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New Bioremediation Technologies to Remove Heavy Metals and Radionuclides using Fe(III)-, Sulfate- and Sulfur- Reducing Bacteria

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1. Introduction

Microbial mineral formation and dissolution converged to produce a new field of research on bacterial-metal interaction developed within the last decade, called geomicrobiology. This new field tries to elucidate the role that microbes play or have played in specific geological processes and gives information about the earliest geochemical signals of life on earth. Furthermore, an understanding of bacterial-metal interactions provides the basis of improved models of metal cycling and the environmental impact of such transformations.With the need for new and low-cost technologies to remove heavy metals and radionuclides polluting the environment, the knowledge of the mechanisms, by which microorganisms interact with metals, has been recently developed (Lloyd et al. 2002; Barton et al. 2003; Lloyd 2003).

Iron and manganese are the two most abundant reactive metals in the earth's-crust, and the origin of life is initially connected to the ability of iron to readily cycle between Fe(III) and Fe(II) states. Some of the earliest geochemical signals of life on earth are the conversion of Fe(II) dissolved in the archaeon seas to Fe(III) oxides deposits. This conversion is possibly a result of the Fe(II) oxidizing microorganisms.

Today, Fe(III) is very abundant at the earth's surface, but is very insoluble at neutral pH and so microorganisms, which require iron to support growth, have developed siderophores which are the evolutionary response to the appearance of O_2 in the atmosphere and responsible the concomitant oxidation of Fe(II) to Fe(III).

A wide variety of microorganisms is capable of dissimilatory Fe(III) reduction which is the early form of microbial respiration. These bacteria use molecular hydrogen, lactate, pyruvate or acetate as their electron donor and

Fe(III) as electron acceptor. Many of them are also able to use Mn(IV) as electron acceptor, reducing it to Mn(II).

In this first group of bacteria, the growth is coupled to the reduction of Fe(III) and Mn(IV). In the second group, some metals like selenium and arsenic, can be used by some bacteria to support growth, but the other heavy metals are toxic and lethal for the bacteria, hence they have developed detoxification strategies in which the reduction of the metal gives a less toxic element (Most toxic heavy metals are less soluble and toxic when reduced than oxidized).

The need to remediate extensive metal contamination of groundwater and soils from heavy metals and radionuclides has stimulated an increased interest to find new metalresistant microorganisms and new bioremediation processes. Indeed, laboratory microorganisms, such as *Escherichia coli*, are not good candidates to be used in bioremediation processes, as they are not adapted to heavy metals contamined environments.

The aim of this article is to provide an overview of the development of technologies, using the activity of Fe(III)-, sulfate- and sulfur- anaerobic bacteria to remove heavy metals and metalloids from ground waters and soils.

2. Microbial Reduction of Metals by Fe(III)-reducing Bacteria

Fe(III) reduction has been highly conserved during evolution (Lonergan et al. 1996). A wide diversity of microorganisms are able to reduce Fe(III) or Mn(IV) (Lloyd 2003). Nevertheless, the present chapter will focus on the *Geobacter* sp. and *Desulfuromonas* sp., included in the Geobacteraceae group. Indeed, the Geobacteraceae group is divided in two sub-groups: the *Geobacter* cluster and the *Desulforomonas* cluster (Lonergan et al. 1996).

2.1 *Geobacter*

Geobacter species are microorganisms able to colonize habitats with elevated metal concentrations. Dissimilatory Fe(III) reduction is a well-known environmental process in various environment, such as sediments, shallow aquifers and in the deep surface. A recent study (Cummings et al. 2003) has clearly shown that various phylotypes of *Geobacter* sp. could be isolated from pristine and metal-contaminated sites. The persistence of *Geobacter* species is highly important, since it provides a glimpse of its use in the bioremediation processes of heavy metal-contaminated sites. Moreover, Childers et al. (2002) demonstrated that some *Geobacter* sp. accesses Fe(III) oxides by chemotaxis. These findings pinpoint the reason why among the Fe(III)-reducing bacteria, *Geobacter* sp. are the most abundant community in sediment environments, suggesting that they could be considered a kind of natural environmental clean-up bacteria and new tools for bioremediation processes.

