BIOREMEDIATION OF HEAVY METAL POLLUTION EXPLOITING CONSTITUENTS, METABOLITES AND METABOLIC PATHWAYS OF LIVINGS. A REVIEW

Pavel KOTRBA[a1,b] and Tomáš RUML[a2,*]

[a] *Department of Biochemistry and Microbiology, Prague Institute of Chemical Technology, Technická 3, 166 28 Prague, Czech Republic; e-mail:* [1] *pavel.kotrba@vscht.cz,* [2] *tomas.ruml@vscht.cz*

[b] *Research Institute of Innovative Technology for the Earth, 9-2 Kizugawadai, Kizu-cho, Soraku-gun, Kyoto 619-0292, Japan; e-mail: pavelk@rite.or.jp*

Received May 22, 2000
Accepted July 31, 2000

Removal of heavy metals from the soil and water or their remediation from the waste streams "at source" has been a long-term challenge. During the recent era of environmental protection, the use of microorganisms for the recovery of metals from waste streams as well as employment of plants for landfill applications has generated growing attention. Many studies have demonstrated that both prokaryotes and eukaryotes have the ability to remove metals from contaminated water or waste streams. They sequester metals from soils and sediments or solubilize them to aid their extraction. The proposed microbial processes for bioremediation of toxic metals and radionuclides from waste streams employ living cells and non-living biomass or biopolymers as biosorbents. Microbial biotransformation of metals or metalloids results in an alteration of their oxidation state or in their alkylation and subsequent precipitation or volatilization. Specific metabolic pathways leading to precipitation of heavy metals as metal sulfides, phosphates or carbonates possess significance for possible biotechnology application. Moreover, the possibility of altering the properties of living species used in heavy metal remediation or constructing chimeric organisms possessing desirable features using genetic engineering is now under study in many laboratories. The encouraging evidence as to the usefulness of living organisms and their constituents as well as metabolic pathways for the remediation of metal contamination is reviewed here. A review with 243 references.

Key words: Heavy metals; Bioremediation; Metallothioneins; Metalloproteins; Phytochelatins; Metal chelates, Biosorption; Bioprecipitation; Biotransformation.

During the last decades an increasing attention has been paid to hazards arising from the contamination of the environment with heavy metals. Once released into the environment, metallic species tend to persist indefinitely, circulating in the ecosystems and eventually accumulating through the food chain. This represents a problem of great economic and public-health significance. Consequently, environmental awareness is growing in the public and industry, and environmental legislation becomes progressively stricter, leading to a need for effective and low-cost technologies. Lower cost and higher efficiency at low metal concentrations make biotechnological processes more attractive in comparison with physico-chemical methods for heavy metal removal. Microbial remediation of metals, however, is still rather a research issue with few large-scale applications.

1. METALS AND LIVINGS

Metals and metalloids include all the elements except H, B, C, N, P, O, S, halogens, and the noble gases. Metalloids are only Si, Ge, As, Sb, Se and Te. Alkali metals and alkaline earth metals in groups Ia and IIa, together with Al and metals of groups IIIb, IVb, and Vb comprise the non-transition metals. Transition elements with incompletely filled d or f orbitals are in periods 4, 5, and 6 of the Periodic Table[1] with the exception of the IIb group metals (Zn, Cd, Hg) that are classified as transition metals. The lanthanides and actinides *i.e.* rare earth elements constitute inner transition series and occur in a wide variety of oxidation states (from +1 up to the group number) as the atoms are stabilized by various irregular electron configurations. Metal ions are classified as type-A, type-B and transition metal cations[2]. Type-A metal cations possess the electron configuration of an inert gas while type-B metal cations contain 10 outer shell electrons corresponding to Ni^0, Pd^0, and Pt^0. The transition metal cations have 1 to 9 (or 13) outer shell electrons (refs[2,3], Table I).

TABLE I
Classification of metal cations (ref.[2]) and ligands according to the HSAB scheme (refs[9,30])

Metal cations		
Type-A metals	Transition metals	Type-B metals
Li^+, Na^+, K^+, Be^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Al^{3+}, Sc^{2+}, La^{2+}, Ti^{4+}, Zr^{4+}, Th^{4+}	V^{2+}, Cr^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Ti^{3+}, V^{3+}, Cr^{3+}, Mn^{3+}, Fe^{3+}, Co^{3+}	Cu^+, Ag^+, Au^+, Tl^+, Ga^+, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Sn^{2+}, Tl^{3+}, Au^{3+}, In^{3+}, Bi^{3+}
Hard acids	Intermediate	Soft acids
All type-A cations, Cr^{3+}, Mn^{3+}, Fe^{3+}, Co^{3+}, UO^{2+}, VO^{2+}	All divalent transition metal cations, Zn^{2+}, Pb^{2+}, Bi^{3+}	All Type-B cations, except Zn^{2+}, Pb^{2+}, Bi^{3+}
Ligands		
Hard bases	Intermediate	Soft bases
H_2O, OH, PO_4^{3-}, ROH, R_2O, R-NH_2	aniline, pyridine, SO_3^{2-}, N_2, N_3^-, NO_3^-	R_2S, RSH, SCN, $S_2O_3^{2-}$, CN^-, CO, $CH_2{=}CH_2$

From a biological point of view, metals and metal ions can be sorted according their environmental impact or toxicity. The 65 metallic elements of densities greater than 5 g cm^{-3} are called "heavy" and exert toxic effect on the livings[3,4]. However, the toxicity of a particular metal ion depends on the organism, the metal concentration, and its distribution in the environment and subsequently in the body fluids and/or tissues. The heavy metal concentration in the organism is generally proportional to the environmental level of the metal[5]. Thus the concentration of a given heavy metal ion in the organism should be clearly defined prior to evaluating its toxicity impact by the *via*bility expressed as the ability to survive and reproduce. The classification as essential and inessential metals[3] is based on their toxicity at very low concentrations (the essential ones) or benefic to the organism at adequate levels. Despite these general rules, some microorganisms can sustain the replacement of an essential metal ion by the usually non-biological one (*e.g.* K^+ for Cs^+ (refs[6,7])).

At least one-third of the known enzymes requires metal ions for activity[8]. For example, iron is present in catalases, oxidases, cytochromes, and other proteins as well as in hemoglobin and myoglobin. Cobalt is a component of vitamin B_{12}, and of several enzymes such as methionine synthetase, ribonucleotide reductase, and methyl malonyl CoA mutase. Molybdenum is required by nitrogenase, nitrate reductase, and xanthine oxidase. Manganese is required for DNA replication (referenced in[9]). Nickel is a component of, *e.g.*, Ni-Fe hydrogenase[10], and urease[11]. Copper plays a catalytic role in nitrite reductase[12] and many oxidases, *e.g.*, cytochrome C oxidases[13] and ascorbate oxidase[14]. Magnesium is required for enolase activity, phosphate transfer enzymes (referenced in[9]), DNA polymerase[15] as well as for the DNase activity[16]. Mg^{2+} is also a component of the cell wall of the Gram-negative bacteria playing a critical role in maintaining the integrity of the cell envelope[9,17]. Zinc is necessary for the function of alkaline phosphatase, glyoxalase[9], alcohol dehydrogenase[18], and many other metalloproteins, including zinc-finger peptides, transcription factors known to regulate the activity of many genes[19]. Calcium is a major metallic component of the cell wall of Gram-positive bacilli and their spores[17]. It is also a component of the mineral matrix of bones and teeth[20] and plays a role as signal messenger in mammals, plants[21], and prokaryotes[22]. Sodium and potassium ions play a role in establishing the membrane potential. These ions, therefore, mediate nervous signal transmission in mammals[21]and also create the "ion motive force" that is used for ATP synthesis in some prokaryotes[23,24].

Contrasting with the above examples, many other metal ions do not possess any essential biological functions, *e.g.*, lead, aluminum, cadmium, tin, and mercury, but they can still be accumulated[5,25] and bound to many proteins and other cellular components or compartments[9,26–28]. An excess of either essential or inessential metal ions leading to their binding to compounds of biological significance may result in various clinical syndromes[28]. Wilson's disease (caused by an excess of copper), thalassaemia (caused by an excess of iron) or "itai-itai" diseases (due to cadmium contamination of the food) represent examples of such syndromes.

It is conceivable that the exposure of a given organism to an excess of heavy metals leads to an imbalance of the natural equilibrium and thus to more or less obvious changes in the stability of a given biotope[5]. Extensive pollution of the environment by toxic metals and radionuclides arises mainly from antropogenic activities (Table II). Natural processes such as volcanic activities are quite poor contributors[29].

1.1. Metal Binding Biomolecules

It has been observed many times in various peptides, proteins and other biological materials that alkali and alkali earth metal ions bind most effectively to oxygen as electron donor, while transition metals bind equally well to nitrogen and oxygen[9]. The mutual affinity depends on the

TABLE II
Anthropogenic sources of toxic metals and metalloids (ref.[29]) in tons per year

Metal	Fuel, power and metallurgy	Agriculture	Manufacturing	Waste disposal
Arsenic	45.952	7.730	7.550	13.635
Cadmium	16.444	3.666	2.450	25.466
Chromium	344.853	92.680	50.610	81.629
Copper	26.953	411	33.740	68.875
Mercury	6.879	5.245	1.145	2.918
Nickel	239.901	60.246	7.440	60.969
Lead	325.884	192	9.300	58.570
Selenium	70.355	6.566	4.250	6.252
Zinc	162.871	824.935	85.015	168.815

acid-basic properties of both electron donor atoms and the metal ions. The metal can be classified as a Lewis acid as it tends to attain enough electrons to reach an inert state, whereas the ligand is a Lewis base as it is capable to provide electron pairs to be shared with the metal ion. Ligand–metal binding relies on the formation of coordination bonds. The propensity of a particular metal ion to bind to a ligand may be predicted on the basis of the hard and soft acids and bases (HSAB) theory[30]. The terms soft and hard (and intermediate) reflect the degree of mobility or polarizability of the electrons of an element (Table I). Thus hard acid is characterized by a small size, low polarizability, high electropositivity, and a large positive charge or oxidation state. It has few not easily excitable outer electrons and it usually forms ionic or electrostatic bonds. Hard bases tend to have low polarizability, high electronegativity, and large negative charges. They are small, have high energy and inaccessible vacant orbitals. They usually form ionic or electrostatic bonds. Soft acids and bases have properties opposite to those of their "hard" counterparts and tend to form a stable coordination represented by a σ and π bonds of the ligand that is a σ-donor and π-acceptor (the negative charge distribution remains unchanged[31]). The exceptional transition cations (Zn^{2+}, Cd^{2+}, and $Hg^{+,2+}$) usually cannot form the π interaction with the ligand as a consequence of their d^{10} state[31]. The rule was established that hard acids tend to form strong bonds with hard bases and soft acids with soft bases[3,9,30]. In general, soft acids (metals) prefer phosphorus to nitrogen and sulfur to oxygen as ligand atoms. The opposite is true for hard acids (Table I). However, in a mixture of hard and soft metal ions, the latter may show a higher affinity for hard ligands[3]. This rather exceptional pattern is of great importance especially for the biosorption of soft (heavy) metals. It could be explained by a rapid ligand exchange with the oxygen of water that is characteristic of hard metals[3,9,30]. Very soft acids and bases are often poisonous because of their very strong binding to the corresponding bases and acids, respectively (referenced in[9]). For example, $Hg^{+,\,2+}$ and Cd^{2+} rank among the most toxic metal ions as they strongly bind to the sulfhydryl groups of peptides and proteins inhibiting their biological function by replacing the essential (biologicaly active) metals or blocking the reactive amino acid residues. Cyanides, trivalent arsenic compounds and/or carbon monoxide may form strong coordination bonds with the metals of metalloproteins (and metal containing cofactors). This reaction inhibits the biological function of the metal by blocking orbitals of biological importance and it may even result in the removal of metals from metalloproteins[9].

