aimed to fill this knowledge gap and developed protocols for genome shuffling and verification of the mutants. At beginning, we attempted the mutagenesis using UV radiation as mutagenic agent. However, the UV radiation dries the agar plates and *G. metallireducens* did not grow on agar plates with poor water content. Further, growth of *G. metallireducens* on agar plate and selection of single colonies was a very time-consuming procedure. Therefore, we changed the mutagenic agent to nitrosoguanidine, as the latter worked also in liquid medium where rapid growth rate can be attained.

Random mutagenesis methods must be coupled to a suitable screening to identify the mutants that have acquired the desired characteristics. In our work, we chose the mutants capable of growing rapidly under high concentration of Cr(VI). We assumed that the fastest growing colonies under such conditions were those that have acquired the highest Cr(VI) resistance capability.

We have found the best conditions to apply the genome shuffling to *G. metallireducens* (see Chapter 5). The phases of the process were observed by optical microscopy. The mutants obtained had an increased Cr(VI) tolerance. While the wild type could not grow in media containing more than 2.5 mM Cr(VI), the genome shuffled mutant tolerated up to 5 mM Cr(VI). This seemingly small increase was higher than that obtained by sub-cultured *G. metallireducens* over 12 months in NB iron oxide spiked with Cr(VI) 2.5 mM. The addition of higher Cr(VI) concentrations were no permissive for *G. metallireducens*. In these subcultures, no increasing of resistance was observed, despite the long-time exposure and strong selective pressure.

In general the double increase of the tolerance from 2.5 to 5.0 Cr(VI) is not very significative. However, the increase was achieved by only one genome shuffling round. Due to time constraints, we could not perform further cycles of genome shuffling. However, it was expected that Cr(VI) would increase much further by additional rounds of genome shuffling (Bajwa et al., 2010; John et al., 2008; D. Shi et al., 2009; Y. Zhang et al., 2002).

In the second part of our work, we selected several Cr(VI)-resistant mutants and characterized them through microbial biofilm voltammetry in potentiostat-controlled electrochemical cells, to determine whether the increased resistance to Cr(VI) toxicity correlates with a faster EET rate (Shen et al., 1993). The working electrode (anode) was poised at oxidative potential (0,2 V vs SCE). Results showed that one of the mutant, called M23 had an increased electrode-respiring capability. However, the mix of

the genome shuffling mutants showed smaller electroactivity although higher Cr(VI)-resistant capability.

This is consistent with previous research on other DMRB such as *Shewanella oneidensis*, where metal resistance was related to respiratory rate, i.e., faster respiring bacteria resisted to higher metal concentration (Burns, 2010). Further experimental work is required to clarify the correlation between electroactivity and metal-reduction capability. It appears that there is little overlapping between these two functions and EET and Cr(VI) reduction are run by the same proteic machinery.

While it is thermodynamically possible for chromate reduction to generate enough energy to support respiration, true chromate respiration remains to be discovered (D. R. Lovley & Phillips, 1994). Studies simulating field conditions have supported these results, confirming (1) the ubiquity of indigenous Cr-reducing microorganisms in a variety of soil, both contaminated and clean, (2) the importance of irrigation to maintain reducing conditions and (3) the proportionality between organic matter loading and Cr(VI) reduction(Cifuentes, Lindemann, & Barton, 1996), suggesting that Cr(VI) bioremediation by reduction in soil holds great promise in cases where re-oxidation can be permanently prevented. In *Geobacter metallireducens* and *Shewanella oneidensis*, when soluble Pu(IV)(EDTA) was provided as terminal electron acceptor, both the cultures rapidly reduced it to Pu(III)(EDTA) with nearly complete reduction within 20 to 40 min, depending on the initial concentration. However, neither *Geobacter* or *Shewanella* were able to use Pu(IV) as a terminal electron acceptor to support growth, consistently to our Cr(VI) reduction results. In *Geobacter uraniireducens* 34 c-type cytochrome genes were upregulated in cell grown on uranium contaminated sediments. Some of them were genes that are homologous to cytochromes required for optimal Fe(III) and U(VI) reduction by *G. sulfurreducens* (Mouser et al., 2009).

The removal of Cr(VI) from groundwater following the in situ stimulation of metal reduction is often concomitant with substantial increases in the growth and activity of dissimilarity metal-reducing microorganisms in the family *Geobacteraceae*, consistently with what happens when U(VI) is bio-remediated. The mechanism used by *Geobacter spp.* to reduce Cr(VI) is far to be clear. Instead, U(VI) reduction is much better known. Early studies with *Geobacter metallireducens* (previously named GS15) suggested that U was reduced extracellularly to uraninite under conditions promoting cell growth (D. R. Lovley, 1991). The development of genetic tools in *Geobacter sulfurreducens* (Coppi et al., 2001) motivated molecular studies to elucidate

the biological mechanism behind this reaction. Because *c*-cytochromes are abundant in the cell envelope of *Geobacter* bacteria, studies focused on identifying extra-cytoplasmic *c*-cytochromes that could function as dedicated U reductases (E. Shelobolina et al., 2007). However, mutations were often pleiotropic (H. U. Kim et al., 2008) and showed no defect or only partial defects in the cell's ability to remove U(VI). Interpretation was also difficult due to inconsistencies in the reported mutant phenotypes, with some mutations reportedly abolishing U(VI) removal activities, yet mutant cells showing extensive mineralization (E. Shelobolina et al., 2007). Furthermore, these studies consistently showed that the U precipitated inside the cell envelope. Uranium is not known to be essential for the synthesis of any cell component or for any cellular biological reaction, yet can be reduced and precipitated nonspecifically by the abundant low-potential electron donors of the cell envelope of Gram-negative bacteria (Wall & Krumholz, 2006). This is predicted to compromise the integrity of the cell envelope and its vital functions. Because of this, the environmental relevance of these early studies is questionable.

This fact is one of most important factor making the genome shuffling a better approach. One of our mutant, M23, showed higher electroactivity with respect to the parent strain. This success implies that random mutagenesis is a feasible strategy to obtain metallo-tolerant electroactive mutants. It is likely that the genome shuffling resulted in multiple mutations on different genic loci. The resulting microorganisms can be used in bioremediation of soil and groundwater at high Cr(VI) concentration, but also in MFCs fed with metal-contaminated wastewater for simultaneous Cr(VI) reduction and energy recovery. The loci are not still been characterized. However, possible candidates of mutation sites are all the cytochromes, reductase, membrane channel, uptake proteins and genes related to EPS. Pilin and flagellin play also a crucial role on insoluble Fe(III) reduction (Tremblay et al., 2012), thus both the proteins can be part of the list of candidates.

Metabolic analysis of *G. metallireducens* showed its ability to obtain energy through dissimilatory reduction of Fe and Mn (D. R. Lovley et al., 1987), while the ability to reduce Cr (VI) was found later as well by Lovley together with manganese, uranium and other metals (D. R. Lovley, 1993). This metal reduction (besides Fe and Mn) occurs through metabolism or co-metabolism. *G. metallireducens* was the first organism found to able to completely oxidize organic compounds to carbon dioxide using iron oxides as the electron acceptor (D. R. Lovley et al., 2004). *G. metallireducens* can oxidize short chain fatty acids, alcohols and mono-aromatic compounds such as toluene and phenol using iron as its electron acceptor (D. Lovley, 2013)(Malvankar et al., 2012). *G. metallireducens* also takes part in carbon, nutrient and geochemistry cycles and in metal bioremediation, transforming

soluble and harmful contaminants into insoluble and harmless forms (Childers et al., 2002).

The genome of *G. metallireducens* has been sequenced and is available in gene data bank: it is a 4.01 Mbp genome consisting of a 3,997,420 bp circular chromosome (encoding 3,621 genes with a GC content of 59.51%) and a 13,762 bp plasmid (encoding 13 genes with a GC content of 52.48%)(Aklujkar et al., 2009). In the chromosome are encoded genes of housekeeping pathways like metabolism enzymes, cell structure proteins, chemotaxis sensor, flagella and pili (Copeland et al., 2005). In the plasmid are encoded a gene for a toxin and the relative protein resistance, known as RelE/StbE system. The genes encoding the flagella synthesis are induced only when iron oxide is sensed and soluble electron acceptor are in low concentration (Childers et al., 2002). *G. metallireducens* was originally thought to be immotile because they were grown under laboratory and favourable conditions. When soluble metals were replaced with less favourable iron oxide, *G. metallireducens* synthesized flagella to move towards and reduce the metal through direct electron transfer by outer membrane cytochromes (D. R. Lovley et al., 2004) and pili (D. R. Lovley, 1993)(Reguera et al., 2005a). As cited before, the chromosome of *G. metallireducens* contains genes that allow the ability of chemotaxis and flagella synthesis. Chemotaxis allows *G. metallireducens* to detect compounds and evaluate the environment and together with motility, allows *G. metallireducens* to move where the environmental conditions have higher nutrient and metal concentrations (Childers et al., 2002).

Although *G. sulfurreducens* is a better known model, *G. metallireducens* shows greater metabolic versatility, and comparative genomic analysis suggests that metabolism, physiology and regulation of gene expression in *G. metallireducens* may be dramatically different from other *Geobacteraceae* (Lloyd et al., 2003)**.** Moreover, *G. metallireducens* has a well-documented resistance and adaptation to solid metals as terminal electron acceptors. Thus, we chose *G. metallireducens* as parent strain for our work. Differently from *G. sulfurreducens, G. metallireducens can* reduce Cr (VI), together with manganese, uranium and other metals (D. R. Lovley, 1993). This metal reduction (besides Fe and Mn) occurs through metabolism or co-metabolism. *G. metallireducens* was the first organism found to able to completely oxidize organic compounds to carbon dioxide using iron oxides as the electron acceptor (Liu et al., 2002). Indeed, *G. metallireducens* can oxidize short chain fatty acids, alcohols and mono-aromatic compounds such as toluene and phenol using iron as its electron acceptor (Gorby & Lovley, 1991). *G. metallireducens* also takes part in carbon, nutrient and geochemistry cycles and in metal bioremediation,

transforming soluble and harmful contaminants into insoluble and harmless forms (Childers et al., 2002).

Due to its versatility and its ubiquity, *G. metallireducens* has been applied to bioremediation of soil and groundwater, as it is able to bio-reduce metals and priority pollutants, such as Cr(VI) (Lloyd et al., 2003), Pu(VI) (Icopini et al., 2009)and Hg(II)(Wiatrowski & Barkay, 2006).The bioreduction operated by *Geobacteraceae* lowers the toxicity of the metal, as it is transformed to an insoluble and precipitated form, which prevent it from spreading in the groundwater, and help cleaning up the contaminated site (Hau & Gralnick, 2007). *Geobacteraceae* and other DMRB have been used to augment the natural attenuation rate (i.e., the rate at which the indigenous bacterial community removes the pollutant) and could be applied to contaminated sites such as abandoned mines, flooded land, and dismissed military facilities. In comparably short time, usually of magnitude of months or years, toxic metal species could be detoxified. *G. metallireducens* has been tested in concurrent bioremediation by biodegradation and bioleaching processes (K. Lee et al., 2012). In another study aimed to stimulate microbially-mediated reduction of Cr(VI) in a Cr(VI) contaminated aquifer in United States, a poly-lactate compound (HRC) was injected in 2004 (Chakraborty et al., 2010; Faybishenko et al., 2008). Cr(VI) concentrations rapidly declined to below the detection limit and remained so for more than three years after the injection. Researcher isolated an iron-reducing *Geobacter metallireducens*-like isolate, a sulphate-reducing *Desulfovibrio vulgaris*-like strain and a nitrate-reducing *Pseudomonas stutzeri*-like isolate among the bacterial community. All of these isolates were capable of reducing Cr(VI) anoxically.

Among the limiting factors for field application of *G. metallireducens* is the low resistance of the bacteria used to Cr(VI). For example, the low resistance can be explained in term of cytotoxicity and concentration: chromate is actively transported across biological. In contrast, most cells are impermeable to Cr(III) probably because it forms water insoluble compounds and accumulates inside the cells. In general DMRB show higher resistance because of their capability to produce ferrous iron [Fe(II)], which reductively transforms heavy metals in contaminated groundwater. The bacterial reduction of indigenous ferric iron Fe(III)] to Fe(II) has been proposed as a means of establishing redox reactive barriers in the subsurface. The reduction of Fe(III) to Fe(II) can be accomplished by stimulation of indigenous dissimilatory metal-reducing bacteria (DMRB) or injection of DMRB into the subsurface. The microbially produced Fe(II) can chemically react with contaminants such as Cr(VI) to form insoluble Cr(III) precipitates. Thus, for this capability that protect themselves against the heavy metals, DMRB are the ideal starting point to apply modifications, and

G. metallireducens was the only member of the group known to be able to Cr(VI) extracellular direct reduction and tolerate Cr(VI).