Various species of *Geobacter* have been isolated and characterized. In the early 1990's, Lovley and co-workers (1993a) characterized *Geobacter metallireducens*, a strict anaerobic bacterium, able to reduce various metals such as Mn(IV) or U(VI). *Desulfuromonas acetoxidans* is the closest relative of *G. metallireducens*. On the other side, *Geobacter sulfurreducens*, isolated from an hydrocarbon contaminated ditch, by Caccavo et al. (1994), was the first bacterium described that is able to couple the oxidation of hydrogen (or acetate) to Fe(III) reduction. Various heavy metals, such as Cr(VI) and more particularly Tc(VII), are reduced by *G. sulfurreducens* and *G. metallireducens* (Lloyd et al. 2000; Liu et al. 2002). More recently, *Geobacter hydrogenophilus*, *Geobacter chapellei* and *Geobacter grbiciae* (Coates et al. 2001) and *Geobacter bremensis* sp. *nov*. and *Geobacter pelophilus* sp. *nov*. (Straub and Buchholz-Cleven 2001) were also isolated.

Mechanisms of the reduction of Fe(III) and Mn(IV) have been extensively studied, using *Geobacter* species as a model (Lloyd 2003). Cytochromes are heme enzymes involved in the electron transfer chain coupled to metal reduction (Fe(III) for example). Metals, such as gold, silver, mercury and chromate, considered as electron acceptors, were reduced by *G. metallireducens* c-type cytochromes (Lovley et al. 1993a; Coates et al. 1996). Moreover, Lloyd (2003) has showed that c-type cytochromes of *G. metallireducens* transfer electrons to soluble Au(III) (Lovley et al. 1993a).

The first study, reporting the purification and characterization of a c-type cytochrome from *G. sulfurreducens*, indicated that a small molecular weight periplasmic protein (9.6 kDa) functions as an Fe(III)-reductase (Seeliger et al. 1998). However, another Fe(III)-reductase, described by Gaspard et al. (1998), is a molecular weight c-type cytochrome associated with peripheral outer membrane. Investigations by the Lovley's group (Lloyd et al. 1999c) demonstrated that the periplasmic 9.6 kDa cytochrome c was not an electron shuttle to Fe(III). The 9.6 kDa cytochrome closest relative was the three-hemic cytochrome c_7 from *Desulforomonas acetoxidans* (Seeliger et al. 1998) which is a multihemic, low potential cytochrome c homologous to the cytochrome c_3 isolated from sulfate reducing bacteria. This cytochrome was cloned and expressed in *Escherichia coli* and is able to reduce metals *in vitro* (Londer et al. 2002). Its structure was elucidated at 1.45Å (Pokkuluri et al. 2004). Other c-type cytochromes were also characterized (Magnuson et al. 2000 and 2001; Leang et al. 2003). Up to date, more than 100 c-type cytochromes could be found in the *G. sulfurreducens* genome (Methe et al. 2003). Lloyd et al. (2003) have reported the biochemical and genetic analysis of one small periplasmic c-type designated as PpcA.

Interestingly, a c_7 cytochrome of another species of *Geobacter* (*G. metallireducens*) which is highly homologous to *G. sulfurreducens*, was also purified and characterized, with an apparent molecular weight of 9.5 kDa and triheme per molecule, homologous with *D. acetoxidans* cytochrome c_7 (Afkar and Fukumori 1999).

Other proteins, such as hydrogenases, may be involved in the reduction of Tc(VII). A direct enzymatic reduction or a Fe(II)-mediated reduction of Tc(VII) by Fe(III)-reducing bacteria has been highlighted (*G. sulfurreducens*) by Lloyd et al. (2000).

While *Geobacter* is able to reduce metals and radionuclides, there have been a few reports that pinpoint their potential contributions to a bioremediation process (Lovley 1995; Lovley 1997). The scientific community is just beginning to decipher the physiology and metabolism of *Geobacter* species, and we are at the discovery stage of their potent use in the bioremediation process. Several reports indicate that the adding of electron donors *in situ* stimulate the microbial reduction by *Geobacter* community. Microbial reduction of U(VI) to insoluble U(IV) of uranium-contaminated sub-surface sediments was assayed by Finneran et al. (2002). It was found that the nitrate content of the sediments had a negative impact on the reduction of Fe(III) to Fe(II) and U(VI) to U(IV) by *G. metallireducens*, since nitrate has to be reduced first (Finneran et al. 2002). At the same time, a reduction of uranium in samples from U(VI)-contaminated aquifer sediments (Holmes et al. 2002) and from the aquifer itself (Anderson et al. 2003) amended with acetate, was clearly associated with a reduction of Fe(III) by the *Geobacter* community.

More recently, a U(VI)- and Tc(VII)-contaminated aquifer was *in situ* reduced while *Geobacter* was stimulated with electron donors, even if the site was highly nitrate concentrated (Istok et al. 2004). Members of Geobacteraceae are not able to grow at high salinities, nevertheless, a high U(VI) concentration in a saline aquifer sediments could be reduced in water by the addition of acetate (Nevin et al. 2003). The groundwater geochemistry of contaminated aquifers, amended with electron donors, was monitored using bio-markers: microbial biofilms including *Geobacter* and nitrate-reducing microorganisms (Peacock et al. 2004).