1.2. Metal Chelates

Metal chelates are formed when the metal binding molecule forms coordinate bonds with the metal involving more than one pair of shared electrons (multidentate ligand), thus creating a ring structure[9,31]. The coordination number of a metal corresponds to the maximum number of coordination bonds, it is frequently 4 and 6 and less often 2 and 8. A chelate complex may be bidentate, tridentate, *etc.*, depending on the number of chelate rings in the structure. Individual stabilities of particular coordination bonds combine in synergistic manner. The well-known Irving–Williams series attributes the stability of a chelate–metal complex to the atomic number of a metal ion of the same oxidation state[9,32]. The Irving–Williams order of stability Mn < Fe < Co < Ni < Cu > Zn (with Cu being the most strongly bound) corresponds to many experimental data obtained studying (nonproteinous) biopolymer–metal interactions and well describes the reaction of transition metals with a multidentate ligand[9]. Other parameters, such as steric hindrance and the character of the ligand (see above) would also significantly affect the stability of a particular complex.

2. BIOREMEDIATION OF METAL(LOID) POLLUTION

The bulk of studies on the use of living materials for environmental restoration have focused primarily on microbial degradation of organic compounds. However, the potential of the microbial biomass as a way to remove [heavy] metal ions and metalloids from waste effluents and soils has been recognized early and it is systematically reported since the middle 80's. A number of experimental and theoretical studies have been the subject of several excellent reviews: The biological treatment of effluents or metal loaded areas (soils) is often an economically attractive method of a great commercial potential as compared to physico-chemical methods[33,34]. Depending on the nature of contaminated matrix two principal treatments are used. To avoid the undesired natural mobilization of toxic metal(loid)s and their subsequent entry into the food chain[5], metals immobilized in soil, silt, or solid waste need first to be solubilized to transfer the metal to a water phase which is then collected and further treated. This may be achieved by microbial leaching[35–37] or by leaching with metallophores[36]. Metal(loid)s can then be removed from liquid streams through sequestration by various sorbents of biological origin[27,37–41]. In addition, the bioconversion of mobile metal(loid) species in both liquid or solid matrixes

into less soluble forms or instead into volatile forms that will escape to air represents two other alternative approaches[33,35,40,41]. Nowadays an increasing number of reports has also been devoted to the phytoremediation of metal(loid) contaminated soils[34,42,43].

Outlined here are some of biological processes (either in use, or under study at present) that are of importance for the restoration of the metal(loid) contaminated environment. Some specific desirable features of livings that may aid the bioremediation of metal pollution are described and discussed. Special emphasis is placed on the contribution and promising exploitation of the microbial biomass for metal recovery from liquid effluents.

2.1. Metal Binding Biomolecules for the Bioremediation of Metals

The majority of microbial, algal, and plant cell wall material, such as extracellular and capsular polysacharides (cellulose, chitin, chitosan, alginate, pectine, fucoidan, carrageenan, peptidoglycans, glycoproteins, lipopolysacharides, teichoic acid, teichuronic acid), lipopolysacharides (emulsan) and low-molecular weight organic acids (citrate, oxalate acid, phytate) bear oxygen (of hydroxy, carboxy, sulfate, phosphate, and hydroxamate functionalities) and nitrogen (mostly acetamido and amino groups) as major components of metal-binding ligands. In addition, some metal binding compounds such as biopigments (flavins, flavonoids, polyhydroxyantraquinones, tannin, melanin) are produced by microbes in response to various stress agents (*e.g.*, following metal exposure; referenced in[9,27,38,39]). Metal binding by such compounds is fortuitous but of great significance for metal decontamination as it represents the first barrier against a variety of metallic species. Moreover, these ligands are the predominant metal binding sites when dead biomass is used as a biosorbent (see below).

Virtually all amino acids, peptides, and proteins are capable of binding metal ions at least *via* carboxyl and α-amino groups or *via* nitrogen and oxygen atoms of the peptide bond. However, the metal binding centers in peptides and proteins are mainly contributed by the side chains of non-hydrophobic amino acid (thoroughly reviewed in ref.[44]). In a comprehensive study, Rulíšek and Vondrášek[26] (screening the metal binding sites of approximatelly 100 metalloproteins and 3 000 smaller transition metal complexes) scored the preferred coordination geometries of Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+} in metalloproteins. From this analysis it was concluded that the preferred arrangement is octahedral for Co^{2+} and Ni^{2+}, tetrahedral for Zn^{2+}, square planar for Cu^{2+}, and linear for Hg^{2+}, whereas

Cd^{2+} tends to bind in both tetrahedral and octahedral arrangements. As shown below with metallothioneins, metalloproteins might play an important role in the bioremediation of heavy metal pollution. A metal-specific or otherwise improved bioprocess for metal removal from the environment has been developed through metal binding site engineering so to alter metal ion selectivity[45], an approach that has been recently employed to improve the performance of cytochrome P450 in organics degradation[46].

2.1.1. Metallothioneins and Related Peptides

In many organisms, poisonous levels of free heavy metal ions are detoxified *via* sequestration by intracellular ligands such as metallothioneins[47–49]. Margoshes and Vallee[48] first used the name metallothionein (MT) for a cadmium-binding protein from mammalian kidney. Similar proteins were subsequently isolated from other sources and were subdivided into distinct classes on the basis of structural similarities (Fig. 1). Peptides synthesized in a template independent manner and made up of γ-Glu-Cys repetitions (Fig. 3) are referred as phytochelatins (PCs) and *iso*-phytochelatins (*iso*-PCs)[57–59] and are considered as a very special group of MTs. In addition, proteins sharing similarity with classes I and II MTs in both primary structure and predicted function – metallothionein-like (MT-like) proteins have recently been identified in a variety of plants[59,60]. According to a classic definition, MT-like proteins should rather be designated as peptides, because their relative molecular weights are lower than 10 kDa (see below). However, as they are commonly referred to MT or MT-like proteins in literature, the same nomenclature will be kept here.

2.1.1.1. Class I and II Metallothioneins

Class-I MTs are defined as polypeptides whose primary structure is related to mammalian MTs, while those of class II display none or only a very distant sequence similarity to mammalian MTs (ref.[49]). However, Cys-X-Cys (where X is any amino acid residue other than Cys) and Cys-Cys sequence motifs are characteristic of and invariant in both class I and class II MTs (Fig. 1).

2.1.1.1.1. Class I Metallothioneins – Mammalian MTs

Although class I metallothioneins have been isolated from organisms other than mammals[49], the most thoroughly studied mammalian MTs are discussed here. Mammalian MTs are intracellular peptides of 61 or 62 amino

MT of class I: Human, *Homo sapiens sapiens* (MT-1A)[50]

```
MDPNCSCATGGSCTCTGSCKCKECKCTSCKKSCCSCCPMS.CAKCAQGCICKGASEKCSCCA
```

Mouse, *Mus musculus*[51]

```
MDPNCSCSTGGSCTCTSSCACKNCKCTSCKKSCCSCCPVSGCSKCAQGCVCKGAADKCTCCA
     * *      * *   * *   * * *   ** * *     * *    * *       * **
```

MT of class II: Yeast, *Saccharomyces cerevisiae* (CUP1)[52]

```
MFSELINFQNEGHCQCQCGSCKNNEQCQKSCSCPTGCNSDDKCPCGNKSEETKKSCCSGK
                * * * *      * * * *      * *           **
```

Wheat, *Tricium aestivum* (E_C)[53]

```
.MGCNDKCGCAVPCPGGTGCRCTSARSDAAAGEHTTCGCGEHCGCNPCACGREGTPSGRANRRA..NCSCGAACNCASCGSTTA
    *   * *   *      * *                 * *   * *  * *                    * *   * * *
```

MT-like; type 1: Pea, *Pisum sativum* ($PsMT_A$)[54]

```
....MSGCGCGSSCNCGDSCKCNKRSSGLSYS.EMETTETVILGVGPA.KIQFEGAEMSAASE.DGGCKCGDNCTC.DPCNCK
```

Arabidopsis thaliana (MT1a)[55]

```
. MADSNCGCGSSCKCGDSCSCEKNY.....................................NKECD.NCSCGSNCSCGSNCNC
        * *   * *   * *                                      *  * * *  * *   * *
```

MT-like; type 2: *Arabidopsis thaliana* (MT2a)[55]

```
MSCCGGNCGCGSGCKCGNGCGGCKMYPDLGFSGETTTTETFVLGVAPAMKNQYEASGESNNAESD.ACKCGSDCKC.DPCTCK
  * *    * *   * *   *   *                                    * *   * *    *
```

MT-like; odd: Douglas-fir, *Pseudothsuga menziesii*[56]

```
MSSDGKDCGCADPTQCDKKGNSLGVEMVETSYDYNMNMSFGFEYEMETVA.....................AEN.GCKSGASSKYSN..RCM
         * *      *                                                  *           *
```

FIG.1

Amino acid sequences and classification of metallothioneins (MTs) and MT-like proteins; dots are included for optimum alignment of homologous sequences; asterisks indicate the distribution cysteines. The Lys(30)-Lys(31) bridge separating the N-terminal β-domain and the C-terminal α-domain of class I MTs is underlined. The first eight amino acids that are cleaved from CUP1 after translation are written in italic. For details on the MTs of class III (phytochelatins), see Fig. 3

acid residues lacking aromatic amino acids and histidine. Their molecular mass is 6–7 kDa. Mammalian tissues usually contain two major fractions, *i.e.* MT-1 and MT-2, differing at neutral pH by a single negative charge. Within these fractions, isoforms specified by letters as MT-1a, MT-1b could be resolved by high-performance liquid chromatography[49].

The expression of mammalian MTs is induced by an excess of heavy metal ions such as Cd^{2+} or Cu^{2+} (refs[49,61]). However, certain hormones, cytokines, growth factors, tumor inducers and chemical and physical stress may also induce MT expression[49,61]. This indicates that MTs play more than a simply protective role. For example, the fact that DNA damage caused by oxidative stress is reduced in the presence of MTs but it is enhanced when MTs expression is suppressed[62] supports such a protective ability of the sulfhydryl groups of these peptides. The induction of MTs is transcriptionally regulated by *cis*-acting metal regulatory elements and *trans*-acting factors[47,60]. The existence of a large number of factors and conditions that modulate MT synthesis during organism development[63,64], regeneration and reproduction, suggests that these act not only in detoxification of harmful chemicals[61,62] but have other, likely primary roles in the metal homeostasis and in the formation of pools of essential heavy metals.

Mammalian MTs consist of two domains designated β (N-terminal) and α (C-terminal). These two domains (Fig. 2) coordinate 7 divalent metal ions[49,65–67] into tetrahedral tetrathiolate clusters[49] comprising a total 20 Cys residues that are shared by different MT sequences (Figs 1 and 2). The β- and α-domains bind three and four bivalent metal ions, respectively. On the other hand, either domain binds 6 equivalents of monovalent ions such as Cu^{+} or Ag^{+} (refs[65,66]). A certain selectivity of MT domain for metal ions and the independent assembly of metal thiolate clusters was observed in early works on MTs[66–68].

More recent studies on the affinity of metal ions to MT and its individual domains revealed much stronger binding of Cd^{2+} to the α- than to the β-domain[69,70]. The affinity of Cd^{2+} for the α-domain appeared to be 12-fold higher than for the entire MT (ref.[70]). The binding affinity for the Cd^{2+} to β-domain was estimated to be 140-fold weaker as compared to the α-domain[69]. MT purified from Cd^{2+}-treated mammalian cells contained five Cd^{2+} and two Zn^{2+} ions preferentially located in the β-domain[65,67] (Fig. 2). Semiempirical calculations derived from X-ray and NMR data revealed that individual metal binding sites in either domain differed in their affinity for[71]. The relatively low affinity of two particular Cd^{2+} metal binding centers in the β-domain that bind two Cd^{2+} allows exchange with Zn^{2+}.

In vitro studies have shown that Cu^+ is preferentially bound by the β-domain in intact MTs (ref.[68]), indicating higher stability of Cu^+ coordination by this domain. Moreover, the Cu_6β-domain but not the Cd_3β-domain could be expressed as a stable polypeptide in *E. coli*[72]. These and the above mentioned differences (in terms of metal binding properties) between β- and α-domains point to a prevalent role of the β-domain in the homeostasis of essential metals and to a main role of the α-domain in the detoxification of poisonous metal species.

2.1.1.1.2. Class II Metallothioneins

Metallothioneins isolated from non-animal sources such as the yeasts *Saccharomyces cerevisiae*, *Candida glabrata*, *Candida albicans*[59,73], algae[74], cyanobacteria (*Synechococcus* sp.[75]) or plants (*Tricum aestivum*[53], *Zea mays*[76]) are of class II. Thus far, the only lower organisms possessing a class I MT is the ascomycete *Neurospora crassa*[49].