To our best knowledge, this is the first time genome shuffling has been applied to DMRB or electrochemically active microorganism and one of the first times to strict anaerobes. In fact the first application of genome shuffling to the strict anaerobe *Clostridium diolis* was published only in 2009 (Otte et al., 2009). In this work, genome shuffling has been applied to the 1,3propandiol producer bacterium to improve its productivity. NTG (1h) was the mutagenic agent generating strains with superior substrate and product tolerance levels. These improved strains were then used for genome shuffling and selection for 1,3-propanediol and organic acid tolerance. After four rounds of genome shuffling and selection, 80% improved strain compared to the yield from the parental wild-type strain were obtained. Our results, instead, show that *G. metallireducens* treated by the same mutagenic agent improved its Cr(VI) tolerance by 100% and its electroactivity by 200% after only one genome shuffling round. This yield of improvement is compatible with what reported in literature, where the tolerance improvement percentage varies from 62.2 to 300%.

Chromium is toxic at few mM concentrations, and adaptation by natural selection may be more difficult. In our best knowledge, the only *Geobacteraceae* known to be resistant to Cr(VI) is *G. metallireducens*, while among the other DMRB families here we cite *S. oneidensis.* . In this study, we focused on Cr(VI) reduction to Cr(III), which represent the most common microbial detoxification route for Cr(VI)- contaminated soils and groundwater. Wild type *G. metallireducens* and DMRB in general have low resistance to Cr(VI) toxicity. This limits the effectiveness of bioremediation treatment and prevents rapid decontamination of highly polluted sites, such as industrial and military sites. Since we do not have full understanding of *G. metallireducens* physiology and regulation, it is not possible to apply a direct mutagenesis strategy, or at least, it will be very time-consuming. In our work, we attempted to increase resistance to Cr(VI) toxicity using a random mutagenesis method, genome shuffling.

Results show that genome shuffling can be applied to the strict anaerobe and electroactive microorganisms *G. metallireducens* generating Cr-tolerant mutants The mutants were tested by biological, molecular, and electrochemical assays, showing an improved Cr(VI) toxicity resistance.

In particular, the tolerance of the mutant strains to Cr(VI) was twofold the tolerance of the wild type, while the electroactivity of the mutant was heavily compromised in term of capacity of electron transfer. The mutants obtained could have interesting application for Cr(VI) bioremediation. However, the

biological mechanism behind the increase Cr(VI) tolerance remains elusive and further work is required.

The present work can also have some implications for soil bioremediation. Our results refer to simplified laboratory systems, where Cr(VI) or the electrode are the only extracellular electron acceptor. In the field, various chemical species contribute to Cr(VI) reduction through abiotic pathways. Although the biological reduction of Cr(VI) by metal-reducing bacteria has been proved, Cr(VI) reduction's in an anaerobic environment is controlled by abiotic kinetic factors. Even when the biological reduction rate reaches its maximum, the influence of Fe(II) at pH > 5.5 and S(–II) at pH < 5.5 turns out to be a decisive factor that determines the fate of Cr(VI) under anaerobic conditions (Fendorf et al., 2000). Therefore, it is Fe(II) and sulphides that are the main reducers of Cr(VI) in the anaerobic horizons of hydromorphic soils. Their influence depends on the environment's pH in many respects. The microbial activity influences the Cr(VI) cycle indirectly by producing Fe(II) and S(–II), which are chemical reducers and result from the biological reduction of Fe(III) and sulphates(Wielinga et al., 2001). Moreover, Fe(III) reduced to Fe(II) by DMRB, so the presence of Cr-tolerant microorganisms such as *Geobacter metallireducens* M23 (as long as they maintain a good Fe(III) reducing activity) can be beneficial to Cr(VI) overall reduction to Cr(III).

The in situ stimulation of Fe(III) oxide reduction by *Geobacter* bacteria leads to the concomitant precipitation of hexavalent uranium [U(VI)] from groundwater and by direct reduction though conductive pili (Cologgi et al., 2011). Hexavalent chromium is reduced by the same mechanisms. It should be mentioned in this context, that to our knowledge a microbial respiration with Cr(VI) cannot serve as sole source of energy for microbial growth. However, a recent study revealed that resting cells of *Shewanella oneidensis* reduce Cr(VI) for the most part using the same outer membrane cytochromes that also catabolically reduce ferric iron (Belchik et al., 2011). Hence, chromium toxicity might just be too high to support microbial growth under anaerobic conditions with Cr(VI) as electron acceptor (Ramírez-Díaz et al., 2008a), so the different electrochemical behaviour of the high chromium concentration mutant is not a absurd because of the different mechanisms related to the chromium resistance and the metal reducing activity. Indeed, the reduction of chromium allows the cell to localize the precipitation process at least partly to the cell surface and to thereby keep part of the toxic reduction substrates and products from entering the cell. Despite its promise for the bioremediation, the biological mechanism behind this reaction remains elusive and further work is required. Regarding to an infield use of the *G. metallireducens* generated, a technology such as genome shuffling that use the natural cellular machinery to lead an

improved phenotype, a strains produced by this technology might not be considered as GMO, although the derived bacterium is less characterized than a GMO (Ahmed, 2003).

For all the experiments, CA showed a biofilm growth of *G. metallireducens* on graphite electrode. The multiple peaks visible in both CV and DPV analyses also confirmed the complexity of the *G. metallireducens* surface. Multiple cytochromes and redox proteins have been previously implicated in outer membrane-based electron transfer in proteomic and labelling studies (Nevin et al., 2009a). As most proteins implicated in electron transport by *G. metallireducens* contain multiple hemes or redox centres, the detected redox centres could reflect individual hemes, domains that act as a single center, or individual proteins (Wolf et al., 2009). Recent work with the multiheme cytochrome MtrC (L. Shi et al., 2009) showed that multiheme proteins do not demonstrate classic, individual redox behaviour for each heme but rather act as a cluster with a broader midpoint. In another study (Clarke et al., 2011), the same protein was observed to behave as two pentaheme domains with broad midpoint potentials. Future work with specific mutants lacking key redox proteins in *G. sulfurreducens* will aid in identifying the origin of these peaks. In fact, there are many differences between the cytochrome in their native environment, the periplasmic membrane, and as pure molecules.

The CV relative to mutants and WT appear to have differenences. Specifically, we could not observe the appearance of a sigmoid curve in the CV, which indicated that catalytic electron transfer was established (Figure 3.8.C). In general, the ohmic current observed in the mutants was higher than in the wild type. This suggests that the mutants have higher electron transfer resistance, thus are not able to produce high current. Further experiments are required to determine the Cr(VI) reduction kinetics, and to verify if the Cr(VI) respiration rate of the mutants is higher than in the WT. However, the significant current produced is much higher than the one produced by a non-electroactive strain. One possible explanation is that the mixed consortia contains both mutants and wild type, and the mutants were all different each other. Indeed, after the selection of single colonies in solid medium, the isolated showed different behaviours.

In the first part of the PhD work, we have focused on the optimization of the electrochemical parameters and growth conditions of *G. metallireducens, G. sulfurreducens* and *S. oneidensis* (data not shown). Our electrochemical results show how the response of an intact film to a range of applied potentials can be measured systematically and analysed to produce data that are easily compared.

Since one of the goals was to characterize the catalytic behavior of the system, low scan rates (1 mV s^{-1}) were chosen, since they permitted reactions with a time constant on the order of ~1 s to be active as turnover processes at each imposed potential step. At high levels of electron donor and low scan rates, catalytic voltammograms should therefore be representative of steady-state conditions. In addition, minimization of ohmic current aided in identification of inflection points in derivative analysis. For a system such as this, with what appears to be sluggish interfacial electron transfer kinetics, the potential difference between anodic and cathodic peaks for a given redox couple could change significantly with even modest changes in scan rate. We have also used electrochemical techniques under non-turnover conditions (Jain et al., 2011) to better elucidate these electron transfer kinetics for a more complete understanding of the interplay between microbial catalytic abilities and interfacial electron transfer. The catalytic wave consistently observed for *G. metallireducens* is an independent demonstration that interior oxidative processes of this organism are linked via a continuous pathway to surfaces and that the entire collection of attached organisms (i.e., the biofilm) behaves as an adsorbed catalyst. The midpoint potential of the catalytic wave at −0.15 V supports a model with a dominant rate-limiting electron transfer reaction and shows that *G. metallireducens* respiration rate does not increase when cells are provided with an electron acceptor with a potential greater than 0 V. The latter result implies that the final step of electron transfer (e.g., between a terminal external protein and the electrode) is not rate limiting, as this process can always be accelerated by additional applied potential. The midpoint and the limiting current potential found in this thesis work are consistent with *G. metallireducens* being adapted for the reduction of iron oxides with a potential between −0.2 and 0 V versus SHE in the environment and suggest that cells do not derive any additional energetic benefit from higher-potential electron acceptors.

Based on the results reported here, voltammetric methods previously developed to characterize electron transfer phenomena by enzymes adsorbed at carbon electrodes can be extended to the characterization of viable biofilms. By choosing the appropriate conditions, these methods are not destructive and allow in vivo determination of electron transfer from whole cells to electrodes under conditions that are comparable to those encountered in natural environments. Both thermodynamic and kinetic parameters can be determined and used to define the phenotype of an organism for comparison with other strains or mutants. These methods can be applied to well-defined pure cultures, as well as to complex microbial communities, and could allow for quantitative comparisons in the development of better microbial catalysts based on direct electron transfer between bacteria and electrodes.

Potentiostat-controlled electrodes at a sufficiently positive potential are nearly equivalent to MFC anodes in which the electron acceptor is non-limiting, reducing the technical complexity and simplifying the conceptual model of electron transfer. With respect to actual MFC anodes, the electrode potential is higher, thus the power measured in electrochemical cells can be considered as the maximum power producible by the electroactive microorganisms under investigation. However, at high oxidative potentials, conformational change, unfolding, and irreversible process could alter the catalytic abilities of enzymes, decreasing the anodic current (Perez-Roa et al., 2006). Electrochemical techniques are the method to study the electron transfer reactions in the Fe(III)-reducing bacterium. Electrochemical data can then complement these biochemical studies. Several electrochemical techniques have been used to characterize redox proteins including cytochromes. Large numbers of redox proteins are electrochemically active. However, direct electron exchange between a redox protein and an electrode is usually hindered by the peptide chain adjoining the active redox center of the protein.

5.Conclusions and future directions

We applied successfully genome shuffling to the DMRB, electroactive bacterium *Geobacter metallireducens.* We developed protocol for genome shuffling using nitrosoguanidine and coupled the mutagenesis with culture-based and electrochemical-based selection methods. Our results show that genome shuffling applied to *Geobacter metallireducens* can increase its Cr(VI)-tolerance to 5 mM (vs. 2.5 mM for the parent strain) after a single round of genome shuffling. In fact, the shuffling of the mutations occurred in the different genomes of the *G. metallireducens* mutagenized cells resulted in a two-fold increase of the tolerance.

Genome shuffling procedure generated also mutants that couple Cr(VI) resistance to higher electroactivity. By applying a second screening based on solid Fe(III) reduction, we selected a strain that possess both tolerance to Cr(VI) and high electroactivity. The electroactivity of the mutant *G. metallireducens* M23 was approximately two-fold higher than the parent strain when grown in potentiostat-controlled electrochemical cells. Notably, the voltammetric fingerprint of M23 was different from the parent strain, in which most of the EET occur at higher potential.

Unfortunately, we spent most of the time in developing the genome shuffling protocol. *G. metallireducens* is a strict anaerobe and grows slowly in anaerobic tubes. Due to these time constraints, we could not provide an explanation of the increased resistance to Cr(VI) toxicity. Additionally, we could not determine if the resistance to Cr(VI) and the higher electroactivity are related. Further investigation is needed to understand the mechanism behind the improved phenotype of the mutant.

However, this is to our best knowledge the first successful attempt of genome shuffling modification to increase the resistance to metal toxicity in a DMRB strain. These results may be of interest to those researchers that seek to improve electroactivity of model electroactive microorganisms for energy recovery applications. Additionally, the availability of a relatively simple method to improve metal tolerance in environmental microorganisms might help the engineers and the microbiologist that work in highly contaminated sites, where low concentration of metal-reducing organisms (due to metal toxicity) slow down the bioremediation process.