A genomic approach could be useful in the bioremediation process, since *Geobacter sulfurreducens* has been sequenced (Methe et al. 2003). A genetic system has been recently developed (Coppi et al. 2001) in which *Geobacter* could be replaced by *Ralstonia eutropha*, able to neutralize Cadmium *via* the expression of a mouse-metallo-fusion protein (Lovley and Lloyd 2000). Recombinant indigeneous soil microorganisms, expressing metallothioneins (cysteine rich proteins able to bind heavy metals), could be used in the polluted soils (Valls et al. 2000). Indeed, they described a recombinant *Ralstonia eutropha* strain able to support and adsorb high Cd^{2+} concentration in soils.

Although the important role of *Geobacter* in the geochemistry of the sub-surface environment has been clearly described (Lovley 1997), but their potential uses in *in situ* or *ex situ* bioreaction configurations have not been yet developed. Therefore, biochemical (molecular biology and genomics) and ecological approaches, leading to improving methods for using *Geobacter* as bioremediation agent, will undoubtedy make an impact in the future of the environmental biotechnology.

2.2 *Desulfuromonas*

Bacteria that are able to grow by linking the oxidation of acetate to the reduction of elemental sulfur have been known, since Pfennig and Biebl (1976) described the isolation of *D. acetoxidans*. Recently two other species, *D. palmitatis* and *D. thiophila* have also been described (Coates et al. 1995; Finster et al. 1997).

Desulfuromonas, a sulfur reducing bacterium, is strictly anaerobic, gram negative, flagellated and rod-shaped. It acquires its energy from sulfur respiration and completely oxidizes acetate with S to carbon dioxide via the citric acid cycle (Widdel and Pfennig 1991). Reduction of S produces hydrogen sulfide (H_2S) which can react with heavy metal ions to form less toxic insoluble metal sulfides (Kim et al. 2001). Furthermore, these bacteria are also able to enzymatically reduce and precipitate these heavy metals (Aubert et al. 1998a,b). Several studies, focused on the bioenergetic metabolism of sulfur reducing bacteria, have led to the characterization of various metalloproteins and in particular, multiheme low potential cytochromes (Bruschi 1994; Bruschi et al. 1997), the most abundant being the cytochrome c_7. The biological function of cytochrome c_7 is not clearly established, but it has been proposed to have a role as an electron transfer protein in the sulfur metabolism of this bacterium, acting as a terminal reductase in the metabolic pathway by directly reducing elemental sulfur to sulfide; it has also been suggested that cytochrome c_7 could be involved in the dissimilatory reduction of Fe(III) and Mn(IV) to obtain energy growth by these bacteria (Roden and Lovley 1993).

The three dimensional structure of this triheme cytochrome determined by nuclear magnetic resonance shows that the orientation of the three heme groups is similar to that of the tetrahemic cytochrome with the heme 2 lacking (Banci et al. 1996).

The three heme groups have negative redox potentials ranging from –102 to –177 mV. Electrochemistry experiments have demonstrated the direct reduction of Fe(III), Mn(IV), V(V) (Lojou et al. 1998b; Lojou and Bianco 1999) by multihemic cytochrome whereas mitochondrial c-type cytochromes did not exhibit any activity (Lojou et al. 1998a).

The interaction between Cr(VI) and cytochrome c_7 was chosen as a model for the reduction of metals by c_3-type cytochromes, as the three dimensional structure of the oxidized and the reduced states of this cytochrome have been solved using NMR studies (Banci et al. 1996). ^{1}H NMR experiments have been performed using reduced cytochrome c_7 (by a catalytic amount of hydrogenase representing the smallest amount necessary to reduce its physiological partner (Brugna et al. 1998): the c_3-type cytochrome) and various amounts of Cr(VI). Figure 1 shows a single binding site near heme IV, the heme with the highest reduction potential (Dolla et al. 1991; Assfalg et al. 2002). An electron flow involving the three hemes and the protein chain is proposed to explain the reaction which is potentially important for the construction of biosensors.

Moreover, several multihemic cytochrome c of higher molecular weight (50, 65 and 250 kDa) have been characterized by Bruschi et al. (1997), and Pereira et al. (1997) exhibiting several domains and high thermal stability (Giudici et al. 2003). The genome sequence of the bacteria, presently under study, reveals a very high number of multihemic cytochromes as observed in *Geobacter sulfurreducens* genome. Considering these similarities with the presence of multihemic domains and low potential redox, these cytochromes could be related to cytochrome c_7 and show also a metal reduction activity and could be used to select high performance metal-reductase bacteria or to develop biosensors.