A well-known class-II member is CUP1, the MT of *S. cerevisiae*. It is mainly responsible for copper tolerance and its structural gene is located in *CUP1* locus (chromosome VIII) that may amplify upon Cu^{2+} exposure[52]. Multiple tandem repeats of *CUP1* are then maintained to strengthen resistance. *CUP1* transcription is induced by Cu^+ and Ag^+ but not by Cd^{2+} or

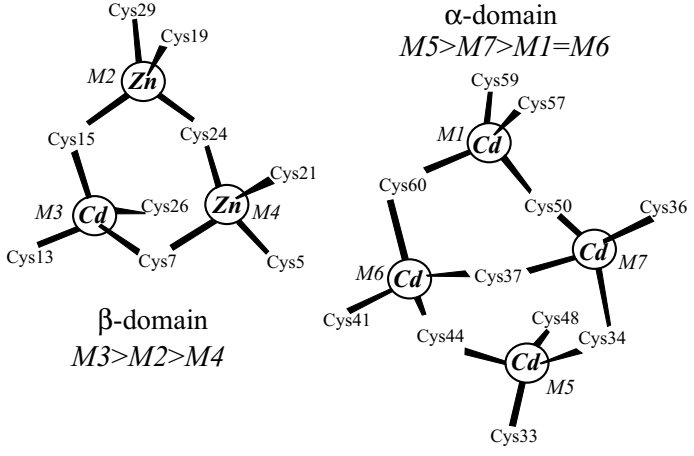

FIG. 2

Coordination of divalent metal ions in the β- and α-domains of class I metallothionein (MT). Mammalian MTs isolated from organisms exposed to Cd^{2+} usually contain 5 Cd^{2+} and 2 Zn^{2+} atoms[65,67] bound in tetrahedral tetrathiolate clusters[49]. The affinity of particular metal binding centers (M1 to 7) for Cd^{2+} (ref.[69]) is indicated

Zn^{2+}, and it is driven by a Cu^{+}-binding[77] *trans*-acting factor named ACE1 (ref.[78]). The CUP1 binds, however, both Cd^{2+} and Zn^{2+} (4 equivalents per molecule) besides 8 equivalents per molecule of monovalent ions. In the case of yeast protein there is no evidence of the formation of two separate metal binding domains. In contrast to mammalian MTs, processing of the primary translation product results in the removal of 8 amino acids from the N-terminus (Fig. 1). The processed CUP1 (6.6 kDa) contains 12 cysteines of a total of 53 amino acid residues[52].

It is noteworthy that CUP1 seems to be solely involved in metal tolerance. On the contrary, other members of class II MTs, such as the wheat Zn^{2+}-binding E_C protein (Fig. 1), function exclusively in metal homeostasis during seed development[53,59,60]. Its expression only depends on the stage of development and its level does not change as a function of the extracellular Zn^{2+} concentration.

2.1.1.2. Metallothionein-Like Proteins

Most plant MT-like proteins consist of about 63 to 83 amino acids[59,60]. The analysis of cDNAs isolated from various sources revealed that the bulk of plant MT-like proteins exhibit more than 50% sequence homology and can be sorted into two groups on the basis of predicted location of Cys residues into two groups[59,60]. In type 1, there is exclusively the Cys-X-Cys motif whereas in type 2 there are the Cys-Cys and the Cys-X-X-Cys motifs are both highly represented within the N-terminal domain (Fig. 1). Some recently described sequences, however, could not be classified as 1 or 2 (ref.[59]) as their Cys distributions differ from that of either types (Fig. 1). In plant MT-like proteins, terminal Cys-rich domains are often separated by a central region (approximatelly 40 amino acids long) without any Cys residues. The presence of this region represents a principal difference from mammalian and fungal metallothioneins as well as from the wheat E_c protein (Fig. 1) and its maize homologue[79]. Both “long” MT-like proteins and “short” MT-like proteins (45 amino acids) are abundant in plants albeit the latter ones with lower frequency. The latter possess “full-size” Cys-rich domains, but the “central spacer” (containing one aromatic amino acid residue usually Tyr) is shortened to 7 amino acids. The role of the central spacer region in MT-like protein is still obscure (referenced in[59]).

The identification of abscisic acid (ABA) as well as ethylene-response elements in the genomic clones of MT-like protein genes confirmed the existence of a direct connection between plant germination, development or tissue type and transcription of an MT-like protein gene previously ob-

served *in vivo*[59]. The differential expression of MT-like proteins and their transcription in response to various stress conditions is reminiscent to the homeostatic (and protective) role played by MTs of classes I and II. Despite extensive studies, the role of MT-like proteins in heavy metal tolerance is still rather unclear. Evidence that MT-like proteins may protect plants from heavy metal poisoning comes from transgenic tobacco seedlings (*Nicotiana tabacum*) overexpressing the type-2 MT-like protein of *N. glutinosa*[80]. On the other hand, transcription of MT-like genes seems to be organism- and metal-specific in wild-type plants grown in the presence of high of heavy metal concentrations[59]. The most pronounced effects are elicited by Cu^{2+}. For example, elevated external Cu^{2+} levels resulted in a decrease of a type-2 MT-like mRNA in *Brassica juncea* whereas Zn^{2+} had a reverse effect[81]. On the contrary, the exposure of *Arabidopsis* seedlings to Cu^{2+} upregulated an MT-like type-2 mRNA, but Cd^{2+} and Zn^{2+} had only a slight effect[55]. We would like to emphasize that, little is known about the regulatory mechanisms controlling the exprssion of MT-like genes and the only *cis*-acting element showing homology to the well-known metal-response elements has been identified in tomato[82] (*Lysopersicon esculentum*).

There are few, mostly indirect, data on the metal binding ability of plant MT-like proteins. It is known that many plant MT-like proteins are capable of complementing the heavy metal hypersensitivity of yeast (*S. cerevisiae*)[55] and cyanobacteria (*Synechococcus* PCC 7942) (ref.[83]). The MT-like protein of *Pisum sativum* ($PsMT_A$) has metal-to-protein stoichiometries ranging from 4.3 to 6, from 4 to 5 and from 3 to 3.5 in the case of Zn^{2+}, Cd^{2+}, and Cu^{+}, respectively[84]. The stabilities of Zn^{2+}-, Cd^{2+}-, and Cu^{+}-$PsMT_A$ closely resemble those of the corresponding equine renal MT complexes.

2.1.1.3. Phytochelatins

Phytochelatins (PCs) are synthesized in a template independent manner. In contrast to MTs, however, PCs exhibit some features characteristic of MTs and therefore they are sometimes referred to as class-III metallothioneins. The most common PC amino acid sequence is $(\gamma\text{-Glu-Cys})_n\text{Gly}$, abbreviated as PC_n, where *n* ranges from 2 to 11 depending on the source. PCs were originally found in yeasts (*e.g. Schizosaccharomyces pombe*) and plants[57–59]. Peptides containing a C-terminal amino acid (Xaa) other than glycine are referred to as *iso*-phytochelatins (Fig. 3), abbreviated *iso*-PC_n (Xaa)[58]. At present, PC, *iso*-PC_n (β-Ala), *iso*-PC_n (Ser), and *iso*-PC_n (Glu) have all been detected in plants. The occurrence of PC forms lacking the terminal glycine was noted both in the yeast *S. pombe* and in plants[58,59]. As PCs and *iso*-PCs

have basically the same biosynthetic origin and mode of action the abbreviation PC will be used throughout this work.

As mentioned above, the mammalian MTs are induced both by heavy metal ions and by various stress factors[49,61]. On the contrary, metals and metalloids have been reported as the only factors that induce PC synthesis. The importance of PCs in heavy metal (in particular Cd^{2+}) detoxification *in vivo* is well documented although it does not represent a general mechanism of heavy metal tolerance[43,58,59,85,86] and a role of PCs in metal homeostasis can not be excluded as well[59].

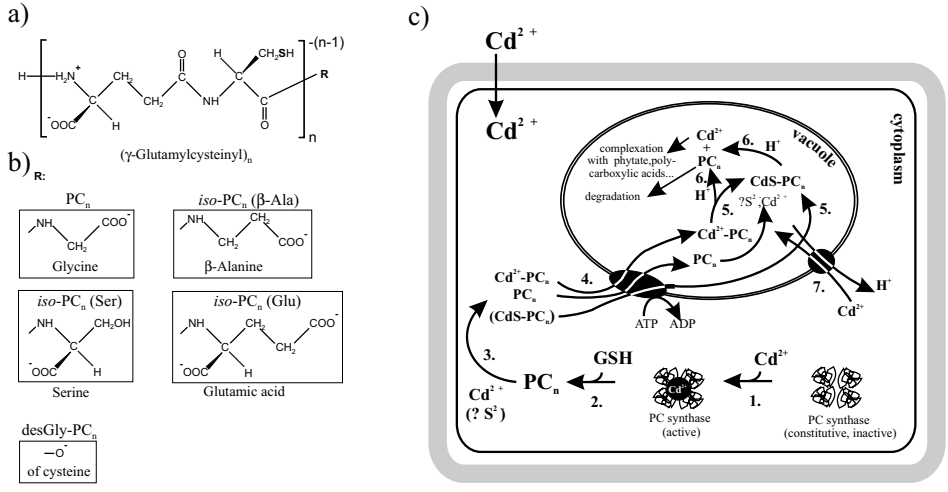

FIG. 3

a Chemical structure of the γ-glutamylcysteinyl repeating unit invariant in both phytochelatins (PCs) and *iso*-PCs. The number of repeats (*n*) vary from 2 to 11, depending on the source[58,59]. b Basic structural difference between *iso*-PCs and PCs is in C-terminal amino acid residues (R) which is different from glycine[58,59]. c General model describing phytochelatins (PCs) synthesis in response to Cd^{2+} and intracellular localization of the Cd^{2+}–PC complex. Cd^{2+} ions entering the cytoplasm 1. activate PC synthase which 2. builds up PCs. Subsequently, PCs 3. sequester Cd^{2+} ions and form an LMW Cd^{2+}–PC complex (a HMW CdS–PC complex can be formed in the cytoplasm). Cd^{2+}-PC complexes and apo-PCs are 4. transported through tonoplast in an ATP-dependent manner (CdS–PC complexes are also translocated to the vacuole, but with a much lower efficiency). In the vacuole, the Cd^{2+}–PC complex 5. accommodates S^{2-} and forms HMW CdS–PC, which may reside in the vacuole. Alternatively, Cd^{2+}–PC and/or CdS–PC complexes 6. dissociate in the acidic vacuolar sap, Cd^{2+} can be bound by vacuolar ligands and PC degrade. The Cd^{2+} ion may enter vacuole also *via* a low-afinity H^+/Cd^{2+}antiporter 7.

PC synthesis is induced exclusively by metals and metalloids. The Cd^{2+} ion is the most potent inducer of PC synthesis and cells start PC synthesis with a lag period of 5–15 min after exposure to Cd^{2+} (refs[57–59]).

An enzyme capable of PC synthesis was isolated from *Silene cucubalus* and characterized by Grill *et al.*[87]. This enzyme, γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase, E.C.2.3.2.15), is generally expressed in a constitutive manner in all PC and *iso*-PC producing species[57–59]. The plant PC synthase is a metal-dependent enzyme and the purified apoprotein is activated *in vitro* by a spectrum of metals analogous to that inducing PC synthesis *in vivo*[87].

Grill *et al.* described PC synthesis by the following equation:

$$(\gamma\text{-Glu-Cys})_n\text{Gly} + (\gamma\text{-Glu-Cys})_n\text{Gly} \rightarrow (\gamma\text{-Glu-Cys})_{n+1}\text{Gly} + (\gamma\text{-Glu-Cys})_{n-1}\text{Gly};$$

where $n = 1,2,3,....$

The (γ-Glu-Cys) unit is primarily provided by GSH (or its homologues); however, PC synthase is able to use also PCs as (γ-Glu-Cys) donor.

In fission yeast, two ATP-independent pathways of PC synthesis were described[88]. The first one closely resembles that found in *S. cucubalus* while the other is based on the addition of γ-Glu-Cys to $(\gamma\text{-Glu-Cys})_n$ followed by the addition of the terminal Gly residue most likely by glutathione synthetase. It has been shown that the *gsh2* gene of *S. pombe* encodes a bifunctional enzyme possessing both glutathione synthetase (E.C.6.3.2.3) and PC synthetase activity[89].