References

Abbassi, B. E., & Shquirat, W. D. (2008). Kinetics of indigenous isolated bacteria used for ex-situ bioremediation of petroleum contaminated soil. *Water, Air, and Soil Pollution, 192*(1-4), 221-226.

Acharya, U. R., Mishra, M., Mishra, I., & Tripathy, R. R. (2004). Potential role of vitamins in chromium induced spermatogenesis in swiss mice. *Environmental Toxicology and Pharmacology, 15*(2), 53-59.

Afkar, E., Reguera, G., Schiffer, M., & Lovley, D. R. (2005). A novel geobacteraceae-specific outer membrane protein J (OmpJ) is essential for electron transport to fe (III) and mn (IV) oxides in geobacter sulfurreducens. *BMC Microbiology, 5*(1), 41.

Ahmed, F. E. (2003). Genetically modified probiotics in foods. *Trends in Biotechnology, 21*(11), 491-497.

Aklujkar, M., Krushkal, J., DiBartolo, G., Lapidus, A., Land, M., & Lovley, D. (2009). The genome sequence of geobacter metallireducens: Features of metabolism, physiology and regulation common and dissimilar to geobacter sulfurreducens. *BMC Microbiology, 9*(1), 109.

Al Hasin, A., Gurman, S. J., Murphy, L. M., Perry, A., Smith, T. J., & Gardiner, P. H. (2009). Remediation of chromium (VI) by a methane-oxidizing bacterium. *Environmental Science & Technology, 44*(1), 400-405.

Alam, M., Hossain, M. A., Yonge, D. R., Peyton, B. M., & Petersen, J. N. (2006). Bioreduction of hexavalent chromium in flow-through quartz sand columns. *Journal of Environmental Engineering, 132*(3), 358-366.

Allen, T. D., Kraus, P. F., Lawson, P. A., Drake, G. R., Balkwill, D. L., & Tanner, R. S. (2008). Desulfovibrio carbinoliphilus sp. nov., a benzyl alcohol-oxidizing, sulfate-reducing bacterium isolated from a gas condensate-contaminated aquifer. *International Journal of Systematic and Evolutionary Microbiology, 58*(6), 1313-1317.

Alper, H., Miyaoku, K., & Stephanopoulos, G. (2005). Construction of lycopene-overproducing E. coli strains by combining systematic and combinatorial gene knockout targets. *Nature Biotechnology, 23*(5), 612-616.

Armstrong, F. A., Camba, R., Heering, H. A., Hirst, J., Jeuken, L. J., Jones, A. K., et al. (2000). Fast voltammetric studies of the kinetics and energetics of coupled electron-transfer reactions in proteins. *Faraday Discussions, 116*, 191-203.

Atsumi, S., Wu, T., Eckl, E., Hawkins, S. D., Buelter, T., & Liao, J. C. (2010). Engineering the isobutanol biosynthetic pathway in escherichia coli by comparison of three aldehyde reductase/alcohol dehydrogenase genes. *Applied Microbiology and Biotechnology, 85*(3), 651-657.

Baczynski, T. P., & Pleissner, D. (2009). Bioremediation of chlorinated pesticide–contaminated soil using anaerobic sludges and surfactant addition. *Journal of Environmental Science and Health Part B, 45*(1), 82-88.

Bailey, J. E. (1991). Toward a science of metabolic engineering. *Science, 252*(5013), 1668-1675.

Bajwa, P. K., Pinel, D., Martin, V. J., Trevors, J. T., & Lee, H. (2010). Strain improvement of the pentose-fermenting yeast< i> pichia stipitis</i> by genome shuffling. *Journal of Microbiological Methods, 81*(2), 179-186.

Banci, L., Bertini, I., Bruschi, M., Sompornpisut, P., & Turano, P. (1996). NMR characterization and solution structure determination of the oxidized cytochrome c7 from desulfuromonas acetoxidans. *Proceedings of the National Academy of Sciences, 93*(25), 14396-14400.

Belchik, S. M., Kennedy, D. W., Dohnalkova, A. C., Wang, Y., Sevinc, P. C., Wu, H., et al. (2011). Extracellular reduction of hexavalent chromium by cytochromes MtrC and OmcA of shewanella oneidensis MR-1. *Applied and Environmental Microbiology, 77*(12), 4035-4041.

Bento, F. M., Camargo, F. A., Okeke, B. C., & Frankenberger, W. T. (2005). Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. *Bioresource Technology, 96*(9), 1049-1055.

Beukes, J., Pienaar, J., Lachmann, G., & Giesekke, E. (1999). The reduction of hexavalent chromium by sulphite in wastewater. *Water S. A., 25*(3), 363-370.

Bobiles, S. C., Librojo-Basilio, N. T., & Varca, L. M. (2009). Surfactant-aided bioremediation of chlorpyrifos in the soil. *Asia Life Sciences, 19*(1)

Bond, D. R., & Lovley, D. R. (2003). Electricity production by geobacter sulfurreducens attached to electrodes. *Applied and Environmental Microbiology, 69*(3), 1548-1555.

Bopp IV, C. J., Lundstrom, C. C., Johnson, T. M., Sanford, R. A., Long, P. E., & Williams, K. H. (2010). Uranium 238U/235U isotope ratios as indicators of reduction: Results from an in situ biostimulation experiment at rifle, colorado, USA. *Environmental Science & Technology, 44*(15), 5927-5933.

Borsodi, A. K., Micsinai, A., Kovács, G., Tóth, E., Schumann, P., Kovács, A. L., et al. (2003). Pannonibacter phragmitetus gen. nov., sp. nov., a novel alkalitolerant bacterium isolated from decomposing reed rhizomes in a hungarian soda lake. *International Journal of Systematic and Evolutionary Microbiology, 53*(2), 555-561.

Bradley, P. M., & Chapelle, F. H. (1998). Microbial mineralization of VC and DCE under different terminal electron accepting conditions. *Anaerobe, 4*(2), 81-87.

Bragg, J. R., Prince, R. C., Harner, E. J., & Atlas, R. M. (1994). Effectiveness of bioremediation for the exxon valdez oil spill. *Nature, 368*(6470), 413-418.

Brim, H., Osborne, J. P., Kostandarithes, H. M., Fredrickson, J. K., Wackett, L. P., & Daly, M. J. (2006). Deinococcus radiodurans engineered for complete toluene degradation facilitates cr (VI) reduction. *Microbiology, 152*(8), 2469-2477.

Bro, C., Regenberg, B., Förster, J., & Nielsen, J. (2006). In silico aided metabolic engineering of< i> saccharomyces cerevisiae</i> for improved bioethanol production. *Metabolic Engineering, 8*(2), 102-111.

Burkhardt, E. (2010). *Interaction of Fe (III)-Reducing Bacteria with Heavy Metals in Contaminated Soils,*

Burns, J. L. (2010). Molecular mechanisms of microbial iron respiration by shewanella oneidensis MR-1.

Busalmen, J. P., Esteve-Núñez, A., Berná, A., & Feliu, J. M. (2008). C-Type cytochromes wire Electricity-Producing bacteria to electrodes. *Angewandte Chemie, 120*(26), 4952-4955.

Butler, J. E., Young, N. D., Aklujkar, M., & Lovley, D. R. (2012). Comparative genomic analysis of geobacter sulfurreducens KN400, a strain with enhanced capacity for extracellular electron transfer and electricity production. *BMC Genomics, 13*(1), 471.

Butler, J. E., Young, N. D., & Lovley, D. R. (2010a). Evolution of electron transfer out of the cell: Comparative genomics of six geobacter genomes. *BMC Genomics, 11*(1), 40.

Butler, J. E., Young, N. D., & Lovley, D. R. (2010b). Evolution of electron transfer out of the cell: Comparative genomics of six geobacter genomes. *BMC Genomics, 11*(1), 40.

Cabrera, G., Pérez, R., Gomez, J., Abalos, A., & Cantero, D. (2006). Toxic effects of dissolved heavy metals on< i> desulfovibrio vulgaris</i> and< i> Desulfovibrio</i> sp. strains. *Journal of Hazardous Materials, 135*(1), 40-46.

Caccavo, F., Lonergan, D. J., Lovley, D. R., Davis, M., Stolz, J. F., & McInerney, M. J. (1994). Geobacter sulfurreducens sp. nov., a hydrogen-and acetate-oxidizing dissimilatory metal-reducing microorganism. *Applied and Environmental Microbiology, 60*(10), 3752-3759.

Cai, H., Eramo, A. G., Evans, P. J., Fricke, R., & Brennan, R. A. (2010). In situ bioremediation of perchlorate in vadose zone soil using gaseous electron donors: Microcosm treatability study. *Water Environment Research, 82*(5), 409-417.

Calvo, C., Manzanera, M., Silva-Castro, G., Uad, I., & González-López, J. (2009). Application of bioemulsifiers in soil oil bioremediation processes. future prospects. *Science of the Total Environment, 407*(12), 3634-3640.

Cardenas, E., Wu, W., Leigh, M. B., Carley, J., Carroll, S., Gentry, T., et al. (2008). Microbial communities in contaminated sediments, associated with bioremediation of uranium to submicromolar levels. *Applied and Environmental Microbiology, 74*(12), 3718-3729.

Cervantes, C., Campos-García, J., Devars, S., Gutiérrez-Corona, F., Loza-Tavera, H., Torres-Guzmán, J. C., et al. (2001). Interactions of chromium with microorganisms and plants. *FEMS Microbiology Reviews, 25*(3), 335-347.

Chai, L., Huang, S., Yang, Z., Peng, B., Huang, Y., & Chen, Y. (2009). Cr (VI) remediation by indigenous bacteria in soils contaminated by chromium-containing slag. *Journal of Hazardous Materials, 167*(1), 516-522.

Chakraborty, D., Ranganathan, S., & Sinha, S. (2010). Carbothermic reduction of chromite ore under different flow rates of inert gas. *Metallurgical and Materials Transactions B, 41*(1), 10-18.

Chen, D., Wang, G., & Li, J. (2007). Interfacial bioelectrochemistry: Fabrication, properties and applications of functional nanostructured biointerfaces. *The Journal of Physical Chemistry C, 111*(6), 2351-2367.

Chen, K., Kao, C., Chen, C., Surampalli, R. Y., & Lee, M. (2010). Control of petroleum-hydrocarbon contaminated groundwater by intrinsic and enhanced bioremediation. *Journal of Environmental Sciences, 22*(6), 864-871.

Childers, S. E., Ciufo, S., & Lovley, D. R. (2002). Geobacter metallireducens accesses insoluble fe (III) oxide by chemotaxis. *Nature, 416*(6882), 767-769.

Chiu, A., Shi, X., Lee, W., Hill, R., Wakeman, T., Katz, A., et al. (2010). Review of chromium (VI) apoptosis, cell-cycle-arrest, and carcinogenesis. *Journal of Environmental Science and Health, Part C, 28*(3), 188-230.

Christians, F. C., Scapozza, L., Crameri, A., Folkers, G., & Stemmer, W. P. (1999). Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling. *Nature Biotechnology, 17*(3), 259-264.

Cifuentes, F., Lindemann, W., & Barton, L. (1996). Chromium sorption and reduction in soil with implications to bioremediation. *Soil Science, 161*(4), 233-241.

Clarke, T. A., Edwards, M. J., Gates, A. J., Hall, A., White, G. F., Bradley, J., et al. (2011). Structure of a bacterial cell surface decaheme electron conduit. *Proceedings of the National Academy of Sciences, 108*(23), 9384-9389.

Coco, W. M., Levinson, W. E., Crist, M. J., Hektor, H. J., Darzins, A., Pienkos, P. T., et al. (2001). DNA shuffling method for generating highly recombined genes and evolved enzymes. *Nature Biotechnology, 19*(4), 354-359.

Cohen, M. D., Kargacin, B., Klein, C. B., & Costa, M. (1993). Mechanisms of chromium carcinogenicity and toxicity. *CRC Critical Reviews in Toxicology, 23*(3), 255-281.

Cohen, S. M., & Halper, S. R. (2002). Dipyrromethene complexes of iron. *Inorganica Chimica Acta, 341*, 12-16.

Cologgi, D. L., Lampa-Pastirk, S., Speers, A. M., Kelly, S. D., & Reguera, G. (2011). Extracellular reduction of uranium via geobacter conductive pili as a protective cellular mechanism. *Proceedings of the National Academy of Sciences, 108*(37), 15248-15252.