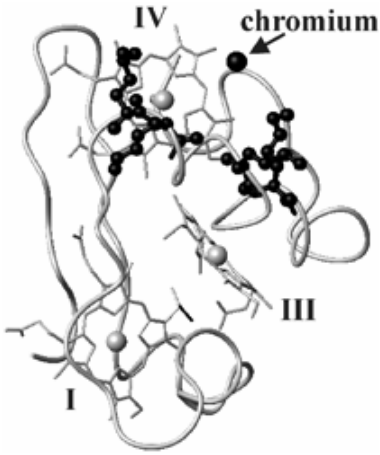

Fig. 1. The Cr(III) binding site on cytochrome c_7 from *Desulfuromonas acetoxidans*. The Cr(III) ion is shown as a black sphere, and the hemes are labeled by roman numbers (Assfalg et al. 2002)

3. Microbial Interaction with Toxic Metals by Sulfate-reducing Bacteria

In contrast to the first group of bacteria in which the metal is used as a terminal acceptor in the metabolism, sulfate-reducing bacteria (SRB) are not able to use the metal to support growth. SRB are strict anaerobic bacteria, requiring a redox potential of less than -200 mV (Postgate 1984) and are naturally present in waters and soils. These microorganisms are found in various sites contaminated with metals, metalloids and pollutants, which are lethal to other bacteria. The first isolated SRB was *Spirullum desulfuricans* (reclassified as *Desulfovibrio* included in Desulfovibrionaceae group) in 1895 (Beyerinck 1895). At the end of the 1980's, the role of SRB on the bioremediation of technetium was highlighted (Pignolet et al. 1989). Now-a-days, SRB are of increasing economic and industrial importance, since the European criteria, regarding the heavy

metal rejected in the environment, are more drastic. The ability to reduce metals to a less toxic form, associated with its precipitation, is a potentially useful process for bioremediation.

SRB are able to couple the oxidation of organic compounds or H_2 with the reduction of sulfate as electron acceptor. During this process, the dissimilatory sulfate reduction, leads to the production of H_2S which is dissimilated into the environment and can reduce heavy metals. SRB, in addition to the chemical indirect reduction due to the production of H_2S, can also reduce metals *via* enzymatic pathway involving c_3-type cytochromes (Lovley and Phillips 1992; Lovley et al. 1993a,b). *Desulfovibrio desulfuricans* can not only reduce the soluble toxic form of U(VI) to insoluble U(IV) (Lovley and Phillips 1992; Tucker et al. 1996), but also Cr(VI), Mo(VI), Se(VI) (Tucker et al. 1998), Pd (Lloyd et al. 1998) and Tc(VII) (Lloyd et al. 1999a,b). The metal-reductase activity of the c_3 cytochrome has been decribed in the case of several heavy metals, such as U(VI), Cr(VI), Fe(III) (Lovley et al. 1993b; Lovley and Phillips 1994; Lojou et al. 1998a,b; Michel et al. 2001; Elias et al. 2004), Pd (Lloyd et al. 1998) and Tc (Lloyd et al. 1999a,b). All of these recent studies emphazised a wide metal reduction activity among SRB associated with cytochrome c_3, which are periplasmic proteins. When exposed to heavy metal ions, bacteria grown in the presence of high Cr(VI) concentration accumulate precipitates of trivalent chromium at its cell surface (Fig. 2) (Goulhen et al. 2005). These findings are consistent with a direct electron transfer to the metal by cytochromes and hydrogenases, which are periplasmic proteins.

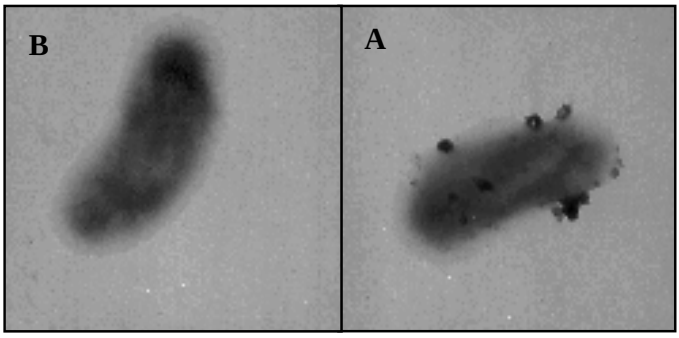

Fig. 2. Electron micrographs of unstained preparations of *D. vulgaris* Hildenborough grown in the absence of Cr(VI) (panel A) or in the presence of 250 μM Cr(VI) (panel B)