Two different Cd^{2+}–PC complexes from plants and yeasts could be found based on molecular weight determinations carried out by gel filtration[57–59]. These are referred to as low molecular weight (LMW, 2 to 4 kDa) and high molecular weight (HMW, 6 to 9 kDa) complexes. Native LMW complexes are frequently composed by two or three PC_n of various lengths; within such complexes Cd^{2+} is coordinated by four Cys residues with a Cd–S bond length of 2.52 ± 0.02 Å (ref.[90]). A HMW CdS–PC complex was characterized as a crystallite of approximatelly 12–20 Å coated by PCs (refs[91,92]). An SH group : Cd^{2+} ratio lower than 1, as described in some HMW complexes, indicates a different coordination geometry and a higher capacity for the metal ion. The mechanism by which the sulfide is accommodated into such complex is not known yet. Very little data is available on the *in vivo* formation of PC complexes with metals other than Cd^{2+}. However, the formation of Cu^{2+}, Pb^{2+}, Hg^{2+}, and Ag^{+}–PC complexes *in vitro* has been described[59]. The enzymes involved in GSH synthesis are localized in the cytoplasm,

chloroplasts, and mitochondria, while PC synthase activity is abundant in the cytoplasm. However, most of intracellular Cd^{2+} is vacuolar both in plants and yeasts[58,59]. Figure 3 shows a widely accepted model of PC synthesis and membrane transport of PC metal complexes (LMW Cd–PC, or even HMW CdS–PC (refs[93])) across the tonoplast *via* specific ATP-dependent transporters[94,95]. The LMW complex would then incorporate S^{2-} and form an HMW complex that is stable in the acidic vacuolar sap. LMW and/or HMW complexes may reside in the vacuole as stable metal–peptide complexes or dissociate and exchange Cd^{2+} with other vacuolar ligands (Fig. 3).

2.1.1.4. Significance of Metal Bioremediation by Metallothioneins

The potential of MTs, PCs and MT-like proteins for the bioremediation of metal pollution has been pointed out in many reviews[27,33,41,42]. Earlier attempts to produce metallothioneins[68,69,72,84,96–98] or other metal binding peptides, such as polyhistidine peptide fusions[99], in *E. coli* were basically directed towards the *in vitro* study of the metal-binding properties of the recombinant peptides and proteins. An enhanced ability of *E. coli* to accumulate heavy metal ions due to the production of metallothioneins[96–98], polyhistidine and CUP1-polyhistidine fusions[100] in the cytoplasm was also reported. However, the intracellular expression of novel metal-binding sites (MBSs) could not be easily made instrumental to the engineering of microorganisms for bioremediation. It should also be stressed here that the expression of these intracellular heavy metal ligands often led to an increase in metal tolerance. Therefore, the maintenance of cell viability and metal ion transport at otherwise toxic metal concentrations seems to be responsible for the resistance rather that true heavy metal accumulation. The efficiency of such metabolically sponsored intracellular heavy metal accumulation is low for bioremediation but it could be improved through the engineering of potent metal ion transporters into the bacteria of interest.

Indeed, the co-expression of specific bacterial Hg^{2+}-transporters (MerT/MerP proteins, described below) with either yeast metallothionein (CUP1) or the pea MT-like protein $PsMT_A$ (as a fusion to glutathione S-transferase) in *E. coli* resulted in an approx 18-fold selective increase of the wild-type Hg^{2+} accumulation[101] as well as in an increased resistance to elevated metal concentrations[102]. In this system, Hg^{2+} accumulation was not affected by Na^+, Mg^{2+}, and Cd^{2+}, extreme levels of pH or ionic strength nor by cyanide or EDTA[101], *i.e.* the factors that interfere with many metal

cleanup processes. Moreover, engineered *E. coli* cells were highly effective at low metal concentrations, being capable of lowering Hg^{2+} level from 2 ppm to about 5 ppb in a hollow fiber bioreactor with cycling of Hg^{2+} contaminated medium[103].

A successful attempt to enhance both the tolerance to and the accumulation of Cd^{2+} with *B. juncea* seedlings overexpressing the *E. coli*-borne glutathione synthetase gene *gshII* (ref.[104]) (related to *gsh2*, see the text above) has recently been reported. Transgenic plant shoots accumulated up to 3-fold higher amounts of the metal as compared to wild-type plants. Cd^{2+} tolerance, PC levels and metal accumulation capacity positively correlated with *gshII* expression level.

The importance of PCs in Cd^{2+} detoxification in plants and yeasts[57–60] as well as the protective role of overexpressed MT-like proteins against cadmium toxicity[80] have been well documented. Nevertheless, a high diversity of data on the levels of the metal-binding biopolymers induced in wild type and tolerant ecotypes and on the poor or none PC synthesis occurring in response to some metals can be found in literature[59]. It could thus be concluded that overproduction of PCs and MT-like proteins is neither a general mechanism for detoxification nor universal mechanism for the induction of increased tolerance to heavy metal ions. On the other hand, PC deficient plants are hypersensitive to heavy metals[105,106]. Further research devoted to metal fixation within the plant body and to the evolution of tolerant plants should be directed towards understanding the mutual link between genes involved in control of metal tolerance[85,86] and PC synthesis. Ultimately, the evaluation of such relationships would allow to construct hyperaccumulator plants suitable for bioremediation, hypertolerant plants for green highly contaminated areas and/or plants with a reduced metal uptake in edible parts.

The importance of such an approach is underlined by failure of many previous attempts to obtain transgenic plants for bioremediation simply through the introduction of MT genes. Transgenic plants overexpressing various mammalian or yeast MTs exhibited increased cadmium tolerance[107–112]. The possibility of constructing corresponding hyperaccumulator plants bearing the wheat metal ion transporter encoded by the *LCT1* gene has been recently reported[113], documenting thus that the production of plants or yeasts with improved heavy metal tolerance could be an important issue. Unfortunately, the expression of either mammalian (α-domain of MT[108,114–116]) or yeast (CUP1[112]) MTs in plants driven by the constitutive 35S promoter of the cauliflower mosaic virus did not significantly increase Cd^{2+} accumulation in the upper part of plant. In some cases,

MT overexpression resulted in a decrease of up to 70% in Cd^{2+} translocation from roots to shoots as compared to wild-type plant[115,116].

The intracellular production of MTs, MT-like proteins or PCs might potentially stimulate heavy metal accumulation. However, the introduction of MTs as novel metal binding site on the bacterial surface seems to be a more straightforward approach for the production of biomass with improved metal binding properties[117–120] (see Chap. 2.1.1.2. for details).

Of course environmentally robust and acceptable bacteria other than *E. coli* should be used to make both Hg^{2+}-accumulation *via* MerT/MerP/[CUP1 or $PsMT_A$] and surface MT display approaches instrumental to the bioremediation process.

2.1.2. Biosorption of Metals

Biosorption is a rapid, reversible, fortuitous process independent of cell metabolism. The mechanism of metal biosorption is a complex issue and it involves mainly ion exchange, chelation and adsorption by physical forces, ion entrapment in inter- and intrafibrillar capillarities and inner spaces of the structural polysaccharide network[39]. It occurs both in living and dead biomass.

2.1.2.1. Natural Biomass as a Biosorbent

Dead biomass derived from waste biomass of algae, moss, yeast, molds or bacteria is usually referred to as a biosorbent. It may be used in either an unmodified, chemically modified or immobilized form to obtain particles with mechanical properties appropriate for sorption columns[38,39]. There is a wide diversity of biosorbents and some of the highest metal binding capacities are listed in Table III.

The selectivity of biosorbents with a known spectrum of metal binding sites or the preference for a metal in a mixture in wastewater can be semi-empirically evaluated on the basis of the HSAB (ref.[30]) theory or the Irving–Williams principle[33]. However, the architecture of the cell wall that also contributes for metal binding properties, is rather complex and thus the evaluation of sorption characteristics (isotherms) in single- and multi-metal systems is required for a conclusive judgment[38,39]. It is generally agreed that ion exchange is the prevalent mechanism for metal biosorption. The carboxyl group is the main ligand in fungi, algae[130,131] and Gram-positive bacteria[132]. The sulfate group of algae and the acetamide and amine groups of fungal cell walls account for only 10% of the total metal

TABLE III
Uptake of metals by biosorbents from various sources[a]

Metal	Biomass type	Organism	Metal uptake $\mu mol\ g_{dry\ wt}^{-1}$	Reference
Cd^{2+}	brown marine alga	*Ascophyllum nodosum*	1.900	120
	water plant	*Potamogeton luteus*	1.100	121
	bacterium	*Bacillus* sp.		
	AMT-Bioclaim™		900	122
	yeast	*Candida tropicalis*	530	123
	fungus	*Rhizopus arrhizus*	240	124
Cu^{2+}	bacterium	*Bacillus* sp.		
	AMT-Bioclaim™		2.400	122
	water plant	*Potamogeton luceus*	1.280	121
	yeast	*Candida tropicalis*	1.270	123
	fungus	*Rhizopus arrhizus*	253	125
Ni^{2+}	brown marine alga	*Fucus vesiculosus*	680	126
	yeast	*Candida tropicalis*	340	123
	fungus	*Rhizopus arrhizus*	305	124
Pb^{2+}	bacterium	*Bacillus* sp.		
	AMT-Bioclaim™		2.900	122
	fungus	*Absidia orchidis*	1.700	127
	water plant	*Potamogeton luceus*	1.360	121
	brown marine alga	*Sargassum natans*	1.300	126
Zn^{2+}	bacterium	*Bacillus* sp.		
	AMT-Bioclaim™		2.110	122
	water plant	*Potamogeton luceus*	1.000	121
	yeast	*Candida tropicalis*	460	123
	fungus	*Rhizopus arrhizus*	310	125
UO_2^{2+}	filamentous bacteria	*Strept. longwoodensis*	1.850	128
	fungus	*Rhizopus arrhizus*	920	125
	yeast	*Sacharomyces cerevisiae*	590	129

[a] The biomass is in its natural state, chemically modified or immobilized.

binding sites (referenced in[39]). On the other hand, the acetamide groups of chitin and amino functionalities of chitosan seem to be of critical importance for highly efficient biosorption of UO_2^{2+} and MoO_4^{2-}, respectively[38,39]. The contribution of hydroxyl groups to metal complexation is mostly attributed to those in α-D-mannans of fungal cell walls and to the D-mannuronic and L-gulonic acids in *cis*-oriented algal polysaccharides[27,38]. The spectrum of metal-binding biomolecules (and specific groups) in the microbial cell wall may be altered by culture conditions. For example, melanin containing biomass that is produced by stressed fungi in the stationary phase[27] or the sulfhydryl enriched biomass (produced by certain steroid-transforming fungi[38]) could be the biosorbents of choice.

The most important features of an effective biosorbent are its metal(s) binding capacity, its affinity for metal(s) (determining the kinetics of the process and thus the remaining concentration of metal ions in the effluent) and the effective operating time of the sorption column (metal breakthrough). The ionic form of the biosorbent (especially for carboxyl groups) is also important. For example, treatment of *Rhizopus arrhizus* with $NaHCO_3$ to exchange tightly bound H^+ for Na^+ ions in metal binding sites led to a more than 3-fold increase in metal binding capacity[133]. Maintenance of the sorption capacity in multiple sorption–desorption cycles is also desirable.

Some attempts to commercialize biosorption processes were made in the early 90's. For example, the BIO-FIX (US Bureau of Mines, UT, U.S.A.) are polysulfone beads containing immobilized non-*via*ble biomass from various sources including cyanobacteria (*Spirulina*), yeasts, algae and plants (*Lemna* sp., *Shagnum* sp.)[41,134]. The removal of heavy metals from wastewater with such a matrix was efficient at both low (*e.g.*, 50 ppb of Cd^{2+}, 220 ppb of Ni^{2+}) and high (*e.g.*, 1.2 ppm of Cd^{2+}, 42 ppm of Ni^{2+}) metal levels[134]. The sorbent could be reused for more than 120 sorption-desorption cycles after regeneration by acidic treatment. The content of Cd^{2+} in the effluent was below 10 ppb while that of Ni^{2+} was reduced to 30 ppb. *Bacillus* sp. biomass immobilized onto a polyethyleneimine ion exchanger *via* glutaraldehyde crosslinking is employed in the AMT-Bioclaim(tm) process (VistaTech Partnership Ltd., UT, U.S.A.)[124,135]. Effluents with 10 to 50 ppb of metal are obtained with this sorbent, which is regenerated by alkali treatment. The AlgaSORB(tm) (Bio-Recovery Systems Inc., NM, U.S.A.) process employs an unspecified algal biomass immobilized in a silica matrix[136].