Compton, R. G., & Banks, C. E. (2007). *Understanding voltammetry* World Scientific.

Comte, S., Guibaud, G., & Baudu, M. (2008). Biosorption properties of extracellular polymeric substances (EPS) towards cd, cu and pb for different pH values. *Journal of Hazardous Materials, 151*(1), 185-193.

Conrad, M. E., Brodie, E. L., Radtke, C. W., Bill, M., Delwiche, M. E., Lee, M. H., et al. (2010). Field evidence for co-metabolism of trichloroethene stimulated by addition of electron donor to groundwater. *Environmental Science & Technology, 44*(12), 4697-4704.

Copeland, A., Lucas, S., Lapidus, A., Barry, K., Detter, J., Glavina, T., et al. (2005). Complete sequence of geobacter metallireducens GS-15. *Unpublished,*

Coppi, M. V., Leang, C., Sandler, S. J., & Lovley, D. R. (2001). Development of a genetic system forGeobacter sulfurreducens. *Applied and Environmental Microbiology, 67*(7), 3180-3187.

Costa, M., & Klein, C. B. (2006). Toxicity and carcinogenicity of chromium compounds in humans. *CRC Critical Reviews in Toxicology, 36*(2), 155-163.

Coursolle, D., Baron, D. B., Bond, D. R., & Gralnick, J. A. (2010). The mtr respiratory pathway is essential for reducing flavins and electrodes in shewanella oneidensis. *Journal of Bacteriology, 192*(2), 467-474.

Crameri, A., Dawes, G., Rodriguez Jr, E., Silver, S., & Stemmer, W. P. (1997). Molecular evolution of an arsenate detoxification pathway by DNA shuffling. *Nature Biotechnology, 15*(5), 436-438.

Crameri, A., Raillard, S., Bermudez, E., & Stemmer, W. P. (1998). DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature, 391*(6664), 288-291.

Dai, M., & Copley, S. D. (2004). Genome shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by sphingobium chlorophenolicum ATCC 39723. *Applied and Environmental Microbiology, 70*(4), 2391-2397.

Dai, M., Ziesman, S., Ratcliffe, T., Gill, R. T., & Copley, S. D. (2005). Visualization of protoplast fusion and quantitation of recombination in fused protoplasts of auxotrophic strains of< i> escherichia coli</i>. *Metabolic Engineering, 7*(1), 45-52.

Daprato, R. C., Löffler, F. E., & Hughes, J. B. (2007). Comparative analysis of three tetrachloroethene to ethene halorespiring consortia suggests functional redundancy. *Environmental Science & Technology, 41*(7), 2261-2269.

Dayan, A., & Paine, A. (2001). Mechanisms of chromium toxicity, carcinogenicity and allergenicity: Review of the literature from 1985 to 2000. *Human & Experimental Toxicology, 20*(9), 439-451.

De Flora, S., Bagnasco, M., Serra, D., & Zanacchi, P. (1990). Genotoxicity of chromium compounds. A review. *Mutation Research/Reviews in Genetic Toxicology, 238*(2), 99-172.

De Flora, S., Camoirano, A., Bagnasco, M., Bennicelli, C., Corbett, G., & Kerger, B. (1997). Estimates of the chromium (VI) reducing capacity in human body compartments as a mechanism for attenuating its potential toxicity and carcinogenicity. *Carcinogenesis, 18*(3), 531-537.

Desai, J. D., & Banat, I. M. (1997). Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews, 61*(1), 47-64.

Dheilly, A., Linossier, I., Darchen, A., Hadjiev, D., Corbel, C., & Alonso, V. (2008). Monitoring of microbial adhesion and biofilm growth using electrochemical impedancemetry. *Applied Microbiology and Biotechnology, 79*(1), 157-164.

Ding, Y. R., Hixson, K. K., Giometti, C. S., Stanley, A., Esteve-Núñez, A., Khare, T., et al. (2006). The proteome of dissimilatory metal-reducing microorganism< i> geobacter sulfurreducens</i> under various growth conditions. *Biochimica Et Biophysica Acta (BBA)-Proteins and Proteomics, 1764*(7), 1198-1206.

Dumas, C., Basseguy, R., & Bergel, A. (2008). Electrochemical activity of< i> geobacter sulfurreducens</i> biofilms on stainless steel anodes. *Electrochimica Acta, 53*(16), 5235-5241.

Durot, M., Bourguignon, P., & Schachter, V. (2009). Genome-scale models of bacterial metabolism: Reconstruction and applications. *FEMS Microbiology Reviews, 33*(1), 164-190.

El-Naggar, M. Y., Wanger, G., Leung, K. M., Yuzvinsky, T. D., Southam, G., Yang, J., et al. (2010). Electrical transport along bacterial nanowires from shewanella oneidensis MR-1. *Proceedings of the National Academy of Sciences, 107*(42), 18127-18131.

Erable, B., Duţeanu, N. M., Ghangrekar, M. M., Dumas, C., & Scott, K. (2010). Application of electro-active biofilms. *Biofouling, 26*(1), 57-71.

Esteve-Núnez, A., Núnez, C., & Lovley, D. R. (2004). Preferential reduction of fe (III) over fumarate by geobacter sulfurreducens. *Journal of Bacteriology, 186*(9), 2897-2899.

Faraoni, A., Santucci, R., Campanella, L., Tranchida, G., & Brunori, M. (1990). Voltammetric studies on the electrochemical behaviour of membrane-entrapped hemes. *Biology of Metals, 3*(2), 122-124.

Farhadian, M., Vachelard, C., Duchez, D., & Larroche, C. (2008). In situ bioremediation of monoaromatic pollutants in groundwater: A review. *Bioresource Technology, 99*(13), 5296-5308.

Faybishenko, B., Hazen, T. C., Long, P. E., Brodie, E. L., Conrad, M. E., Hubbard, S. S., et al. (2008). In situ long-term reductive bioimmobilization of cr (VI) in groundwater using hydrogen release compound. *Environmental Science & Technology, 42*(22), 8478-8485.

Fendorf, S., Wielinga, B. W., & Hansel, C. M. (2000). Chromium transformations in natural environments: The role of biological and abiological processes in chromium (VI) reduction. *International Geology Review, 42*(8), 691-701.

Finneran, K. T., Housewright, M. E., & Lovley, D. R. (2002). Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments. *Environmental Microbiology, 4*(9), 510-516.

Flemming, H., & Wingender, J. (2010). The biofilm matrix. *Nat Rev Micro, 8*(9), 623-633.

Franks, A. E., & Nevin, K. (2010). Microbial fuel cells, a current review. *Energies, 3*, 899-919.

Franks, A. E., Nevin, K. P., Jia, H., Izallalen, M., Woodard, T. L., & Lovley, D. R. (2009). Novel strategy for three-dimensional real-time imaging of microbial fuel cell communities: Monitoring the inhibitory effects of proton accumulation within the anode biofilm. *Energy & Environmental Science, 2*(1), 113-119.

Fredrickson, J. K., Romine, M. F., Beliaev, A. S., Auchtung, J. M., Driscoll, M. E., Gardner, T. S., et al. (2008). Towards environmental systems biology of shewanella. *Nature Reviews Microbiology, 6*(8), 592-603.

Fredrickson, J. K., Kostandarithes, H. M., Li, S., Plymale, A. E., & Daly, M. J. (2000). Reduction of fe (III), cr (VI), U (VI), and tc (VII) byDeinococcus radiodurans R1. *Applied and Environmental Microbiology, 66*(5), 2006-2011.

Fricke, K., Harnisch, F., & Schröder, U. (2008). On the use of cyclic voltammetry for the study of anodic electron transfer in microbial fuel cells. *Energy & Environmental Science, 1*(1), 144-147.

Fry, J. C., Parkes, R. J., Cragg, B. A., Weightman, A. J., & Webster, G. (2008). Prokaryotic biodiversity and activity in the deep subseafloor biosphere. *FEMS Microbiology Ecology, 66*(2), 181-196.

Gad, S. C. (1989). Acute and chronic systemic chromium toxicity. *Science of the Total Environment, 86*(1), 149-157.

Gadd, G. M. (2010). Metals, minerals and microbes: Geomicrobiology and bioremediation. *Microbiology, 156*(3), 609-643.

Galushko, A. S., & Schink, B. (2000). Oxidation of acetate through reactions of the citric acid cycle by geobacter sulfurreducens in pure culture and in syntrophic coculture. *Archives of Microbiology, 174*(5), 314-321.

Ganguli, A., & Tripathi, A. (2002). Bioremediation of toxic chromium from electroplating effluent by chromate-reducing pseudomonas aeruginosa A2Chr in two bioreactors. *Applied Microbiology and Biotechnology, 58*(3), 416-420.

Godos, I. d., Blanco, S., García-Encina, P. A., Becares, E., & Muñoz, R. (2009). Long-term operation of high rate algal ponds for the bioremediation of piggery wastewaters at high loading rates. *Bioresource Technology, 100*(19), 4332-4339.

Gong, J., Zheng, H., Wu, Z., Chen, T., & Zhao, X. (2009). Genome shuffling: Progress and applications for phenotype improvement. *Biotechnology Advances, 27*(6), 996-1005.

Gorby, Y. A., & Lovley, D. R. (1991). Electron transport in the dissimilatory iron reducer, GS-15. *Applied and Environmental Microbiology, 57*(3), 867-870.

Gorby, Y. A., Yanina, S., McLean, J. S., Rosso, K. M., Moyles, D., Dohnalkova, A., et al. (2006). Electrically conductive bacterial nanowires produced by shewanella oneidensis strain MR-1 and other microorganisms. *Proceedings of the National Academy of Sciences, 103*(30), 11358-11363.

Gregory, K. B., & Lovley, D. R. (2005). Remediation and recovery of uranium from contaminated subsurface environments with electrodes. *Environmental Science & Technology, 39*(22), 8943-8947.

Gusmão, V. R., Chinalia, F. A., Sakamoto, I. K., & Varesche, M. B. A. (2007). Performance of a reactor containing denitrifying immobilized biomass in removing ethanol and aromatic hydrocarbons (BTEX) in a short operating period. *Journal of Hazardous Materials, 139*(2), 301-309.

Hansel, C., Wielinga, B., & Fendorf, S. (2003). Structural and compositional evolution of Cr/Fe solids after indirect chromate reduction by dissimilatory iron-reducing bacteria. *Geochimica Et Cosmochimica Acta, 67*(3), 401-412.

Hansen, G., Shillito, R. D., & Chilton, M. (1997). T-strand integration in maize protoplasts after codelivery of a T-DNA substrate and virulence genes. *Proceedings of the National Academy of Sciences, 94*(21), 11726-11730.

Hanson, K., & Desai, A. J. (1996). Intergeneric protoplast fusion between acinetobacter sp. A3 and pseudomonas putida DP99 for enchanced hydrocarbon degradation. *Biotechnology Letters, 18*(12), 1369-1374.

Harnisch, F., & Freguia, S. (2012). A basic tutorial on cyclic voltammetry for the investigation of electroactive microbial biofilms. *Chemistry–An Asian Journal, 7*(3), 466-475.

Hau, H. H., & Gralnick, J. A. (2007). Ecology and biotechnology of the genus shewanella. *Annu.Rev.Microbiol., 61*, 237-258.

Hernandez, M., & Newman, D. (2001). Extracellular electron transfer. *Cellular and Molecular Life Sciences CMLS, 58*(11), 1562-1571.

Holmes, D. E., Chaudhuri, S. K., Nevin, K. P., Mehta, T., Methé, B. A., Liu, A., et al. (2006a). Microarray and genetic analysis of electron transfer to electrodes in geobacter sulfurreducens. *Environmental Microbiology, 8*(10), 1805-1815.

Holmes, D. E., Chaudhuri, S. K., Nevin, K. P., Mehta, T., Methé, B. A., Liu, A., et al. (2006b). Microarray and genetic analysis of electron transfer to electrodes in geobacter sulfurreducens. *Environmental Microbiology, 8*(10), 1805-1815.

Holmes, D. E., Mester, T., O'Neil, R. A., Perpetua, L. A., Larrahondo, M. J., Glaven, R., et al. (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*(5), 1422-1435.

Hunter, W. J., & Shaner, D. L. (2009). Nitrogen limited biobarriers remove atrazine from contaminated water: Laboratory studies. *Journal of Contaminant Hydrology, 103*(1), 29-37.

Huvinen, M., Mäkitie, A., Järventaus, H., Wolff, H., Stjernvall, T., Hovi, A., et al. (2002). Nasal cell micronuclei, cytology and clinical symptoms in stainless steel production workers exposed to chromium. *Mutagenesis, 17*(5), 425-429.