In order to develop potentially new bioremediation processes, it is required to select the most efficient and heavy metal-resistant strains. Thus, it is highly important to select strains from various contaminated sites and to evaluate their potent activity. Nevertheless, the SRB community behaviour (regarding adaptation) in contaminated sites is poorly documented and has to be evaluated to decipher the response of the SRB in changing environment. A study has recently focused on the high diversity and characterization of SRB in a groundwater uranium contaminated

site (Chang et al. 2001). *Desulfotomaculum* sp. was predominant in this site and *Desulfovibrio* sp. was isolated from a parcel exhibiting lower uranium concentrations (Chang et al. 2001). Models, able to forecast the activity of SRB regarding heavy metals, are to be developed, since, for example, copper has more inhibitory effects than zinc on SRB (Utgikar et al. 2001; Utgikar et al. 2003). Interestingly, the cultivation of *D. vulgaris* in the presence of Cu(II) and Hg(II) increases the lag phase and final biomass yield. Toxic metal adaptation appeared to be an ATP-dependent mechanism (Chang et al. 2004).

A *Desulfovibrio* strain highly resistant to copper (about 10 fold normal level) was recently isolated. The *pco* gene, well-known to play an important role in copper resistance, was present on a plasmid of that particular strain which could be used as an interesting bioremediation tool (Karnachuk et al. 2003).

Various SRB, including *Desulfovibrio* and *Desulfomicrobium* species, were evaluated regarding their enzymatic reduction of Cr(VI). Intact cells of *D. norvegicum* showed the best Cr(VI)- reducing activity (up to 500 µM Cr(VI)) compared to *D. escambiense*, D. vulgaris Hildenborough, *D. gigas*, *D. desulfuricans*, a strain named BRGM isolated from a gold mine (France) and new strains isolated from black smokers (Pacific ocean) (Michel et al. 2001; Michel et al. 2003a). The Cr(VI) acts as a stressing agent at high concentrations, leading to an increasing bacterial cells fragility, since bacteria become long filament (default in cell division) and c-type cytochromes could be found in culture supernatant (Michel et al. 2001; Bruschi et al. 2003). The effects of Cr(VI) on bioenergetic metabolism were monitored using isothermal microcolorimetry (Chardin et al. 2002). An extension of the lag growth phase and deep changes in the bacterial metabolism of lactate were observed in the presence of high Cr(VI) concentration. The growth was inhibited with a concomitant energy production, which suggests that lactate is catabolized for lowering the redox potential to maintain survival conditions for sulphate-reducing bacteria. Indeed, Cr(VI) reduction is a protective escape to keep the bacterial environment favourable (Chardin et al. 2002; Bruschi et al. 2003). Microcalorimetry could be a potent criterion to evaluate the effects of the metal concentration on bacteria and to choose the best strain needed to decontaminate a polluted environment (Bruschi et al. 2003).

As metal reduction can also be achieved enzymatically, the metal reductase activity of purified cytochromes c_3 and hydrogenases have been studied. On the basis of amino-acid sequence and three dimensional comparisons of multihemic cytochromes, characterized by bishistidinyl axial iron coordination and low redox potentials, we have proposed that all these cytochromes belong to the cytochrome c_3 superfamily and that they have a common ancestral origin (Bruschi et al. 1992; Bruschi et al. 1994; Bruschi 1994). As we have demonstrated that the all the cytochrome c_3 tested and the cytochrome c_7 have a metal reductase activity, we could propose that the other multihemic cytochromes c described in sulfate and sulfur reducing bacteria, as they possess the common tetraheme motif as building block, could have also a similar metal

reductase activity (Czjzek et al. 1996; Czjzek et al. 2002). In order to pinpoint the SRB strain demonstrating the highest metal reductase activity, Michel *et al.* (2001) compared the chromate reductase activity of various c-type cytochromes, concluding that c_3-cytochrome from *D. norvegicum* presented the highest activity (Table 1). The monohemic cytochrome c_{553} from *D. vulgaris*, characterized by a higher redox potential and mitochondrial cytochrome c, was also tested and showed no metal reductase activity. This result suggests that a negative redox potential is necessary for enzymatic reduction. The Cr(VI) reductase activity of site directed mutagenesis mutants of cytochrome c_3, named respectively H22M and H35M (the histidine residue of the sixth axial ligand heme 1 and 2 respectively has been replaced by a methionine residue), have been tested. A decrease of 15% in the chromate reductase activity is observed for mutant H22M suggesting that heme 1 is crucial for chromate reduction.