2.1.2.2. The Improvement of Metallosorption by the Display of Engineered Cell Surface Metal Binding Sites

The anchoring of particular amino acid sequences to a biosorbent could lead to an increased binding capacity, and possibly selectivity, for metal ions. Biosorbents can be enriched with amino acids classified by the HSAB principles as transit metal ligands stronger than those naturally present on the microbial surfaces (see above). This approach has been used for the display of various short metal binding peptide sequences[137–144] or metallothioneins of either mammalian[117–119] or fungal[117] origin on the surface of *E. coli*. It resulted in a dramatic increase of cadmium bioaccumulation in the apparent absence[118] of extensive intracellular uptake.

The surface display of yeast CUP1 (ref.[117]) *i.e.* a class II MT mammalian MT isoform 1A (ref.[117]) (HMT1A), or the HMT1A α-domain[118] fused to the outer membrane protein LamB all resulted in 15–20 fold increase of the natural ability of *E. coli* to bind cadmium. Histidine is another amino acid residue present in MTs that possesses a high affinity for transition metals. The surface exposure of two hexahistidine moieties fuses to the LamB protein led to an over 10-fold increase in Cd^{2+} adosorption by *E. coli* cells[137]. Other potent anchors that have been successfully employed for the exposure of novel metal binding sites on the surface of Gram-negative bacteria are OmpC (ref.[138]), OmpA (ref.[139]), the peptidoglycan-associated protein (PAL) the Lpp-OmpA fusion protein (reviewed in[140]) and the fimbrial structural protein FimH (ref.[141]). Recently, chimeric hexahistidine constructs, particularly $His_3GluHis_6$ and His_6 have been successfully anchored to the surface of Gram-positive *Staphylococcus species*, thus conferring Ni^{2+} and Cd^{2+} binding capacity to these organisms[142]. Protein A from *Staphylococcus aureus* and a propeptide sequence from *Staphylococcus hyicus* were used for anchoring metal binding sequences to the surface of non-pathogenic bacteria *Staphylococcus xylosus* and *Staphylococcus carnosus*.

A major advantage of this peptide surface display approach would be selectivity for particular metal ions[28]. Various designs of artificial heavy metal binding sites have been reported in the literature. The synthetic peptide Boc-Cys-Pro-Leu-Cys-OMe, designed as a model of Cys containing metal binding sites, has been shown to bind Hg^{2+}, Zn^{2+} and Cd^{2+} ions using both Cys residues[145]. Binding of Zn^{2+} ions *via* His and Glu residues was described for a model peptide mimicking the metal binding site of ribonucleotide reductase[146]. The peptide Gly-Cys-Gly-Pro-Cys-Gly-Cys-Gly (CP peptide) was selected by screening a set of synthetic peptides rich in histidine and cysteine residues for Cd^{2+} binding[147]. It was shown to bind 3 equivalents of Cd^{2+} *in vitro* (ref.[143]). When displayed on the surface of *E. coli* the CP pep-

tide increased by fourfold the natural Cd^{2+} binding ability of bacterial cells in culture media containing micromolar levels of Cd^{2+} (ref.[143]). The exposure of repetitive metal binding motifs of similar sequence $(Cys\text{-}Gly\text{-}Cys\text{-}Cys\text{-}Gly)_3$ as a fusion with maltose binding protein in *E. coli* led to a novel biosorbent capable to efficiently remove Cd^{2+} and in particular Hg^{2+} down to concentrations below 5 ppb (ref.[144]).

Haymore *et al.* identified several short chelating sequences containing His, Cys, and Asp residues, which could form energetically stable chelating sites with specific metal ions[148]. Amino acid sequences forming stable coordination spheres around transition metals were also identified using a combinatorial peptide library approach[149]. Very powerful technique for selection of peptides with high affinities for given metal ion appears to be the surface display of peptide libraries[139,141,149–151].

It is noteworthy to stress here that future efforts should be directed towards the engineering of metal binding peptides on the surface of environmentally acceptable bacteria such as *Alcaligenes eutrophus* (see below) and *Pseudomonas putida*[27], which have been already employed in existing systems for heavy metal bioremediation. For instance, metal binding peptides introduced on the surface of *Alcaligenes eutrophus* may aid the process of precipitation and crystallization of metal carbonates. This approach could be extended to other biosorbents such as yeasts and molds. The choice/availability of an appropriate carrier for surface display seems to be the only limiting factor. The C-terminal part of α-agglutinin could be such a carrier in yeasts[152].

2.2. Metabolically Sponsored Processes in the Remediation of Heavy Metal Pollution

2.2.1. Bioprecipitation of Metals

Bioprecipitation (biomineralization) follows the initial binding of metal ions to the cells (creating the supersaturated environment). The insoluble metal compound precipitates (and crystallizes) as metal ions combine with anionic species produced by cell metabolism. The process is slow, irreversible and dependent on the temperature and the cellular metabolism. Bioprecipitation is generally non-specific depending on the insolubility of the particular metal compound. The great benefits of the process are (i) a high metal-to-biomass ratio, (ii) the highly crystalline material easy to sepa-

rate, and (iii) the fact that resulting crystallites have a low organic matter content (might be advantageous for the metal recuperation; see below).

2.2.1.1. Metal Phosphate Precipitation by Citrobacter sp.

The exceptional metal (Me^{2+}) accumulation by resting cells of *Citrobacter* sp. is due to bioprecipitation of $MeHPO_4$ at the cell surface caused by an acid-type phosphatase that releases phosphate from an appropriate substrate[27,33] (Fig. 4). *Citrobacter* sp. was originally isolated from a metal polluted soil and its phosphatase activity was attributable to cell survival in the presence of heavy metals[24]. The enzyme, which shares significant sequence similarity with the *phoN* product of other enterobacteria[153,154] is mostly abundant in the periplasm[153,155], especially in polar regions[155]. Two enzyme isoforms were distinguished on the basis of their distinct cation-exchanger binding properties[155]. Both holoenzymes (103–108 kDa) are composed of four subunits of 27 kDa, but they differ in kinetic parameters. The *Citrobacter* sp. acid phosphatase is also produced in metal-free media, its biological function remains unclear but it may relate to a cellular stress response[156,157]. Enzyme production is maximal in the continuous culture under carbon-limiting conditions[27,158] leading to highly active cells well-suited for metal removal. The acid phosphatase of *Citrobacter* sp. is stable, metal and CN^- resistant[24], and operates in a wide range of pH (from 5 up to 9)[24,128] and temperatures (from 2 to 45 °C)[27].

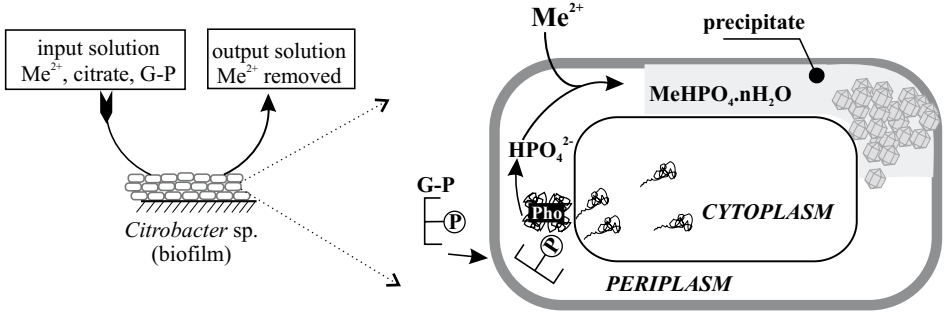

FIG. 4

Bioremediation of metal contamination from aqueous flow using *Citrobacter* sp. immobilized on an inert support. The heavy metal ion (Me^{2+}) entering the periplasm is precipitated as the corresponding hydrogen phosphate[159–164]. The inorganic phosphate is released from glycerol 2-phosphate (G-P) by a periplasmic acid phosphatase[153,155,156] (Pho) produced by *Citrobacter* sp. under carbon-limiting conditions[158]

Heavy metals and radionuclides such as Cd^{2+}, Pb^{2+}, Cu^{2+}, UO_2^{2+}, PuO_2^{2+}, and AmO_2^{2+} can be bioprecipitated as cell-bound metal hydrogenphosphate crystallites[154,159–164]. In addition, Ni^{2+} can be removed *via* interactive ion exchange with a crystalline $HUO_2PO_4 \cdot 4H_2O$ lattice occurs[154,158]. Cells immobilized in polyacrylamide gels[24,132] or used as a biofilm (Fig. 4) on an innert support[27,158] can reach metal-to-biomass ratios of 4 g of Pb^{2+} (ref.[159]), 7 g of Cd^{2+} (ref.[161]), and 9 g of UO_2^{2+} (refs[160,162]) per 1 g of cell dry weight.

Formation of the nucleation site, the rate-limiting step[163] initiating crystallization occurs at the cytoplasmic and the outer membrane[155]. This process was observed in case of UO_2^{2+} biomineralization, the most thoroughly investigated aspect of *Citrobacter* sp. mediated metal remediation. Noteworthy, other phosphatase-producing enterobacteria tested for UO_2^{2+} removal were not as efficient as the heavy-metal-accumulating *Citrobacter* sp. strain. Such superior performance was attributed to higher enzyme levels, different properties of the phosphatases[154,164] and/or to the involvement of additional, species-specific factors[153]. Contrasting with the latter hypothesis, however, *E. coli* transformants overexpressing the PhoN phosphatase showed higher UO_2^{2+} accumulation compared to *Citrobacter* sp.[164]. The bulk of these studies have been performed using glycerol 2-phosphate as a HPO_4^{2-} source. Its replacement with cheaper phosphate source (*e.g.* tributylphosphate)[165] could make this process economically feasible, also allowing the simultaneous degradation of this organic by-product of nuclear fuel reprocessing.

2.2.1.2. Metal Hydroxide and Metal (Bi)carbonate Precipitation by Alcaligenes eutrophus CH34

The ability of *Alcaligenes eutrophus* CH34 to promote the effective bioprecipitation of various heavy metal ions[36,166–168] due to the alkalinization of the periplasmic space and of the outer cell environment. The main feature of the CH34 strain is its ability to supersaturate the cell surface with specific heavy metal ions to be precipitated (Fig. 5). Heavy metals that entered the cytoplasm through the magnesium transporter are exported from the cell interior *via* the metal efflux system that represents the specific mode of metal resistance[36,169–172].

The *A. eutrophus* strain CH34 (recently renamed *Ralstonia eutropha*) was originally isolated from a decantation tank in a zinc factory and exhibits resistance towards an exciting spectrum of heavy metal ions: Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Tl^{+}, Zn^{2+}, and CrO_4^{2-}. Metal-resistance in the CH34 strain is governed by at least seven genes either located on the bacterial

chromosome or on one of two megaplasmids pMOL28 (163 kb) and pMOL30 (238 kbp)[169–172]. The metal-inducible Cd^{2+}, Zn^{2+}, and Co^{2+} efflux system (of the greatest bioremediation significance[36,168]) is encoded by the *czc* operon on plasmid pMOL30. The homologous *cnr* operon (Co^{2+} and Ni^{2+} efflux) is located on the pMOL28 plasmid.

The *czc* operon contains at least eight distinct open reading frames *czcNICBADRS* (ref.[172]). The CzcA, CzcB, and CzcC are components of an efflux protein complex[169,173] (Fig. 5) that functions as a cation–proton antiporter[174], whereas the remaining proteins are involved in transcriptional control of the entire operon[172,175]. The CzcA component (a functional dimer 1 064 amino acids) is essential for cation transport, is able to induce by itself a limited resistance to Zn^{2+} and Co^{2+}, and is thus the central antiporter subunit[169,173]. The N-terminal domain of CzcA contains (predicted) transmembrane α-helixes with charged amino acids that might function as a charge-relay system in proton transfer[169]. The periplasmic CzcB protein (512 amino acid residues) is bound to the outer membrane[173].