Icopini, G. A., Lack, J. G., Hersman, L. E., Neu, M. P., & Boukhalfa, H. (2009). Plutonium (V/VI) reduction by the metal-reducing bacteria geobacter metallireducens GS-15 and shewanella oneidensis MR-1. *Applied and Environmental Microbiology, 75*(11), 3641-3647.

Inoue, K., Leang, C., Franks, A. E., Woodard, T. L., Nevin, K. P., & Lovley, D. R. (2011). Specific localization of the c-type cytochrome OmcZ at the anode surface in current-producing biofilms of geobacter sulfurreducens. *Environmental Microbiology Reports, 3*(2), 211-217.

Inoue, K., Qian, X., Morgado, L., Kim, B., Mester, T., Izallalen, M., et al. (2010). Purification and characterization of OmcZ, an outer-surface, octaheme c-type cytochrome essential for optimal current production by geobacter sulfurreducens. *Applied and Environmental Microbiology, 76*(12), 3999-4007.

Izallalen, M., Mahadevan, R., Burgard, A., Postier, B., Didonato Jr, R., Sun, J., et al. (2008). < i> geobacter sulfurreducens</i> strain engineered for increased rates of respiration. *Metabolic Engineering, 10*(5), 267-275.

Jacques, R. J., Okeke, B. C., Bento, F. M., Teixeira, A. S., Peralba, M. C., & Camargo, F. A. (2008). Microbial consortium bioaugmentation of a polycyclic aromatic hydrocarbons contaminated soil. *Bioresource Technology, 99*(7), 2637-2643.

Jain, A., Gazzola, G., Panzera, A., Zanoni, M., & Marsili, E. (2011). Visible spectroelectrochemical characterization of< i> geobacter sulfurreducens</i> biofilms on optically transparent indium tin oxide electrode. *Electrochimica Acta, 56*(28), 10776-10785.

Jain, A., Zhang, X., Pastorella, G., Connolly, J. O., Barry, N., Woolley, R., et al. (2012). Electron transfer mechanism in< i> shewanella loihica</i> PV-4 biofilms formed at graphite electrode. *Bioelectrochemistry, 87*, 28-32.

James, B. R., & Bartlett, R. J. (1983). Behavior of chromium in soils. VI. interactions between oxidation-reduction and organic complexation. *Journal of Environmental Quality, 12*(2), 173-176.

Jannasch, H. W., Eimhjellen, K., & Farmanfarmalan, A. (1971). Microbial degradation of organic matter in the deep sea. *Science, 171*(3972), 672-675.

Janssen, P. H., Liesack, W., & Schink, B. (2002). Geovibrio thiophilus sp. nov., a novel sulfur-reducing bacterium belonging to the phylum deferribacteres. *International Journal of Systematic and Evolutionary Microbiology, 52*(4), 1341-1347.

Jennette, K. W. (1982). Microsomal reduction of the carcinogen chromate produces chromium (V). *Journal of the American Chemical Society, 104*(3), 874-875.

John, R. P., Gangadharan, D., & Madhavan Nampoothiri, K. (2008). Genome shuffling of< i> lactobacillus delbrueckii</i> mutant and< i> bacillus amyloliquefaciens</i> through protoplasmic fusion for l-lactic acid production from starchy wastes. *Bioresource Technology, 99*(17), 8008-8015.

Kerr, R. A. (1997). Geomicrobiology: Life goes to extremes in the deep earth--and elsewhere? *Science, 276*(5313), 703-704.

Kim, H. U., Kim, T. Y., & Lee, S. Y. (2008). Metabolic flux analysis and metabolic engineering of microorganisms. *Molecular BioSystems, 4*(2), 113-120.

Kim, Y., & Amemiya, S. (2008). Stripping analysis of nanomolar perchlorate in drinking water with a voltammetric ion-selective electrode based on thin-layer liquid membrane. *Analytical Chemistry, 80*(15), 6056-6065.

Kimura, T., Li, Y., Okumura, F., Itoh, N., Nakanishi, T., Sone, T., et al. (2008). Chromium (VI) inhibits mouse metallothionein-I gene transcription by preventing the zinc-dependent formation of an MTF-1-p300 complex. *Biochem.J, 415*, 477-482.

Kirschner, A., Altenbuchner, J., & Bornscheuer, U. T. (2007). Design of a secondary alcohol degradation pathway from pseudomonas fluorescens DSM 50106 in an engineered escherichia coli. *Applied Microbiology and Biotechnology, 75*(5), 1095-1101.

Klimes, A., Franks, A. E., Glaven, R. H., Tran, H., Barrett, C. L., Qiu, Y., et al. (2010). Production of pilus-like filaments in geobacter sulfurreducens in the absence of the type IV pilin protein PilA. *FEMS Microbiology Letters, 310*(1), 62-68.

Konhauser, K. O., Hamade, T., Raiswell, R., Morris, R. C., Ferris, F. G., Southam, G., et al. (2002). Could bacteria have formed the precambrian banded iron formations? *Geology, 30*(12), 1079-1082.

Krushkal, J., Yan, B., DiDonato, L. N., Puljic, M., Nevin, K. P., Woodard, T. L., et al. (2007). Genome-wide expression profiling in geobacter sulfurreducens: Identification of fur and RpoS transcription regulatory sites in a rel gsu mutant. *Functional & Integrative Genomics, 7*(3), 229-255.

Kuhn, T. K., Hamonts, K., Dijk, J. A., Kalka, H., Stichler, W., Springael, D., et al. (2009). Assessment of the intrinsic bioremediation capacity of an eutrophic river sediment polluted by discharging chlorinated aliphatic hydrocarbons: A compound-specific isotope approach. *Environmental Science & Technology, 43*(14), 5263-5269.

Kulik, N., Goi, A., Trapido, M., & Tuhkanen, T. (2006). Degradation of polycyclic aromatic hydrocarbons by combined chemical pre-oxidation and bioremediation in creosote contaminated soil. *Journal of Environmental Management, 78*(4), 382-391.

Kunapuli, U., Jahn, M. K., Lueders, T., Geyer, R., Heipieper, H. J., & Meckenstock, R. U. (2010). Desulfitobacterium aromaticivorans sp. nov. and geobacter toluenoxydans sp. nov., iron-reducing bacteria capable of anaerobic degradation of monoaromatic hydrocarbons. *International Journal of Systematic and Evolutionary Microbiology, 60*(3), 686-695.

Kuyper, M., Toirkens, M. J., Diderich, J. A., Winkler, A. A., Dijken, J. P., & Pronk, J. T. (2005). Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting saccharomyces cerevisiae strain. *FEMS Yeast Research, 5*(10), 925-934.

Ladonin, D. (2002). Heavy metal compounds in soils: Problems and methods of study. *Eurasian Soil Science, 35*(6), 605-613.

LaGrega, M., Buckingham, P., & Evans, J. Environmental resources management (2001) hazardous waste management. *McGraw Hill, New York.Managing Adverse Soil Chemical Environments, 233*, 1202.

Lajoie, C., Zylstra, G., DeFlaun, M., & Strom, P. (1993). Development of field application vectors for bioremediation of soils contaminated with polychlorinated biphenyls. *Applied and Environmental Microbiology, 59*(6), 1735-1741.

Lange, C. C., Wackett, L. P., Minton, K. W., & Daly, M. J. (1998). Engineering a recombinant deinococcus radiodurans for organopollutant degradation in radioactive mixed waste environments. *Nature Biotechnology, 16*(10), 929-933.

Langrrd, S. (1990). One hundred years of chromium and cancer: A review of epidemiological evidence and selected case reports. *American Journal of Industrial Medicine, 17*(2), 189-214.

Leang, C., Coppi, M., & Lovley, D. (2003). OmcB, a c-type polyheme cytochrome, involved in fe (III) reduction in geobacter sulfurreducens. *Journal of Bacteriology, 185*(7), 2096-2103.

Leang, C., & Lovley, D. R. (2005). Regulation of two highly similar genes, omcB and omcC, in a 10 kb chromosomal duplication in geobacter sulfurreducens. *Microbiology, 151*(6), 1761-1767.

Leang, C., Qian, X., Mester, T., & Lovley, D. R. (2010). Alignment of the c-type cytochrome OmcS along pili of geobacter sulfurreducens. *Applied and Environmental Microbiology, 76*(12), 4080-4084.

Lee, J., & Lee, D. (2005). An improved cluster labeling method for support vector clustering. *Pattern Analysis and Machine Intelligence, IEEE Transactions on, 27*(3), 461-464.

Lee, K., Bosch, J., & Meckenstock, R. U. (2012). Use of metal-reducing bacteria for bioremediation of soil contaminated with mixed organic and inorganic pollutants. *Environmental Geochemistry and Health, 34*(1), 135-142.

Léger, C., Elliott, S. J., Hoke, K. R., Jeuken, L. J., Jones, A. K., & Armstrong, F. A. (2003). Enzyme electrokinetics: Using protein film voltammetry to investigate redox enzymes and their mechanisms. *Biochemistry, 42*(29), 8653-8662.

Lennox, J., & Ashe, J. (2009). Biofilms as biobarriers. *The American Biology Teacher, 71*(1), 20-26.

Li, Y., Zhu, S., & Deng, K. (2011). Mössbauer hyperfine parameters of iron species in the course of geobacter-mediated magnetite mineralization. *Physics and Chemistry of Minerals, 38*(9), 701-708.

Liu, C., Gorby, Y. A., Zachara, J. M., Fredrickson, J. K., & Brown, C. F. (2002). Reduction kinetics of fe (III), co (III), U (VI), cr (VI), and tc (VII) in cultures of dissimilatory metal-reducing bacteria. *Biotechnology and Bioengineering, 80*(6), 637-649.

Lloyd, J., Leang, C., HODGES, M. A., Coppi, M., Cuifo, S., Methe, B., et al. (2003). Biochemical and genetic characterization of PpcA, a periplasmic c-type cytochrome in geobacter sulfurreducens. *Biochem.J, 369*, 153-161.

Logan, B. E. (2009). Exoelectrogenic bacteria that power microbial fuel cells. *Nature Reviews Microbiology, 7*(5), 375-381.

Logan, B. E., & Regan, J. M. (2006). Electricity-producing bacterial communities in microbial fuel cells. *Trends in Microbiology, 14*(12), 512-518.

Lovley, D. (2013). Dissimilatory fe (III)-and mn (IV)-reducing prokaryotes. *The prokaryotes* (pp. 287-308) Springer.

Lovley, D. R. (1991). Dissimilatory fe (III) and mn (IV) reduction. *Microbiological Reviews, 55*(2), 259.

Lovley, D. R. (1993). Dissimilatory metal reduction. *Annual Reviews in Microbiology, 47*(1), 263-290.

Lovley, D. R., Holmes, D. E., & Nevin, K. P. (2004). Dissimilatory fe (iii) and mn (iv) reduction. *Advances in Microbial Physiology, 49*, 219-286.

Lovley, D. R., & Phillips, E. J. (1988). Novel mode of microbial energy metabolism: Organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Applied and Environmental Microbiology, 54*(6), 1472-1480.

Lovley, D. R., & Phillips, E. J. (1994). Reduction of chromate by desulfovibrio vulgaris and its c3 cytochrome. *Applied and Environmental Microbiology, 60*(2), 726-728.

Lovley, D. R., Stolz, J. F., Nord, G. L., & Phillips, E. J. (1987). Anaerobic production of magnetite by a dissimilatory iron-reducing microorganism. *Nature, 330*(6145), 252-254.

Mabbett, A. N., Yong, P., Farr, J. P. G., & Macaskie, L. E. (2004). Reduction of cr (VI) by “palladized” biomass of desulfovibrio desulfuricans ATCC 29577. *Biotechnology and Bioengineering, 87*(1), 104-109.

MACKENZIE, R. D., BYERRUM, R. U., DECKER, C. F., HOPPERT, C. A., & LANGHAM, R. F. (1958). Chronic toxicity studies. II. hexavalent and trivalent chromium administered in drinking water to rats. *A.M.A.Archives of Industrial Health, 18*(3), 232-234.

Magnuson, T., Isoyama, N., Hodges-Myerson, A., DAVIDSON, G., Maroney, M., Geesey, G., et al. (2001). Isolation, characterization and gene sequence analysis of a membrane-associated 89 kDa fe (III) reducing cytochrome c from geobacter sulfurreducens. *Biochem.J, 359*, 147-152.