Table 1. Cr(VI) reductase activity of wild type and mutated purified cytochromes (Michel et al. 2001)

Enzymes	Cr(VI) reduction rate (µmol Cr(VI)/min/ µmol enzyme)
Cytochrome c_3 (*Desulfovibrio vulgaris* Hildenborough)	5.08 ± 0.23
Cytochrome c_3 (*Desulfomicrobium norvegicum*)	9.60 ± 0.76
Cytochrome c_7 (*Desulfuromonas acetoxidans*)	5.07 ± 0.23
Cytochrome c_{553} (*Desulfovibrio vulgaris* Hildenborough)	No activity
Cytochrome H35M (*Desulfovibrio. vulgaris* Hildenborough)	5.20 ± 0.25
Cytochrome H22M (*Desulfovibrio vulgaris* Hildenborough)	4.43 ± 0. 3

To test the involvement of the cytochrome c_3 in the *in vivo* U(VI) reduction, a cytochrome c_3 mutant has shown one half of the rate of reduction (Payne et al. 2002). In addition to polyhemic cytochromes, low redox proteins, present in the periplasmic space of SRB, exhibited a metal reductase activity. Lloyd and coworkers (1999a,b) indicated that the Tc(VII)-reductase activity of *D. desulfuricans* was associated with a periplasmic hydrogenase. More recently, it was reported that Tc(VII) could be reduced by the [NiFe] hydrogenase alone or acting with c_3-type cytochrome of *D. fructosovorans* (De Luca et al. 2001). Cr(VI) could be reduced by [Fe], [NiFe], and [NiFeSe] hydrogenases (Michel et al. 2001; Chardin et al. 2003). The highest Cr(VI) rate was observed for purified [Fe] hydrogenase from DvH compared to [Ni-Fe-Se] hydrogenase from *D. norvegicum* (Michel et al. 2001). Moreover, a chromate or oxidative stress applied on DvH cells leads to an overexpression of the periplasmic [Fe] hydrogenase (Fournier et al. 2004).

To summurize, as listed in Table 2, the most frequently reported proteins involved in metal reduction are cytochromes and hydrogenases isolated from Fe(III)-, sulfur- and sulfate- reducing bacteria.

Table 2. c-cytochromes and hydrogenases from Fe(III)-, sulfur- and sulfate- reducing bacteria involved in metal reduction

Organism	Protein	Metal	Reference
G. metallireducens GS-15	c-cytochromes	Fe(III), Au (III), Ag (I), Hg (II), Cr (VI)	Lovley et al. (1993a)
G. metallireducens H-2	c-cytochromes	Fe(III), Cr(VI), Au(III), Ag(I),Hg(II), W(VI), U(VI), V(V), Mo(VI)	Coates et al. (1996)
G. metallireducens 172	c-cytochromes	Fe(III), Cr(VI), Au(III), Ag(I),Hg(II), V(V)	Coates et al. (1996)
G. sulfurreducens	c-cytochrome c-cytochrome (FerA) c-cytochrome (OmcB) cytochrome c_7 (PpcA) hydrogenase	Fe(III) Fe(III) Fe(III) Fe(III), U(VI) Tc(VII)	Gaspard et al. (1998) Magnuson et al. (2000; 2001) Leang et al. (2003) Lloyd et al. (2003) Lloyd et al. (2000)
D. vulgaris Hildenborough	cytochrome c_3 cytochrome c_3 cytochrome c_3 [Fe] hydrogenase	Cr(VI) Fe(III) U(VI) Cr(VI)	Lovley and Philips (1994), Michel et al. (2001) Lojou et al. (1998a,b) Lovley et al. (1993b) Michel et al. (2001)
D. acetoxidans	cytochrome c_7 c-cytochrome cytochrome c_7	Fe(III), Cr(VI), Mn(IV), V(V) Mn(IV), Fe(III) Cr(VI)	Lojou et al. (1998a,b) Roden and Lovley (1993) Michel et al. (2001)
D. fructosovorans	[Fe] hydrogenase cytochrome c_3 [NiFe] hydrogenase	Tc(VII) Tc(VII) Cr(VI)	De Luca et al. (2001) De Luca et al. (2001) Bruschi (unpublished data)
D. desulfuricans	Hydrogenase Hydrogenase	Tc(VII) Pd(II)	Lloyd et al. (1999a) Lloyd et al. (1998)
D. gigas	cytochrome c_3	Fe(III)	Lojou et al. (1998b)
D. norvegicum	[NiFeSe] hydrogenase cytochrome c_3 cytochrome c_3	Cr(VI) Cr(VI) Fe(III)	Michel et al. (2001) Michel et al. (2001) Lojou et al. (1998a,b)

These redox proteins are not acting as terminal electron acceptors for heavy metals. For instance, *D. vulgaris* Hildenborough can reduce Cr(VI) using several enzymes involved in the electron chain transfer, but the reduction of this metal does not support growth (Chardin et al. 2002). Researches, elucidating the mechanisms of bacterial metal reduction, are all the most important, as they will improve the bacterial use conditions during bioremediation processes. Indeed, novel bioremediation approaches could emerge to treat contaminated environments.