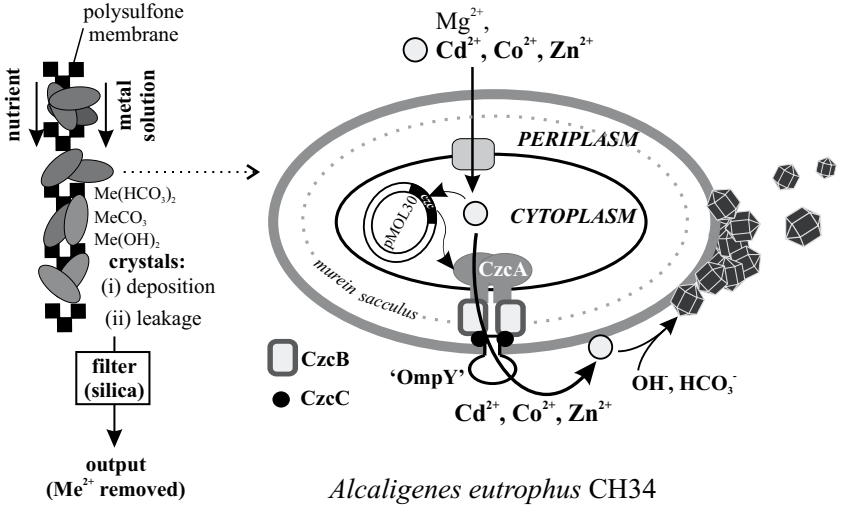

FIG. 5

Bioremediation of metal contamination from aqueous flow using *Alcaligenes eutrophus* CH34. Bacterial cells are immobilized in polysulfone membrane (Zirfon(r)) that separates the nutrient from heavy-metal-contaminated flows[166–168]. Cd^{2+}, Co^{2+} or Zn^{2+} ions (Me^{2+}) entering the cytoplasm *via* the Mg^{2+} transporter[168] induce a specific efflux system (Me^{2+}/H^{+} antiporter) encoded by the *czc* operon on plasmid pMOL30 (refs[169–173]). Heavy metals are pumped out off the cell interior (for details, see the text) and supersaturate the cell surface where they precipitate and crystallize as the corresponding hydroxides or carbonates

It contains two nonessential metal-binding domains (4 histidine residues each[173]) and is necessary for effective Zn^{2+} efflux[172]. The third component of the efflux complex, CzcC (346 amino acids), is localized in the periplasm as an outer membrane-associated protein. It functions as a modifier, switching substrate specificity from Zn^{2+} (and partially Co^{2+}) only to Cd^{2+}, Zn^{2+}, and Co^{2+} efflux probably by anchoring the outer membrane protein OmpY that is involved in transport to the CzcCBA complex[173].

Due to the above described efflux system, high concentrations of metals are accumulated at the outside of cells and are thus bound to cell surface structures. At the same time, the metabolism of *A. eutrophus* increases the extracellular pH and also produces CO_2 that is converted to CO_3^{2-} and HCO_3^-. Subsequently, metal carbonates and bicarbonates precipitate at the cell surface, serving as nucleation sites for the growth of crystal structures (composed of $M(OH)_2$, $M(HCO_3)_2$ and MCO_3) with diameters larger than 10 μm that may be released from the cell surface[166,167] (Fig. 5). The Bacteria Immobilized Composite MEmbrane Reactor (BICMER) concept for the remediation of heavy metals from the wastewater using *A. eutrophus* CH34 was developed at the VITO, Belgium[166–168]. Bacteria were immobilized on Zirfon(r) membranes and used for the construction of a flat sheet reactor or a (continuous) tubular membrane reactor. Using this system (Fig. 5) an initial 150 ppm concentration of Cd^{2+} was reduced below 50 ppb; the decontamination of Zn^{2+}, Cu^{2+} or Ni^{2+} was successful as well.

Another important consequence of the heavy metal resistance of *A. eutrophus* CH34 for bioremediation is the possibility of producing genetically engineered strains of this organism with the ability to degrade organic pollutants as PCBs (refs[176,177]). These organisms may thus be useful for the decontamination of sites polluted by both organic compounds and heavy metals.

2.2.2. Metal Sulfide Precipitation

Hydrogen sulfide is produced by sulfate-reducing bacteria (SRB) such as *Desulphovibrio* and/or *Desulphotomaculum* sp.[35,41]. Mixed cultures being studied to increase the bioremediation potential of these microorganisms[35,178]. It has recently been shown that biofilms made of natural consortia of bacteria that include SRBs are more effective than pure cultures of microbial isolates from these consortia[179] most likely due to fact that bacteria in such consortia are symbiotic with respect to carbohydrate metabolism. Most metal sulfides, such as CdS, ZnS, CuS, FeS, PbS or Ag_2S, are readily formed in solution but their solubility is extremely low. In contrast to the

above-described processes, bacterially promoted metal sulfide precipitation does not entirely fulfill the above stated definition of bioprecipitation, as metal binding to bacteria and subsequent crystal formation does not (necessarily) occur. Amorphous flocks often formed instead. However, using immobilized consorcia containing SRBs, White and Gadd demonstrated that CdS can be accumulated in a superficial layer of the biofilm, either entrapped in a exopolysaccharide form or *via* adhesion of the CdS precipitate by unknown mechanisms[179].

An important consequence of the sulfate-reducing activity produced during the anaerobic decay of the organic matter is the immobilization of metal ions (as sulfides) initially biosorbed to the water sediments[41]. Moreover, the production of sulfide can lead to the H_2S-induced disproportionation of organomercury and organolead compounds into more volatile products and insoluble sulfides[25,180], *e.g.*

$$2\ CH_3Hg^+ + H_2S \rightarrow (CH_3)_2Hg + HgS + 2H^+$$

$$2\ (CH_3)_3Pb^+ + H_2S \rightarrow (CH_3)_4Pb + (CH_3)_2PbS + 2H^+ .$$

The sludge-blanket bioreactor using a selected (but unidentified) consortium of sulfate reducing bacteria, was commercialized at the Budelco BV (non-ferrous plant, Budel–Dorplein, Netherlands) by the Shell Research Ltd. (Sittingbourne, Kent, U.K.)[41,181,182]. Applied to waste ground water contaminated with Zn^{2+} (250 ppm), Cd^{2+} (1.5 ppm), Co^{2+} (250 ppb), and Cu^{2+} (750 ppb) it resulted in metal ion concentrations in the effluent below the 10 ppb level[182].

2.2.3. Biotransformation of Metals

Some microorganisms may sustain growth in metal-contaminated environment by the transformation of metal and metalloid species into less toxic (*e.g.*, insoluble precipitates) or volatile forms. The ability of microbes to transform heavy metals and metalloids by oxidation, reduction, methylation and/or demethylation reactions is encoded by plasmid and/or chromosomal genes[25,170] and could be of great bioremediation significance.

2.2.3.1. Mercury Resistance Encoded by mer Operon Genes

One of the most thoroughly studied heavy metal resistance mechanisms is mercury resistance. It is mediated by a machinery that transports Hg^{2+} into the cytoplasm and reduces it to elemental, volatile Hg^0 that escapes from

the cell with aeration[25,170]. This ability is widespread among Gram-negative and Gram-positive bacteria. Despite differences in the localization (chromosomal *vs* extrachromosomal), organization and transcriptional control of the *mer* operon[140] essentially all species use similar mechanisms. The crucial components that serve as regulatory proteins have been comprehensively reviewed[170]. The other important proteins involved in this process are the specific metal transporters MerT, MerP, and MerC, mercuric reductase (MerA) and organomercurial lyase (MerB). Mercuric reductase (E.C.1.16.1.1) is a dimeric cytoplasmic flavoprotein (FAD) with subunit molecular weight ranging from 54 to 69 kDa (ref.[183]). The enzyme is related to GSH reductase and lipoamide dehydrogenase[25,170]. The postulated mechanism of Hg^{2+} reduction anticipates first, the transfer of electrons from NADPH *via* FAD to reduce the S–S bridge between the active-site cysteines (Fig. 6). The sulfhydryl groups at the active site reduce Hg^{2+} to Hg^0 (refs[25,183]).

Two specific transporters are involved in Hg^{2+} transport across the cytoplasmic membrane (Fig. 6). The first is composed of MerP that binds Hg^{2+}

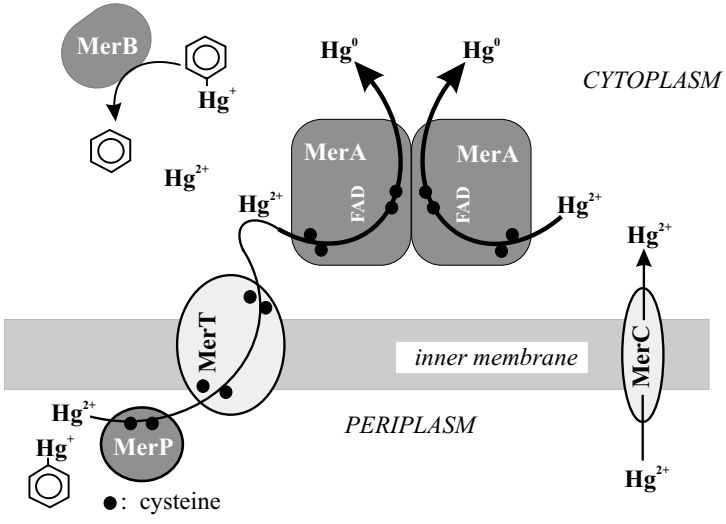

FIG. 6

The enzymes and transporters involved in the mercury resistance mechanism encoded by the *mer* operon[25,170] of Gram-negative bacteria. Hg^{2+} ions enter the cytoplasm *via* MerC and/or MerP/MerT transporters[184–187]. The latter is capable of transporting also organomercury compounds[186]. Hg^{2+} (from organomercury compounds released by organomercurial lyase[22,142], MerB) is reduced by an NADPH-dependent mercuric reductase[25,170,172] (MerA, dimer) to its metallic form that is subsequently evaporated from the cell interior. Cysteine residues involved in the transport and redox reaction[25,172] are indicated

in the periplasm *via* two cysteine residues. It then transfers the metal ion to a cysteine pair in MerT, a transmembrane protein that facilitates Hg^{2+} transport across the membrane[25,184,185]. MerP/MerT has been reported also to transport organomercury (*e.g.* phenylmercuric compounds)[186]. Once the organomercurial is transferred into the cytoplasm, it may be cleaved by organomercurial lyase prior to Hg^{2+} reduction[25]. The other transmembrane protein of the *mer* operon, MerC, that does not require the MerP protein, and can also mediate Hg^{2+} transport into the cytoplasm[187].

Both the whole resistance system[188–191] or some special parts of it[102–104,190,192,193] are considered being a promising tools for bioremediation processes. Continuous cultures of Hg^{2+}-reducing bacteria were able to volatilize Hg^{2+} from contaminated sewage at a rate of 2.5 mg l^{-1} h^{-1} with a removal efficiency of 98% (ref.[188]). Similarly, genetically engineered strains of *Pseudomonas putida*, constitutively overproducing the MerTPAB proteins, exhibited increased resistance to Hg^{2+} and to phenylmercury compounds[191]. The constructed strains combined mercury resistance with benzene and toluene catabolism, however, there was not given remediation data. The expression of bacterial mercuric reductase in transgenic plants[192,193] opens another possible way of removing soil Hg^{2+} by metal volatilization. Transgenic *Arabidopsis thaliana* and *Liriodendron tulipifera* plants expressing the modified *merA* gene both displayed resistance to elevated Hg^{2+} levels and were capable of mercury volatilization at rates an order of magnitude higher than those of control plants. Immobilized Hg^{2+}-reducing *Aeromonas hydrophyla* as well as genetically modified *P. putida* strains expressing the *merA* gene were shown to be capable to remove 95–99% of the Hg^{2+} input thus lowering the metal concentration in the effluent to less than 50 ppb (ref.[190]). Moreover, the test of the leakage of the volatile metal from the culture was extremely encouraging as virtually all released Hg^0 formed droplets of about 1–5 μm in diameter outside the cells and associated with the matrix material (ceramics or alginate)[190].

Hg^{2+}-removal system based on MerT/MerP mediated transport and subsequent binding of themercury ions in the *E. coli* cytoplasm by yeast CUP1 and/or pea $PsMT_A$ (refs[101–103]) was described above (see Chap. 2.1.1.4.).