Mahadevan, R., Bond, D., Butler, J., Esteve-Nunez, A., Coppi, M., Palsson, B., et al. (2006). Characterization of metabolism in the fe (III)-reducing organism geobacter sulfurreducens by constraint-based modeling. *Applied and Environmental Microbiology, 72*(2), 1558-1568.

Maila, M. P., & Cloete, T. E. (2005). The use of biological activities to monitor the removal of fuel contaminants—perspective for monitoring hydrocarbon contamination: A review. *International Biodeterioration & Biodegradation, 55*(1), 1-8.

Majander, K., Anton, L., Antikainen, J., Lång, H., Brummer, M., Korhonen, T. K., et al. (2005). Extracellular secretion of polypeptides using a modified escherichia coli flagellar secretion apparatus. *Nature Biotechnology, 23*(4), 475-481.

Malvankar, N. S., & Lovley, D. R. (2012). Microbial nanowires: A new paradigm for biological electron transfer and bioelectronics. *ChemSusChem, 5*(6), 1039-1046.

Malvankar, N. S., Tuominen, M. T., & Lovley, D. R. (2012). Lack of cytochrome involvement in long-range electron transport through conductive biofilms and nanowires of geobacter sulfurreducens. *Energy & Environmental Science, 5*(9), 8651-8659.

Marsili, E., Baron, D. B., Shikhare, I. D., Coursolle, D., Gralnick, J. A., & Bond, D. R. (2008). Shewanella secretes flavins that mediate extracellular electron transfer. *Proceedings of the National Academy of Sciences, 105*(10), 3968-3973.

Marsili, E., Rollefson, J. B., Baron, D. B., Hozalski, R. M., & Bond, D. R. (2008). Microbial biofilm voltammetry: Direct electrochemical characterization of catalytic electrode-attached biofilms. *Applied and Environmental Microbiology, 74*(23), 7329-7337.

Marsili, E., Sun, J., & Bond, D. R. (2010). Voltammetry and growth physiology of geobacter sulfurreducens biofilms as a function of growth stage and imposed electrode potential. *Electroanalysis, 22*(7-8), 865-874.

Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D., & Keasling, J. D. (2003). Engineering a mevalonate pathway in escherichia coli for production of terpenoids. *Nature Biotechnology, 21*(7), 796-802.

Massoudieh, A., Mathew, A., Lambertini, E., Nelson, K., & Ginn, T. (2007). Horizontal gene transfer on surfaces in natural porous media: Conjugation and kinetics. *Vadose Zone Journal, 6*(2), 306-315.

Mathuna, C. O., O'Donnell, T., Martinez-Catala, R. V., Rohan, J., & O'Flynn, B. (2008). Energy scavenging for long-term deployable wireless sensor networks. *Talanta, 75*(3), 613-623.

McKinlay, J. B., & Zeikus, J. G. (2004). Extracellular iron reduction is mediated in part by neutral red and hydrogenase in escherichia coli. *Applied and Environmental Microbiology, 70*(6), 3467-3474.

Mehta, T., Coppi, M. V., Childers, S. E., & Lovley, D. R. (2005). Outer membrane c-type cytochromes required for fe (III) and mn (IV) oxide reduction in geobacter sulfurreducens. *Applied and Environmental Microbiology, 71*(12), 8634-8641.

Meng-Ying, L., Ji, Z., Peng, L., Jing-Liang, X., & Shun-Peng, L. 细菌生物学特性对其生物膜形成的影响. *PEDOSPHERE, 19*(5), 554-561.

Methe, B., Nelson, K. E., Eisen, J., Paulsen, I., Nelson, W., Heidelberg, J., et al. (2003). Genome of geobacter sulfurreducens: Metal reduction in subsurface environments. *Science, 302*(5652), 1967-1969.

Michalsen, M. M., Peacock, A. D., Smithgal, A. N., White, D. C., Spain, A. M., Sanchez-Rosario, Y., et al. (2009). Treatment of nitric acid-, U (VI)-, and tc (VII)-contaminated groundwater in intermediate-scale physical models of an in situ biobarrier. *Environmental Science & Technology, 43*(6), 1952-1961.

Molokwane, P., & Nkhalambayausi-Chirwa, E. (2009). Microbial culture dynamics and chromium (VI) removal in packed-column microcosm reactors.

Molokwane, P. E., Meli, K. C., & Nkhalambayausi-Chirwa, E. M. (2008). Chromium (VI) reduction in activated sludge bacteria exposed to high chromium loading: Brits culture (south africa). *Water Research, 42*(17), 4538-4548.

Monti, M. R., Smania, A. M., Fabro, G., Alvarez, M. E., & Argaraña, C. E. (2005). Engineering pseudomonas fluorescens for biodegradation of 2, 4-dinitrotoluene. *Applied and Environmental Microbiology, 71*(12), 8864-8872.

Morgado, L., Saraiva, I. H., Louro, R. O., & Salgueiro, C. A. (2010). Orientation of the axial ligands and magnetic properties of the hemes in the triheme ferricytochrome PpcA from< i> G. sulfurreducens</i> determined by paramagnetic NMR. *FEBS Letters, 584*(15), 3442-3445.

Mouser, P. J., Holmes, D. E., Perpetua, L. A., DiDonato, R., Postier, B., Liu, A., et al. (2009). Quantifying expression of geobacter spp. oxidative stress genes in pure culture and during in situ uranium bioremediation. *The ISME Journal, 3*(4), 454-465.

Mukherjee, A. K., & Bordoloi, N. K. (2011). Bioremediation and reclamation of soil contaminated with petroleum oil hydrocarbons by exogenously seeded bacterial consortium: A pilot-scale study. *Environmental Science and Pollution Research, 18*(3), 471-478.

Nealson, K. H., & Myers, C. R. (1992). Microbial reduction of manganese and iron: New approaches to carbon cycling. *Applied and Environmental Microbiology, 58*(2), 439.

Nealson, K. H., & Saffarini, D. (1994). Iron and manganese in anaerobic respiration: Environmental significance, physiology, and regulation. *Annual Reviews in Microbiology, 48*(1), 311-343.

Ness, J. E., Welch, M., Giver, L., Bueno, M., Cherry, J. R., Borchert, T. V., et al. (1999). DNA shuffling of subgenomic sequences of subtilisin. *Nature Biotechnology, 17*(9), 893-896.

Nevin, K. P., Kim, B., Glaven, R. H., Johnson, J. P., Woodard, T. L., Methé, B. A., et al. (2009a). Anode biofilm transcriptomics reveals outer surface components essential for high density current production in geobacter sulfurreducens fuel cells. *PLoS One, 4*(5), e5628.

Nevin, K. P., Kim, B., Glaven, R. H., Johnson, J. P., Woodard, T. L., Methé, B. A., et al. (2009b). Anode biofilm transcriptomics reveals outer surface components essential for high density current production in geobacter sulfurreducens fuel cells. *PLoS One, 4*(5), e5628.

Nevin, K. P., & Lovley, D. R. (2000). Lack of production of electron-shuttling compounds or solubilization of fe (III) during reduction of insoluble fe (III) oxide by geobacter metallireducens. *Applied and Environmental Microbiology, 66*(5), 2248-2251.

Nevin, K. P., & Lovley, D. R. (2002a). Mechanisms for accessing insoluble fe (III) oxide during dissimilatory fe (III) reduction by geothrix fermentans. *Applied and Environmental Microbiology, 68*(5), 2294-2299.

Nevin, K. P., & Lovley, D. R. (2002b). Mechanisms for fe (III) oxide reduction in sedimentary environments. *Geomicrobiology Journal, 19*(2), 141-159.

N'Guessan, A., Elifantz, H., Nevin, K., Mouser, P., Methe, B., & LWoodard, T. K. 394 manley, KH williams, MJ wilkins, JT larsen, PE long, and DR lovley. 395 2010. molecular analysis of phosphate limitation in geobacteraceae during the 396 bioremediation of a uranium-contaminated aquifer. *ISME J, 4*, 253-266.

Nissen, T. L., Kielland-Brandt, M. C., Nielsen, J., & Villadsen, J. (2000). Optimization of ethanol production in< i> saccharomyces cerevisiae</i> by metabolic engineering of the ammonium assimilation. *Metabolic Engineering, 2*(1), 69-77.

O'Flaherty, E. J., Kerger, B. D., Hays, S. M., & Paustenbach, D. J. (2001). A physiologically based model for the ingestion of chromium (III) and chromium (VI) by humans. *Toxicological Sciences, 60*(2), 196-213.

Okoh, A., & Trejo-Hernandez, M. (2006). Remediation of petroleum hydrocarbon polluted systems: Exploiting the bioremediation strategies. *African Journal of Biotechnology, 5*(25)

Otte, B., Grunwaldt, E., Mahmoud, O., & Jennewein, S. (2009). Genome shuffling in clostridium diolis DSM 15410 for improved 1, 3-propanediol production. *Applied and Environmental Microbiology, 75*(24), 7610-7616.

Paje, M. L. F., & Neilan, B. A. (1997). A rhodococcus species that thrives on medium saturated with liquid benzene. *Microbiology, 143*(9), 2975-2981.

Pandey, J., Chauhan, A., & Jain, R. K. (2009). Integrative approaches for assessing the ecological sustainability of in situ bioremediation. *FEMS Microbiology Reviews, 33*(2), 324-375.

Park, H. S., Kim, B. H., Kim, H. S., Kim, H. J., Kim, G. T., Kim, M., et al. (2001). A novel electrochemically active and fe (III)-reducing bacterium phylogenetically related to< i> clostridium butyricum</i> isolated from a microbial fuel cell. *Anaerobe, 7*(6), 297-306.

Park, J. H., & Lee, S. Y. (2008). Towards systems metabolic engineering of microorganisms for amino acid production. *Current Opinion in Biotechnology, 19*(5), 454-460.

Park, J. H., Lee, S. Y., Kim, T. Y., & Kim, H. U. (2008). Application of systems biology for bioprocess development. *Trends in Biotechnology, 26*(8), 404-412.

Parkes, R. J., Linnane, C. D., Webster, G., Sass, H., Weightman, A. J., Hornibrook, E. R., et al. (2011). Prokaryotes stimulate mineral H2 formation for the deep biosphere and subsequent thermogenic activity. *Geology, 39*(3), 219-222.

Parkes, R. J., Cragg, B. A., Bale, S., Getlifff, J., Goodman, K., Rochelle, P. A., et al. (1994). Deep bacterial biosphere in pacific ocean sediments. *Nature, 371*(6496), 410-413.

Pastorella, G., Gazzola, G., Guadarrama, S., & Marsili, E. (2012). Biofilms: Applications in bioremediation. *Microbial Biofilms,* , 73. Caister Academic press.

Patel, J., Zhang, Q., McKay, R. M. L., Vincent, R., & Xu, Z. (2010). Genetic engineering of caulobacter crescentus for removal of cadmium from water. *Applied Biochemistry and Biotechnology, 160*(1), 232-243.

Patnaik, R., Louie, S., Gavrilovic, V., Perry, K., Stemmer, W. P., Ryan, C. M., et al. (2002). Genome shuffling of lactobacillus for improved acid tolerance. *Nature Biotechnology, 20*(7), 707-712.

Patterson, J. W., & Passino, R. (1987). Metals separation and recovery. *Metal Speciation Separation and Recovery,*

Paul, D., Pandey, G., Pandey, J., & Jain, R. K. (2005). Accessing microbial diversity for bioremediation and environmental restoration. *Trends in Biotechnology, 23*(3), 135-142.

Pechova, A., & Pavlata, L. (2007). Chromium as an essential nutrient: A review. *VETERINARNI MEDICINA-PRAHA-, 52*(1), 1.

Penning, H., Sørensen, S. R., Meyer, A. H., Aamand, J., & Elsner, M. (2010). C, N, and H isotope fractionation of the herbicide isoproturon reflects different microbial transformation pathways. *Environmental Science & Technology, 44*(7), 2372-2378.

Pereira, I. A., Pacheco, I., Liu, M., Legall, J., Xavier, A. V., & Teixeira, M. (1997). Multiheme cytochromes from the Sulfur-Reducing bacterium desulfuromonas acetoxidans. *European Journal of Biochemistry, 248*(2), 323-328.

Perez-Roa, R. E., Tompkins, D. T., Paulose, M., Grimes, C. A., Anderson, M. A., & Noguera, D. R. (2006). Effects of localised, low-voltage pulsed electric fields on the development and inhibition of pseudomonas aeruginosa biofilms. *Biofouling, 22*(6), 383-390.