4. Development of Biosensors

Over the last one decade, biosensors have been developed to be used in wide applications. Biosensors offer the potential to measure quickly, cheaply and accurately the contamination degree of environmental sites. There are two major markets for biosensors. The first one is concerned with clinical and health care fields and needs miniaturization and the second one is for environmental purposes for detection and control. Analysis methods could be largely refined with the development of biocaptors, since this kind of approach allows the detection and the direct quantification of a chemical compound in complex media.

Various studies have reported the construction of biosensors, using genetically engineered bacteria (reviewed in D'Souza 2001). More specifically, biosensors for the detection of heavy metals, have been developed (Verma and Singh 2005). These biosensors have used two distinct methods to detect heavy metals ions: (i) proteins (antibodies, enzymes) or (ii) whole cells (genetically modified or not). Various sensors were designed to evaluate the heavy metals concentration, for example, nickel and copper (Forzani et al. 2005), mercury (Hobman et al. 2000), cadmium (Blake et al. 2001), arsenic, iron and lead (Radhika et al. 2005).

We would like to present more specifically biosensors using proteins that exhibit a metal reductase activity. As we have previously demonstrated that the all the cytochrome c_3 tested and the cytochrome c_7 have a metal reductase activity, we could propose that the other multihemic cytochromes c described in sulfate and sulfur reducing bacteria, could have also a similar metal reductase activity. It is to be noticed that all these cytochromes have distinct and low redox potentials hemes and show remarkable properties of thermostability until 125°C for some of them (Florens et al. 1995). Recent studies have demonstrated that hydrogenases and other redox proteins with negative redox potentials (like ferredoxins) can also reduce metals. However, hydrogenases are proteins that are usually sensitive to oxygen and are produced in low amount by bacteria. On the contrary, cytochrome c_3 is stable towards many physico-chemical factors, such as pH, temperature, oxygen, salt, ageing (Bianco et al. 1986; Florens et al. 1995) and are still stable and active once immobilised at the electrode using

various immobilisation techniques. These enzymes are naturally produced at high levels by sulfate reducing bacteria and are also overproduced in specific hosts. Indeed, cytochrome c_3 are better candidates for the construction of biosensors.

Different procedures used for constructing protein/enzyme-modified electrodes have been developed, in particular, adsorption on an electrode surface, covalent attachment, imprisonment in a layer by layer assembly and entrapment within cast films or a dialysis membrane. The performances of such modified electrodes with electroactive proteins or enzymes, attached to their active surface, have been compared (Bianco 2002).

A first approach to test the ability of the cytochromes c_3 family to achieve the remediation of metal contaminated water has been attempted in the case of iron-containing solution (Lojou et al. 1998b). In this study, the ability of poly ester-sulfonic acid ionomer (Eastman AQ-29D), cast on the electrode surface, is able to immobilize the cytochrome.A catalytic current is detected from cyclic voltammetry experiments where Fe(III) serves as the substrate to oxidize the cytochrome.

The membrane electrode technology offers an alternative to the entrapment of cytochromes within a polymer film. This technology has been extensively used in the case of other metals well known for their high toxicity, in particular Cr(VI), V(V) and U(VI) (Lojou et al. 1998a; Lojou and Bianco 1999). A rapid survey of important parameters, such as pH, metal concentration, nature and concentration of the supporting electrolyte, provides significant advantages. Most of the metals, in sediments and soils, are in the form of various insoluble oxides. In this approach, metal oxide and cytochrome c_7 were deposited and entrapped “within” the membrane electrode (Lojou and Bianco 1999).

An amperometric cytochrome c_3-based biosensor was constructed for chromate determination (Michel et al. 2003b). Several processes of enzyme mobilisation have been tested and the best results were obtained with dialysis membranes which allowed the determination of Cr(VI) from 0.2 to 6.84 mg/L with a small amount of cytochrome c_3 (372 ng of protein) required to construct the biosensor.