2.2.3.2. Microbial Methylation of Metals and Metalloids

A variety of bacteria, algae, yeasts, and fungi are capable of converting metal and metalloid ions to organometallic (and organometalloid) compounds that are usually more volatile and may escape from the given biotope[25,35]. Such conversions are quite frequent in nature. Methylation of

Hg, Pb, Sn or Tl species is performed by methylcobalamine, whereas *S*-adenosylmethionine is involved in the methylation of arsenite and selenate. The pathway of the formation of organometallic and organometalloid compounds has been comprehensively reviewed[25,35,194]. For example, selenium biomethylation led to the production of dimethylselenide, dimethyldiselenide, and dimethylselenone products in the soil and in sediments[195]. The remediation of selenium in soils and water is being thoroughly studied[196,197].

The biomethylation of metals and metalloids may also contribute to the activated sludge mediated decontamination of wastewaters (see below). Although biomethylation requires rather anaerobic environment, the aerobic methylation of arsenic, lead, tin, mercury, and/or selenium has been reported[198]. It is assumed, however, that metal(loid) volatilization by the activated sludge microflora is rather a minor process[198].

2.2.3.3. Other Metal Transformations of Bioremediation Significance

A multiple metal(loid)-desolubilization activity was ascribed by Blake *et al.*[189] to a *Pseudomonas maltophyla* strain (O-2) isolated from mercury-contaminated soil. The removal of millimolar concentrations of Hg^{2+}, CrO_4^{2-} Pb^{2+}, Cd^{2+}, SeO_3^{2-}, TeO_3^{2-}, and Ag^{+} was achieved within a week with efficiency higher than 99%. Cleraly, biotransformation reactions involved Hg^{2+}, CrO_4^{2-}, SeO_3^{2-}, and TeO_3^{2-} desolubilization. The Hg^{2+} ion was reduced by an inducible mercuric reductase (see above), elemental Se^0 and Te^0 were produced through reduction of the corresponding tetravalent forms with glutathione reductase, while chromate was reduced to insoluble Cr^{3+} by a membrane bound chromate reductase[189]. The mechanism of precipitation of Pb^{2+}, Ag^{+}, and Cd^{2+} has not been documented at the enzymatic level, but changes in the oxidation numbers of lead and silver (from +2 to +4 and from +1 to 0, respectively) were reported.

Many microorganisms have been shown to catalyze changes in the metal(loid) redox state with the resulting production of in their insoluble forms[25,33,40]. Dissimilatory Fe^{3+}-reducing bacteria such as *Geovibrio ferrireducens*[199], *Geobacter metallireducens* and *Schewanella putrefaciens*[200] are able to reduce UO_2^{2-}, Co^{3+} and $Tc_2O_8^{-}$ to less-soluble species. The $H_2AsO_4^{-}$ anion has been found as a terminal electron acceptor in some bacteria[40]. The trivalent arsenic is soluble but it can combine with sulfide (produced by sulfate-reducing bacteria) to form the insoluble As_2S_3 species (ref.[201]). The natural ability of some bacteria to conserve energy obtained from selenate reduction and form elemental Se^0 may be employed in wastewater

treatment for selenium bioremediation, as shown with a *Thauera selenatis* bioreactor that reached the pilot-plant scale[202]. The process was capable of removing 98% of the selenium present in drainage water.

Both aerobic and anaerobic reduction of CrO_4^{2-} has also been reported[40,203]. The enzymatic activity is generally located (at least partially) at the cell surface of anaerobic bacteria and in the cytoplasm of aerobes[189]. The chromate-reducing ability is ubiquitous among heterotrophic microorganisms[40]. Various organic pollutants such as aromatic can be exploited by bacteria as electron sources for chromate reduction[204,205] resulting in the simultaneous decontamination of organics and chromium. A chromate-reducing strain of *Enterobacter cloacae* was found to be resistant to high levels of chromate (10 mM) and to reduce CrO_4^{2-} to the insoluble Cr^{3+} form[206]. Both fed-batch and dialysis reactors have been proposed for remediation processes exploiting this bacterium[207]. Plants designed for the complete reduction of chromate (inflowing metal concentration of 190 ppm) by *Bacterium dechromaticus* (Romanenko) were built in the Ukraine and Russia in the middle 70's (ref.[208]). The reduction rate of these systems was reported to be 1 g of potassium chromate per g dry weight of bacteria in 72 h. Some other chromate reducing bacteria or mixed cultures were used as immobilized biofilms[25]. However, the use of chromate reducing bacteria seems to have a much more favorable economic impact in the case of chromate reduction and precipitation in the soil than for wastewater cleanup. The latter is more efficiently performed with abiotic processes[40]. The acceleration of chromate reduction by nutrient amendment of the contaminated soil might further improve *in situ* bioremediation as it was reported for the use of glucose[209] or economically more desirable molasses as nutritional supplements[210,211].

2.3. Mixed-Function Consortia for Bioremediation of Heavy Metal and Metalloid Pollution

Many of the above described metal-immobilization mechanisms such as biosorption, metal sulfide precipitation, and biotransformation as well as intracellular metal accumulation operate in the processes that use either natural or artificial biotopes or microbial consortia for waste [water] decontamination from metal(loid)s. Waste streams are either treated directly or more often after an abiotic sewage treatment that can remove up to 40–60% of the metal[33]. Important issues are the input concentrations of metals with the respect to their toxicity. As these processes usually do not employ extremely metal-resistant organisms, they are a more or less sensi-

tive (steady-state) equilibrium. Therefore, the process may break-down when the consortium (or some of its components) is overdosed with a particular metal(loid).

2.3.1. Activated Sludge in Heavy Metal Removal

The activated sludge process is aimed at lowering the organic content of waste (drainage) water by a community of microorganisms contained in a reactor that is constantly supplied with organic matter (waste) and oxygen[198]. Most of the microorganisms present in the activated sludge are bacteria, but other organisms such as cyanobacteria, fungi, yeasts, algae, protozoa, and some metazoa may also play important roles in the wastewater clean-up process[198]. The crucial event in activated sludge is the formation of flocks of 50 to 500 μm in diameter that are composed of a wide spectrum of living or dead (*i.e.*, biopolymers of) bacteria, fungi, protozoa, and metazoa. The major components of flocks are heterotrophic bacteria belonging to the genera of *Pseudomonas* sp., *Achromobacter* sp., *Flavobacterium* sp., *Alcaligenes* sp., *Citrobacter* sp., and *Zooglea* sp. The flocculation accompanying the activated sludge process may also be used for the (auto)immobilization of bacteria potentially important for bioremediation[212].

At present, the main process involved in metal(loid) removal by activated sludge seems to be the biosorption and adsorption (entrapment) of inorganic precipitates[213]. Although, the biotransformation and intracellular accumulation of metal(loid)s are also involved their contribution is assumed to be considerably lower[198]. In an intriguing study, Kodukula *et al.*[214] showed that in activated sludge biosorption the decrease in soluble metal concentration due to biosorption to the microorganisms or to their debris results in the subsequent dissolution of the inorganic precipitate. The partitioning between soluble metal ion and its precipitate is driven by the solubility constant. Metal binding biopolymers in activated sludge consist of various microbial cell wall polysaccharides, bacterial capsules and other excreted polysaccharides, proteins, and nucleic acids released from dead cells[198,215,216]. The formation of highly crosslinked extracellular polysaccharides by the pseudomonad *Zooglea ramigera* is thought to be the main event in flock formation. More recently, hydrophobic interactions between cell-surface proteins[217,218], the role of cations[219] as well as the involvement of lectin-like proteins[220] in bioflocculation have been extensively studied. It appears that the binding of lectin-like proteins to crosslinked polysaccharides stabilizes the biopolymer network of flocks which is further stabilized

through by divalent cations through coordination by biopolymer ligands and the resulting formation of interconnecting bridges.

The production, chemical composition, and metal binding properties of *Z. ramigera* exopolysaccharides were studied in more detail[27]. Depending on the strain the exopolysaccharide is composed by amino sugars, glucose (fibrillar, cellulose-like) and/or by glucose, galactose, and pyruvate. Whole cells of *Z. ramigera* were found to be able to bind, respectively, 2.7, 6.3 and 1.6 moles of Cd^{2+}, Cu^{2+}, and UO_2^{2-} per gram of dry weight[221] (*cf.* the data in Table III).

Quite noteworthy, heavy metal and metalloid removal by the activated sludge is a consequence of the main process taking place in the sludge, namely lowering of the biological oxygen demand (BOD) value of wastewater. The use of activated sludge, as a biosorbent does not necessarily lead to the economically feasible process. Despite encouraging results obtained at the laboratory scale[222], pilot-plant employing activated sludge for the cleanup of wastewaters from the plating industry has been superseded by the chemical precipitation method[223].

2.3.2. Removal of Heavy Metals in Artificial Wetlands and Stream Meanders

Aquatic environments are composed of higher plants that produce organic matter and remove metals by biosorption and by algae produce nutrients to support other heterotrophic organisms in the nearby environment. These organisms, together with aerobic microorganisms remove metals from the water column *via* biosorption and biotransformation, while anaerobic processes (metal sulfide precipitation, biotransformation, and sorption of either organics or inorganics) take place in the sediment[33,35,41]. The "Meander channel" system is operated for the purification of metal-loaded wastewater in the Homestake lead mine (MO, U.S.A.). At least 99% of the input Pb^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, $Fe^{2+/3+}$, and Cd^{2+} are removed from the water column using complex consortia mainly composed of cyanobacteria, algae, and higher plants, including *Potomogeton* and *Typha*[33,41,224] (see also Table III). Final fixation of heavy metal ions occurs *via* sulfide precipitation during the anaerobic decay of settled detritious material. In another acid reduction using microbiology system, called ARUM, process consists of an array of approximatelly 90 to 100 m^3 of "wetlands" separated by permeable dams[225].

Wetland performance is sensitive to environmental changes; it is thus variable and quite unreliable at present[226]. Nevertheless, wetland function

is being further studied[227,228] and modeled[229], and new technologies exploiting wetlands for the remediation of metal pollution are expected soon.

3. CONCLUSIONS

Bioremediation could be a cost-effective alternative to physico-chemical decontamination methods. During the last quarter of this century, we have witnessed an increasing understanding of metal interactions with (micro)organisms that resulted in the design of effective processes for metal bioremediation. Novel processes stemming from recombinant microorganisms or transgenic plants have been proposed based on an important understanding of the molecular mechanisms of metal uptake, binding to biopolymers, and detoxification. However, microbial remediation of metals as well as their phytoremediation is still mainly a basic research problem. The few large-scale applications that have been set up are mainly biosorption and the use of artificial biotopes. Many of the encouraging and potent bioprocesses described above have been developed with genetically engineered *E. coli*, tobacco or *Arabidopsis*, model organisms with no or poor significance for bioremediation technology. Thus, the construction of environmentally robust (micro)organisms possessing the properties that have been shown to be most instrumental for metal remediation remains a future challenge. It should also be stressed, here, that practicable bioremediation technology relies not only on metal interaction with a particular (micro)organism but also on the bioavailability of proper instrumentation allowing growth and survival in contaminated areas and bioreactor set-up (*ex situ* processes). Therefore, organisms with the capacity to grow, survive and properly function in a metal-contaminated environment (in both *ex* and *in situ* remediation processes) are strongly needed. Engineering advances and efficient methods for process screening are similarly required to make the technology robust. *Ex situ* metal remediation processes such as wastewater clean-up may be performed adjusting some parameters and by applying "standard protocols" for *e.g.* biosorption. On the contrary, the *in situ* bioremediation of heavy metal pollution seems to be more restricted by parameters that are difficult to control (*e.g.* climate, soil matrix) and thus processes that are largely independent from such variables need to be rapidly optimized. Present policy regulating the release of genetically modified organisms (GMO) into the environment also considerably limits the use of tailored "wonder-organisms" and may thus slow down program in this research field. However, more favorable laws might be the catalyst of scientific work (that evolves independently anyway) and thus novel GMO

policies could break the barrier that separates the remediation technology market and bioremediation techniques[230].

Notes Added at Editing Proof

Pseudomonas putida was genetically engineered to produce mouse MT and the beta domain of the IgA protease of *Neisseria* in the outer membrane. Resulting highly robust microorganism growing in highly contaminated habitats showed three-fold increased metal-binding capacity[231]. Similarly, engineering of a mouse metallothionein on the cell surface *Ralstonia eutropha* CH34 resulted in an enhanced ability to adsorb Cd^{2+} from the medium[232].