Pessanha, M., Morgado, L., Louro, R. O., Londer, Y. Y., Pokkuluri, P. R., Schiffer, M., et al. (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.

Petri, R., & Schmidt-Dannert, C. (2004). Dealing with complexity: Evolutionary engineering and genome shuffling. *Current Opinion in Biotechnology, 15*(4), 298-304.

Pfleger, B. F., Pitera, D. J., Smolke, C. D., & Keasling, J. D. (2006). Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. *Nature Biotechnology, 24*(8), 1027-1032.

Pham, C. A., Jung, S. J., Phung, N. T., Lee, J., Chang, I. S., Kim, B. H., et al. (2003). A novel electrochemically active and fe (III)-reducing bacterium phylogenetically related to aeromonas hydrophila, isolated from a microbial fuel cell. *FEMS Microbiology Letters, 223*(1), 129-134.

Phillips, E. J., Landa, E. R., & Lovley, D. R. (1995). Remediation of uranium contaminated soils with bicarbonate extraction and microbial U (VI) reduction. *Journal of Industrial Microbiology, 14*(3-4), 203-207.

Pieper, D. H., & Reineke, W. (2000). Engineering bacteria for bioremediation. *Current Opinion in Biotechnology, 11*(3), 262-270.

Potter, M. C. (1911). Electrical effects accompanying the decomposition of organic compounds. *Proceedings of the Royal Society of London.Series B, Containing Papers of a Biological Character, 84*(571), 260-276.

Prpich, G. P., Adams, R. L., & Daugulis, A. J. (2006). Ex situ bioremediation of phenol contaminated soil using polymer beads. *Biotechnology Letters, 28*(24), 2027-2031.

Qian, X., Mester, T., Morgado, L., Arakawa, T., Sharma, M. L., Inoue, K., et al. (2011). Biochemical characterization of purified OmcS, a< i> c</i>-type cytochrome required for insoluble fe (III) reduction in< i> geobacter sulfurreducens</i>. *Biochimica Et Biophysica Acta (BBA)-Bioenergetics, 1807*(4), 404-412.

Qian, X., Reguera, G., Mester, T., & Lovley, D. R. (2007). Evidence that OmcB and OmpB of geobacter sulfurreducens are outer membrane surface proteins. *FEMS Microbiology Letters, 277*(1), 21-27.

Rai, D., Eary, L., & Zachara, J. (1989). Environmental chemistry of chromium. *Science of the Total Environment, 86*(1), 15-23.

Ramage, G., Culshaw, S., Jones, B., & Williams, C. (2010). Are we any closer to beating the biofilm: Novel methods of biofilm control. *Current Opinion in Infectious Diseases, 23*(6), 560-566.

Ramírez-Díaz, M. I., Díaz-Pérez, C., Vargas, E., Riveros-Rosas, H., Campos-García, J., & Cervantes, C. (2008a). Mechanisms of bacterial resistance to chromium compounds. *Biometals, 21*(3), 321-332.

Ramírez-Díaz, M. I., Díaz-Pérez, C., Vargas, E., Riveros-Rosas, H., Campos-García, J., & Cervantes, C. (2008b). Mechanisms of bacterial resistance to chromium compounds. *Biometals, 21*(3), 321-332.

Ramírez-Díaz, M. I., Díaz-Pérez, C., Vargas, E., Riveros-Rosas, H., Campos-García, J., & Cervantes, C. (2008c). Mechanisms of bacterial resistance to chromium compounds. *Biometals, 21*(3), 321-332.

Ramos, J. L., Duque, E., Huertas, M., & Haidour, A. (1995). Isolation and expansion of the catabolic potential of a pseudomonas putida strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *Journal of Bacteriology, 177*(14), 3911-3916.

Ramos, J., Marqués, S., van Dillewijn, P., Espinosa-Urgel, M., Segura, A., Duque, E., et al. (2011). Laboratory research aimed at closing the gaps in microbial bioremediation. *Trends in Biotechnology, 29*(12), 641-647.

Reguera, G., McCarthy, K. D., Mehta, T., Nicoll, J. S., Tuominen, M. T., & Lovley, D. R. (2005a). Extracellular electron transfer via microbial nanowires. *Nature, 435*(7045), 1098-1101.

Reguera, G., McCarthy, K. D., Mehta, T., Nicoll, J. S., Tuominen, M. T., & Lovley, D. R. (2005b). Extracellular electron transfer via microbial nanowires. *Nature, 435*(7045), 1098-1101.

Reguera, G., Nevin, K. P., Nicoll, J. S., Covalla, S. F., Woodard, T. L., & Lovley, D. R. (2006). Biofilm and nanowire production leads to increased current in geobacter sulfurreducens fuel cells. *Applied and Environmental Microbiology, 72*(11), 7345-7348.

Reguera, G., Pollina, R. B., Nicoll, J. S., & Lovley, D. R. (2007). Possible nonconductive role of geobacter sulfurreducens pilus nanowires in biofilm formation. *Journal of Bacteriology, 189*(5), 2125-2127.

Reith, F. (2011). Life in the deep subsurface. *Geology, 39*(3), 287-288.

Renshaw, J. C., Butchins, L. J., Livens, F. R., May, I., Charnock, J. M., & Lloyd, J. R. (2005). Bioreduction of uranium: Environmental implications of a pentavalent intermediate. *Environmental Science & Technology, 39*(15), 5657-5660.

Richter, H., Nevin, K. P., Jia, H., Lowy, D. A., Lovley, D. R., & Tender, L. M. (2009). Cyclic voltammetry of biofilms of wild type and mutant geobacter sulfurreducens on fuel cell anodes indicates possible roles of OmcB, OmcZ, type IV pili, and protons in extracellular electron transfer. *Energy & Environmental Science, 2*(5), 506-516.

Richter, K., Schicklberger, M., & Gescher, J. (2012). Dissimilatory reduction of extracellular electron acceptors in anaerobic respiration. *Applied and Environmental Microbiology, 78*(4), 913-921.

Rittmann, B. E. (2013). The energy issue in urban water management. *Source Separation and Decentralization for Wastewater Management,* , 13.

Ro, D., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., et al. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature, 440*(7086), 940-943.

Robles-González, I. V., Fava, F., & Poggi-Varaldo, H. M. (2008). A review on slurry bioreactors for bioremediation of soils and sediments. *Microb Cell Fact, 7*(5)

Rodgers, P. J., & Amemiya, S. (2007). Cyclic voltammetry at micropipet electrodes for the study of ion-transfer kinetics at liquid/liquid interfaces. *Analytical Chemistry, 79*(24), 9276-9285.

Rodríguez, S. J., & Bishop, P. L. (2007). Three-dimensional quantification of soil biofilms using image analysis. *Environmental Engineering Science, 24*(1), 96-103.

Rodriguez, S., & Bishop, P. L. (2008). Enhancing the biodegradation of polycyclic aromatic hydrocarbons: Effects of nonionic surfactant addition on biofilm function and structure. *Journal of Environmental Engineering, 134*(7), 505-512.

Rollefson, J. B., Levar, C. E., & Bond, D. R. (2009). Identification of genes involved in biofilm formation and respiration via mini-himar transposon mutagenesis of geobacter sulfurreducens. *Journal of Bacteriology, 191*(13), 4207-4217.

Rollefson, J. B., Stephen, C. S., Tien, M., & Bond, D. R. (2011). Identification of an extracellular polysaccharide network essential for cytochrome anchoring and biofilm formation in geobacter sulfurreducens. *Journal of Bacteriology, 193*(5), 1023-1033.

Roller, S. D., Bennetto, H. P., Delaney, G. M., Mason, J. R., Stirling, J. L., & Thurston, C. F. (1984). Electron-transfer coupling in microbial fuel cells: 1. comparison of redox-mediator reduction rates and respiratory rates of bacteria. *Journal of Chemical Technology and Biotechnology.Biotechnology, 34*(1), 3-12.

Rotaru, D. E. H., Franks, A. E., Orellana, R., Risso, C., & Nevin, K. P. (2011). Geobacter: The microbe electric's physiology, ecology, and practical applications. *Advances in Microbial Physiology 59, 59*, 1.

Rowbotham, A. L., Levy, L. S., & Shuker, L. K. (2000). Chromium in the environment: An evaluation of exposure of the UK general population and possible adverse health effects. *Journal of Toxicology and Environmental Health Part B: Critical Reviews, 3*(3), 145-178.

Rui, L., Kwon, Y. M., Fishman, A., Reardon, K. F., & Wood, T. K. (2004). Saturation mutagenesis of toluene ortho-monooxygenase of burkholderia cepacia G4 for enhanced 1-naphthol synthesis and chloroform degradation. *Applied and Environmental Microbiology, 70*(6), 3246-3252.

Santos, C. N. S., & Stephanopoulos, G. (2008). Combinatorial engineering of microbes for optimizing cellular phenotype. *Current Opinion in Chemical Biology, 12*(2), 168-176.

Sardessai, Y., & Bhosle, S. (2002). Tolerance of bacteria to organic solvents. *Research in Microbiology, 153*(5), 263-268.

Sauer, U. (2001). Evolutionary engineering of industrially important microbial phenotypes. *Metabolic engineering* (pp. 129-169) Springer.

Seeliger, S., Cord-Ruwisch, R., & Schink, B. (1998). A periplasmic and extracellular c-type cytochrome ofGeobacter sulfurreducens acts as a ferric iron reductase and as an electron carrier to other acceptors or to partner bacteria. *Journal of Bacteriology, 180*(14), 3686-3691.

Seo, Y., Lee, W., Sorial, G., & Bishop, P. L. (2009). The application of a mulch biofilm barrier for surfactant enhanced polycyclic aromatic hydrocarbon bioremediation. *Environmental Pollution, 157*(1), 95-101.

Shanker, A. K., Cervantes, C., Loza-Tavera, H., & Avudainayagam, S. (2005). Chromium toxicity in plants. *Environment International, 31*(5), 739-753.

Shelobolina, E. S., Vrionis, H. A., Findlay, R. H., & Lovley, D. R. (2008). Geobacter uraniireducens sp. nov., isolated from subsurface sediment undergoing uranium bioremediation. *International Journal of Systematic and Evolutionary Microbiology, 58*(5), 1075-1078.

Shelobolina, E., Coppi, M., Korenevsky, A., DiDonato, L., Sullivan, S., Konishi, H., et al. (2007). Importance of c-type cytochromes for U (VI) reduction by geobacter sulfurreducens. *BMC Microbiology, 7*(1), 16.

Shen, B., Martin, L., Butt, J., Armstrong, F., Stout, C., Jensen, G., et al. (1993). Azotobacter vinelandii ferredoxin I. aspartate 15 facilitates proton transfer to the reduced [3Fe-4S] cluster. *Journal of Biological Chemistry, 268*(34), 25928-25939.

Shi, D., Wang, C., & Wang, K. (2009). Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of saccharomyces cerevisiae. *Journal of Industrial Microbiology & Biotechnology, 36*(1), 139-147.

Shi, L., Richardson, D. J., Wang, Z., Kerisit, S. N., Rosso, K. M., Zachara, J. M., et al. (2009). The roles of outer membrane cytochromes of shewanella and geobacter in extracellular electron transfer. *Environmental Microbiology Reports, 1*(4), 220-227.

Shi, L., Squier, T. C., Zachara, J. M., & Fredrickson, J. K. (2007). Respiration of metal (hydr) oxides by shewanella and geobacter: A key role for multihaem c-type cytochromes. *Molecular Microbiology, 65*(1), 12-20.

Singh, R., Paul, D., & Jain, R. K. (2006). Biofilms: Implications in bioremediation. *Trends in Microbiology, 14*(9), 389-397.

Smith, J. A., Lovley, D. R., & Tremblay, P. (2013). Outer cell surface components essential for fe (III) oxide reduction by geobacter metallireducens. *Applied and Environmental Microbiology, 79*(3), 901-907.

Snow, A. A., Andow, D. A., Gepts, P., Hallerman, E. M., Power, A., Tiedje, J. M., et al. (2005). GENETICALLY ENGINEERED ORGANISMS AND THE ENVIRONMENT: CURRENT STATUS AND RECOMMENDATIONS 1. *Ecological Applications, 15*(2), 377-404.

Sonderegger, M., & Sauer, U. (2003). Evolutionary engineering of saccharomyces cerevisiae for anaerobic growth on xylose. *Applied and Environmental Microbiology, 69*(4), 1990-1998.