5. Development of Bioreactors

A number of bio-processes, based on the activity of sulfate and metal reducing bacteria to prevent heavy metal and metalloid pollution from ground waters and soils, have been developed. The objectives of these studies are to obtain improved biological tools and to develop low-cost effective and reliable technological alternatives for bioremediation.

Chemical treatments for the removal of heavy metals from contaminated materials are chemical extraction with acids and/or chelating agents for soil treatment and precipitation for water cleaning. In industries, the metals,

contained in acid-drainage waters, are most of the time precipitated using lime. Such treatments are expensive, and lead to a large quantity formation of metal-hydroxides (Zinck 1997).

Bioremediation processes are divided in two main groups: one group exploits the enzymatic metal reductase activity of the bacteria (direct reduction) and the second group involves the use of hydrogen sulfide, biologically produced to reduce and precipitate metals (indirect reduction).

The metal precipitation, using H_2S produced by sulfate-reducing bacteria, has been proposed in the '80s (Whang et al. 1982). These kind of approaches lead to the selective metal precipitation, such as copper or zinc, sulfate and acidity removal (Hammack et al. 1993; Foucher et al. 2001). The indirect metal reduction by biologically produced H_2S has already been exploited up to industrial scale, but important innovation can be introduced by improving the technical and economical benefit of currently available configurations.

Various technologies for *in situ* clean-up are available. The direct reduction of the metals would be applied to ground water, using bioreactors (pump and treat) and could be applied to soils after excavation, pulping or heaping and inoculation. These techniques are very expensive and are characterized by low metal extraction efficiencies. The concept of *in situ* zones and bio-barriers, using metal reducing bacteria, is an alternative to pump and treat strategies and a novel application of indirect reduction. The installation of sub-surface zones, where the bacterial growth will be induced by injection of substrates, could be a low cost solution. The migrating metals would be intercepted and immobilized by precipitation with biologically produced H_2S. The capacity of the soils and sediments together with the biofilms to adsorb, filter out and retain inorganic precipitates would be exploited.

Studies on pure cultures are necessary to understand specific mechanisms of metal reduction. However, application in bioremediation could be done by consortia of different microorganisms, containing mainly sulfate reducing bacteria obtained from contaminated soils (White et al. 1997; Vainstein et al. 2003).

Studies, for *in situ* bioremediation of uranium contaminated sites, have been conducted and shown that the microbial community involved contained *Desulfosphorosinus* spp. and *Clostridium* spp. (Suzuki et al. 2003). U(VI) reduction, in the presence of various sulfate concentration, have been proposed by Spear et al. (2000) in order to reach optimal conditions in a bioremediation process. Moreover, treatment of other metals, using anaerobic bioreactors with SRB community culture, has been described, as for example, the bioremediation of (i) phosphogypsum, waste products from fertilizers industries (Rzeczycka et al. 2001; Karnachuk et al. 2002), since the nitrate concentration is not high (Kowalski et al. 2002), or (ii) lead wastes, from car batteries, to PbS (communly named Galena) (Weijma et al. 2002). In the same manner, the reduction of chromate has been described by an enrichment consortium and an isolate of marine sulfate reducing bacteria (Cheung et al. 2003). Pilot plants developed by

Shell research Ltd. and Budelco BV, using an undefined consortium of SRB, have been used successfully to remove Zn and sulfate (White et al. 1997). Here, the metals were precipitated as sulfides. Acetate, produced by sulfate-reducing bacteria, was removed by methanogenic bacteria present in the consortium. This approach was scaled-up and is able to treat 7,000 m^3 per day. Indeed, research on biological approaches of the metal precipitation/immobilisation in contaminated environments are necessary to find out new remediation approaches.

6. Conclusion

The importance of microbial metal reduction has been recently highlighted and studies on several microorganisms, which may serve as models, have been conducted. The use of Fe(III)-, sulfur- and sulfate-reducing bacteria provides challenges in the reduction of metals and radionuclides. Recent advances have been made and thanks to the discovery of new bacteria isolated from contamined sites or extremophilic environments, providing new potent tools in bioremediation processes since the chemistry and biology of polluted sites largely influence the bioremediation method to use. Reduction mechanisms of metals and radionuclides using of Fe(III)-, sulfur- and sulfate-reducing microorganisms, are at the discovery stage. Very little information on the enzymatic metal reduction in natural environments is available. Further studies on the biochemistry and microbial ecology of metal reduction would enhance our understanding of the factors controlling the rate and extent of biotechnological processes. The development of new techniques, such as genomic and proteomic approaches, and the availability of environmentally relevant bacteria annotated genome sequence, promises us undoubtedly significant advances in the environmental technology and more specifically in the understanding of the precise mechanims of bacteria-metal interactions *in situ*.

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