Sophie, J. M., Thomas, G. L., Jordan, B., Duncan, G., Liang, S., Jim, F., et al. (2012). A functional description of CymA, an electron-transfer hub supporting anaerobic respiratory flexibility in shewanella. *Biochemical Journal, 444*(3), 465-474.

Speers, A. M., Cologgi, D. L., & Reguera, G. (2009). Anaerobic cell culture. *Current Protocols in Microbiology,* , A. 4F. 1-A. 4F. 16.

Stearns, D. M. (2000). Is chromium a trace essential metal? *Biofactors, 11*(3), 149-162.

Stemmer, W. P. (1994). Rapid evolution of a protein in vitro by DNA shuffling. *Nature, 370*(6488), 389-391.

Stephanopoulos, G. (2002). Metabolic engineering by genome shuffling. *Nature Biotechnology, 20*(7), 666-668.

Straub, K. L., & Schink, B. (2003). Evaluation of electron-shuttling compounds in microbial ferric iron reduction. *FEMS Microbiology Letters, 220*(2), 229-233.

Stroo, H. F., Major, D. W., & Gossett, J. M. (2010). Bioaugmentation for anaerobic bioremediation of chlorinated solvents. *In situ remediation of chlorinated solvent plumes* (pp. 425-454) Springer.

Sugden, K. D., & Stearns, D. M. (2000). The role of chromium(V) in the mechanism of chromate-induced oxidative DNA damage and cancer. *Journal of Environmental Pathology, Toxicology and Oncology : Official Organ of the International Society for Environmental Toxicology and Cancer, 19*(3), 215-230.

Summers, Z. M., Fogarty, H. E., Leang, C., Franks, A. E., Malvankar, N. S., & Lovley, D. R. (2010). Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. *Science, 330*(6009), 1413-1415.

Sung, Y., Fletcher, K. E., Ritalahti, K. M., Apkarian, R. P., Ramos-Hernández, N., Sanford, R. A., et al. (2006). Geobacter lovleyi sp. nov. strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium. *Applied and Environmental Microbiology, 72*(4), 2775-2782.

Tabak, H. H., Lens, P., van Hullebusch, E. D., & Dejonghe, W. (2005). Developments in bioremediation of soils and sediments polluted with metals and radionuclides–1. microbial processes and mechanisms affecting bioremediation of metal contamination and influencing metal toxicity and transport. *Reviews in Environmental Science and Bio/Technology, 4*(3), 115-156.

Tchobanoglous, G., & Burton, F. L. (1991). *Wastewater engineering treatment, disposal and reuse* McGraw-Hill, Inc.

Torres, C. I., Kato Marcus, A., & Rittmann, B. E. (2008). Proton transport inside the biofilm limits electrical current generation by anode-respiring bacteria. *Biotechnology and Bioengineering, 100*(5), 872-881.

Tremblay, P., Aklujkar, M., Leang, C., Nevin, K. P., & Lovley, D. (2012). A genetic system for geobacter metallireducens: Role of the flagellin and pilin in the reduction of fe (III) oxide. *Environmental Microbiology Reports, 4*(1), 82-88.

Tremblay, P., Summers, Z. M., Glaven, R. H., Nevin, K. P., Zengler, K., Barrett, C. L., et al. (2011). A c-type cytochrome and a transcriptional regulator responsible for enhanced extracellular electron transfer in geobacter sulfurreducens revealed by adaptive evolution. *Environmental Microbiology, 13*(1), 13-23.

Tront, J., Fortner, J., Plötze, M., Hughes, J., & Puzrin, A. (2008). Microbial fuel cell biosensor for< i> in situ</i> assessment of microbial activity. *Biosensors and Bioelectronics, 24*(4), 586-590.

Ueki, T., Leang, C., Inoue, K., & Lovley, D. R. (2012). Identification of multicomponent histidine-aspartate phosphorelay system controlling flagellar and motility gene expression in geobacter species. *Journal of Biological Chemistry, 287*(14), 10958-10966.

Van Dillewijn, P., Nojiri, H., Der Meer, V., Roelof, J., & Wood, T. K. (2009). Bioremediation, a broad perspective. *Microbial Biotechnology, 2*(2), 125-127.

van Maris, A. J., Winkler, A. A., Porro, D., van Dijken, J. P., & Pronk, J. T. (2004). Homofermentative lactate production cannot sustain anaerobic growth of engineered saccharomyces cerevisiae: Possible consequence of energy-dependent lactate export. *Applied and Environmental Microbiology, 70*(5), 2898-2905.

Vargas, M., Malvankar, N. S., Tremblay, P., Leang, C., Smith, J. A., Patel, P., et al. (2013). Aromatic amino acids required for pili conductivity and long-range extracellular electron transport in geobacter sulfurreducens. *MBio, 4*(2)

Venkata Mohan, S., Prasanna, D., Purushotham Reddy, B., & Sarma, P. (2008). Ex situ bioremediation of pyrene contaminated soil in bio-slurry phase reactor operated in periodic discontinuous batch mode: Influence of bioaugmentation. *International Biodeterioration & Biodegradation, 62*(2), 162-169.

Viamajala, S., Peyton, B. M., Apel, W. A., & Petersen, J. N. (2002). Chromate reduction in shewanellaoneidensis MR-1 is an inducible process associated with anaerobic growth. *Biotechnology Progress, 18*(2), 290-295.

Viamajala, S., Peyton, B. M., Sani, R. K., Apel, W. A., & Petersen, J. N. (2004). Toxic effects of chromium (VI) on anaerobic and aerobic growth of shewanella oneidensis MR-1. *Biotechnology Progress, 20*(1), 87-95.

Viamajala, S., Smith, W. A., Sani, R. K., Apel, W. A., Petersen, J. N., Neal, A. L., et al. (2007). Isolation and characterization of cr (VI) reducing< i> Cellulomonas</i> spp. from subsurface soils: Implications for long-term chromate reduction. *Bioresource Technology, 98*(3), 612-622.

von Canstein, H., Ogawa, J., Shimizu, S., & Lloyd, J. R. (2008). Secretion of flavins by shewanella species and their role in extracellular electron transfer. *Applied and Environmental Microbiology, 74*(3), 615-623.

Voordeckers, J. W., Kim, B., Izallalen, M., & Lovley, D. R. (2010). Role of geobacter sulfurreducens outer surface c-type cytochromes in reduction of soil humic acid and anthraquinone-2, 6-disulfonate. *Applied and Environmental Microbiology, 76*(7), 2371-2375.

Wall, J. D., & Krumholz, L. R. (2006). Uranium reduction. *Annu.Rev.Microbiol., 60*, 149-166.

Wang, Y., Li, Y., Pei, X., Yu, L., & Feng, Y. (2007). Genome-shuffling improved acid tolerance and l-lactic acid volumetric productivity in< i> lactobacillus rhamnosus</i>. *Journal of Biotechnology, 129*(3), 510-515.

Weber, F. J., Ooijkaas, L. P., Schemen, R., Hartmans, S., & De Bont, J. (1993). Adaptation of pseudomonas putida S12 to high concentrations of styrene and other organic solvents. *Applied and Environmental Microbiology, 59*(10), 3502-3504.

Wei, P., Li, Z., He, P., Lin, Y., & Jiang, N. (2008). Genome shuffling in the ethanologenic yeast candida krusei to improve acetic acid tolerance. *Biotechnology and Applied Biochemistry, 49*(2), 113-120.

Wei, W., Wu, K., Qin, Y., Xie, Z., & Zhu, X. (2001a). Intergeneric protoplast fusion between kluyveromyces and saccharomyces cerevisiae–to produce sorbitol from jerusalem artichokes. *Biotechnology Letters, 23*(10), 799-803.

Wei, W., Wu, K., Qin, Y., Xie, Z., & Zhu, X. (2001b). Intergeneric protoplast fusion between kluyveromyces and saccharomyces cerevisiae–to produce sorbitol from jerusalem artichokes. *Biotechnology Letters, 23*(10), 799-803.

Wentzel, A., Lewin, A., Cervantes, F. J., Valla, S., & Kotlar, H. K. (2013). Deep subsurface oil reservoirs as poly-extreme habitats for microbial life. A current review. *Polyextremophiles* (pp. 439-466) Springer.

Wetterhahn, K. E., Hamilton, J. W., Aiyar, J., Borges, K. M., & Floyd, R. (1989). Mechanism of chromium (VI) carcinogenesis. *Biological Trace Element Research, 21*(1), 405-411.

Wiatrowski, H., & Barkay, T. (2006). *Reduction of Mercury by Dissimilatory Metal Reducing Bacteria,*

Widdowson, M., Chapelle, F., Casey, C., & Kram, M. (2008). *Estimating Cleanup Times Associated with Combining Source-Area Remediation with Monitored Natural Attenuation,*

Wielinga, B., Mizuba, M. M., Hansel, C. M., & Fendorf, S. (2001). Iron promoted reduction of chromate by dissimilatory iron-reducing bacteria. *Environmental Science & Technology, 35*(3), 522-527.

Williams, K. H., Long, P. E., Davis, J. A., Wilkins, M. J., N'Guessan, A. L., Steefel, C. I., et al. (2011). Acetate availability and its influence on sustainable bioremediation of uranium-contaminated groundwater. *Geomicrobiology Journal, 28*(5-6), 519-539.

Williams, K. H., Wilkins, M. J., N'Guessan, A., Arey, B., Dodova, E., Dohnalkova, A., et al. (2013). Field evidence of selenium bioreduction in a uranium-contaminated aquifer. *Environmental Microbiology Reports,*

Wolf, M., Kappler, A., Jiang, J., & Meckenstock, R. U. (2009). Effects of humic substances and quinones at low concentrations on ferrihydrite reduction by geobacter metallireducens. *Environmental Science & Technology, 43*(15), 5679-5685.

Xiong, Y., Shi, L., Chen, B., Mayer, M. U., Lower, B. H., Londer, Y., et al. (2006). High-affinity binding and direct electron transfer to solid metals by the shewanella oneidensis MR-1 outer membrane c-type cytochrome OmcA. *Journal of the American Chemical Society, 128*(43), 13978-13979.

Xu, B., Jin, Z., Wang, H., Jin, Q., Jin, X., & Cen, P. (2008). Evolution of streptomyces pristinaespiralis for resistance and production of pristinamycin by genome shuffling. *Applied Microbiology and Biotechnology, 80*(2), 261-267.

Yang, C., Liu, N., Guo, X., & Qiao, C. (2006). Cloning of mpd gene from a chlorpyrifos-degrading bacterium and use of this strain in bioremediation of contaminated soil. *FEMS Microbiology Letters, 265*(1), 118-125.

Yu, L., Pei, X., Lei, T., Wang, Y., & Feng, Y. (2008). Genome shuffling enhanced l-lactic acid production by improving glucose tolerance of< i> lactobacillus rhamnosus</i>. *Journal of Biotechnology, 134*(1), 154-159.

Yun, J., Ueki, T., Miletto, M., & Lovley, D. R. (2011). Monitoring the metabolic status of geobacter species in contaminated groundwater by quantifying key metabolic proteins with geobacter-specific antibodies. *Applied and Environmental Microbiology, 77*(13), 4597-4602.

Zhang, C., Fan, F. F., & Bard, A. J. (2008). Electrochemistry of oxygen in concentrated NaOH solutions: Solubility, diffusion coefficients, and superoxide formation. *Journal of the American Chemical Society, 131*(1), 177-181.

Zhang, T., Gannon, S. M., Nevin, K. P., Franks, A. E., & Lovley, D. R. (2010). Stimulating the anaerobic degradation of aromatic hydrocarbons in contaminated sediments by providing an electrode as the electron acceptor. *Environmental Microbiology, 12*(4), 1011-1020.

Zhang, Y., & Miller, R. M. (1992). Enhanced octadecane dispersion and biodegradation by a pseudomonas rhamnolipid surfactant (biosurfactant). *Applied and Environmental Microbiology, 58*(10), 3276-3282.

Zhang, Y., Perry, K., Vinci, V. A., Powell, K., Stemmer, W. P., & del Cardayré, S. B. (2002). Genome shuffling leads to rapid phenotypic improvement in bacteria. *Nature, 415*(6872), 644-646.

Zhou, Z., Yu, M., Yang, H., Huang, K., Li, F., Yi, T., et al. (2008). FRET-based sensor for imaging chromium (III) in living cells. *Chemical Communications,* (29), 3387-3389.

Zhuang, K., Vemuri, G. N., & Mahadevan, R. (2011). Economics of membrane occupancy and respiro-fermentation. *Molecular Systems Biology, 7*(1)