Recently bacterial metal-resistance and metal regulatory proteins (*Synechococcus* PCC 7942 metallothionein and the MerR regulatory protein) were shown to serve as biosensors for heavy metals allowing the detection of 10^{-15} M concentrations of Hg^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} in pure solutions[233]. Very recent advances in the metal mobilization by microbial leaching and metal alkylation, as well as the metal immobilization by production of extracellular matrix[234], reduction[235–237], sulfide precipitation[238,239], of significant importance in heavy metal remediation are summarized in a comprehensive review by Gadd[240].

For a review of the exploitation of recently isolated phytochelatin synthase genes in the heavy metal detoxification[241,242] see Cobbett[243].

REFERENCES

1. Hughes M. N.: *The Inorganic Chemistry of Biological Processes*. Wiley, Chichester 1990.
2. Morgan J. J., Stumm W. in: *Metals and Their Compounds in the Environment* (E. Merian, Ed.), p. 67. VCH Verlag, Weinheim 1991.
3. Gadd G. M.: *FEMS Microbiol. Lett.* **1992**, *79*, 197.
4. Nieboer E., Richardson D. H. S.: *Environ. Pollut.* **1980**, *1*, 3.
5. Nuorteva P.: *Metal Distribution Patterns and Forest Decline*. Yliopistopaino University Press, Helsinki 1990.
6. Jones R. P., Gadd G. M.: *Enzyme Microb. Technol.* **1990**, *12*, 402.
7. Avery S. V., Codd G. A., Gadd G. M.: *J. Gen. Microbiol.* **1991**, *137*, 405.
8. Dedyukhina E. G., Eroshin V. K.: *Process Biochem. (Oxford)* **1991**, *26*, 31.
9. Huber A. L., Holbein B. E., Kidby D. K. in: *Biosorption of Heavy Metals* (B. Volesky, Ed.), p. 249. CRC Press, Boca Raton 1990.
10. Volbeda A., Charon M. H., Piras C., Hatchikian E. C., Frey M., Fontecilla-Camps J. C.: *Nature* **1995**, *373*, 580.
11. Pearson M. A., Michel L. O., Hausinger R. P., Karplus P. A.: *Biochemistry* **1997**, *36*, 8164.
12. Adman E. T., Godden J. W., Turley S.: *J. Biol. Chem.* **1995**, *270*, 27458.

13. Tsukihara T., Aoyama H., Yamashita E., Tomizaki T., Yamaguchi H., Shinzawa-Itoh K., Nakashima R., Yaono R., Yoshikawa S.: *Science* **1995**, *269*, 5227.
14. Messerschmidt A., Ladenstein R., Huber R., Bolognesi M., Avigliano L., Petruzzelli R., Rossi A., Finazzi-Agro A.: *J. Mol. Biol.* **1992**, *224*, 179.
15. Astatke M., Grindley N. D., Joyce C. M.: *J. Mol. Biol.* **1998**, *278*, 147.
16. Shiokawa D., Ohyana H., Yamada T., Tanuma S.: *Biochem. J.* **1997**,*326*, 675.
17. Brock T. D., Madigan M. T., Martinko J. M., Parker J.: *Biology of Microorganisms,* 7th ed. Prentice-Hall, New Jersey 1994.
18. Magoneth E., Hayen P., Delforge D., Delaive E., Remarcle J.: *Biochem. J.* **1992**, *287*, 361.
19. Vallee B. L., Coleman J. E., Auld D. S.: *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 999.
20. Thews G., Mutschler E., Vaupel P.: *Human Anatomy, Physiology and Pathophysiology*. Elsevier, Amsterdam 1985.
21. Voet D., Voet J. G.: *Biochemistry*, 2nd ed. J. Wiley, New York 1995.
22. Norris V., Grant S., Freestone P., Canvin J., Sheikh F. N., Toth I., Trinei M., Modha K., Norman R. I.: *J. Bacteriol.* **1996**, *178*, 367.
23. Bott M., Pfister K., Burda P., Kalbermatter O., Woehlke G., Dimroth P.: *Eur. J. Biochem.* **1997**, *250*, 590.
24. Huder J. B., Dimroth P.: *J. Biol. Chem.* **1993**, *268*, 24564.
25. Gadd G. M. in: *Molecular Biology and Biotechnology of Extremophiles* (R. A. Herbert and R. J. Sharp, Eds), p. 225. Blackie, Glasgow 1992.
26. Rulíšek L., Vondrášek J.: *J. Inorg. Biochem.* **1998**, *71*, 115.
27. Macaskie L. E., Dean A. C. R. in: *Biosorption of Heavy Metals* (B. Volesky, Ed.), p. 199. CRC Press, Boca Raton 1990.
28. Seiler H. G., Sigel H. (Eds): *Handbook on Toxicity of Inorganic Compounds*. Marcel Dekker, Basel 1988.
29. Nriagu J. O., Pacyna J. M.: *Nature* **1988**, *333*, 134.
30. Parr R. G., Pearson R. G.: *J. Am. Chem. Soc.* **1983**, *105*, 7512
31. Klikorka J., Hájek B., Votinský J.: *Obecná a anorganická chemie*, 2nd ed. SNTL/Alfa, Praha 1989.
32. Martin R. B.: *J. Chem. Educ.* **1987**, 402.
33. Gadd G. M. in: *Microbial Control of Pollution* (J. C. Fry, G. M. Gadd, R. A. Herbert, C. W. Jones and I. A. Watson-Craik, Eds), p. 59. Cambridge University Press, Cambridge 1992.
34. Brooks R. R., Chambers M. F., Nicks L. J., Robinson B. H.: *Trends Plant Sci.* **1998**, *3*, 359.
35. White C., Sayer J. A., Gadd G. M.: *FEMS Microbiol. Rev.* **1997**, *20*, 503.
36. Mergeay M.: *NATO ASI Ser., Ser. 3* **1997**, *19*, 65.
37. Unz R. F., Shuttleworth K. L.: *Curr. Opin. Biotechnol.* **1996**, *7*, 307.
38. Volesky B., Holan Z. R.: *Biotechnol. Prog.* **1995**, *11*, 235.
39. Kratochvil D., Volesky B.: *Trends Biotechnol.* **1998**, *16*, 291.
40. Lovley D. R., Coates J. D.: *Curr. Opin. Biotechnol.* **1997**, *8*, 285.
41. Gadd G. M., White C.: *Trends Biotechnol.* **1993**, *11*, 353.
42. Salt D. E., Smith R. D., Raskin I.: *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 643.
43. Chaney R. L., Malik M., Li Y. M., Brown S. L., Brewer E. P., Angle J. C., Baker A. J. M.: *Curr. Opin. Biotechnol.* **1997**, *8*, 279.
44. G. Berthon (Ed.): *Handbook of Metal–Ligand Interactions on Biological Fluids*, Vol. 1. Marcel Dekker, New York 1995.
45. Hellinga H. W. in: *Protein Engineering: Principles and Practise* (J. F. Cleland and C. S. Craik, Eds), p. 369. Wiley–Liss, New York 1996.

46. Kellner D. G., Maves S. A., Sligar S. G.: *Curr. Opin. Biotechnol.* **1997**, *8*, 274.
47. Klaassen C. D., Liu J., Choudhuri S.: *Annu. Rev. Pharmacol. Toxicol.* **1999**, *39*, 267
48. Margoshes M., Vallee B. L.: *J. Am. Chem. Soc.* **1957**, *79*, 4813.
49. Kägi J. H. R.: *Methods Enzymol.* **1991**, *205*, 613.
50. Adams M. D., Kerlavage A. R., Fleischmann R. D., Fuldner R. A., Bult C. J., Lee N., et al.: GenBank accession No. T27991.
51. Glanville N., Durham D. M., Palmiter R. D.: *Nature* **1981**, *292*, 267.
52. Butt. T. R., Ecker D. J.: *Microbiol. Rev.* **1987**, *51*, 351.
53. Kawashima I., Kennedy T. D., Chino M., Lane B. G.: *Eur. J. Biochem.* **1992**, *209*, 971.
54. Evans I. M., Gatehouse L. N., Gatehouse J. A., Robinson N. J., Croy R. R. D.: *FEBS Lett.* **1990**, *262*, 29.
55. Zhou J., Goldsbrough P. B.: *Plant Cell* **1994**, *6*, 875.
56. Chatthai M., Kaukinen K. H., Tranbarger T. J., Gupta P. K., Misra S.: *Plant Mol. Biol.* **1997**, *34*, 243.
57. Rauser W. E.: *Plant Physiol.* **1995**, *109*, 1141.
58. Zenk M. H.: *Gene* **1996**, *179*, 21.
59. Kotrba P., Macek T., Ruml T.: *Collect. Czech. Chem. Commun.* **1999**, *64*, 1057.
60. Robinson N. J., Tommey A. M., Kuske C., Jackson P. J.: *Biochem. J.* **1993**, *295*, 1.
61. Templeton D. M., Cherian M. G.: *Methods Enzymol.* **1991**, *205*, 11.
62. Chubatsu L. S., Meneghini R.: *Biochem. J.* **1993**, *291*, 193.
63. Lau J. C., Cherian M. G.: *Biochem. Cell Biol.* **1998**, *76*, 615.
64. Cherian M. G.: *Environ. Health Perspect.* **1994**, *102 (Suppl. 3)*, 131.
65. Nielson K. B., Atkin C. L., Winge D. R.: *J. Biol. Chem.* **1985**, *260*, 5342.
66. Nielson K. B., Winge D. R.: *J. Biol. Chem.* **1985**, *260*, 8698.
67. Nielson K. B., Winge D. R.: *J. Biol. Chem.* **1983**, *258*, 13063.
68. Nielson K. B., Winge D. R.: *J. Biol. Chem.* **1984**, *259*, 4941.
69. Kurasaki M., Emoto T., Arias A. R. L., Okabe M., Yamasaki F., Oikawa S., Kojima Y.: *Protein Eng.* **1996**, *9*, 1173.
70. Wang Y., Mackay E. A., Kurasaki M., Kägi J. H. R.: *Eur. J. Biochem.* **1994**, *225*, 449.
71. Chang C.-C., Huang P. C.: *Protein Eng.* **1996**, *9*, 1165.
72. Cols N., Romero-Isart N., Bofill R., Capdevila M., Gonzáles-Duarte P., Gonzáles-Duarte R., Atrian S.: *Protein Eng.* **1999**, *12*, 265.
73. Mehra R. K., Winge D. R.: *J. Cell. Biochem.* **1991**, *45*, 30.
74. Reed R. H., Gadd G. M. in: *Heavy Metal Tolerance in Plants: Evolutionary Aspects* (A. J. Shaw, Ed.), p 105. CRC Press, Boca Raton 1991.
75. Huckle J. W., Morby A. P., Turner J. S., Robinson N. J.: *Mol. Microbiol.* **1993**, *7*, 177.
76. White C. N., Rivin C. J.: GeneBank accession No. Z34469, 1994.
77. Dameron C. T., Winge D. R., George G. N., Sansone M., Hu S., Hamer D.: *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6127.
78. Fürst P., Hu S., Hackett R., Hamer D.: *Cell* **1988**, *55*, 705.
79. White C. N., Rivin C. J.: GenGank accession No. Z34469.
80. Suh M. C., Choi D., Liu J. R.: *Mol. Cells* **1998**, *8*, 678.
81. Schäfer H. J., Greiner S., Rausch T., Haag-Kerwel A.: *FEBS Lett.* **1997**, *404*, 216.
82. Whitelaw C. A., Le Huquet J. A., Thurman D. A., Tomsett A. B.: *Plant Mol. Biol.* **1997**, *33*, 503.
83. Robinson N. J., Wilson J. R., Turner J. S.: *Plant Mol. Biol.* **1996**, *30*, 1169.

84. Tommey A. M., Shi J., Lindsay W. P., Urwin P. E., Robinson N. J.: *FEBS Lett.* **1991**, *292*, 48.
85. Verkleij J. A. C., Schat H. in: *Heavy Metal Tolerance in Plants: Evolutionary Aspects* (A. J. Shaw, Ed.), p. 179. CRC Press, Boca Raton 1990.
86. Baker A. J. M., Walker P. L. in: *Heavy Metal Tolerance in Plants: Evolutionary Aspects* (A. J. Shaw, Ed.), p. 156. CRC Press, Boca Raton 1990.
87. Grill E., Löffler S., Winnacker E.-L., Zenk M. H.: *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6838.
88. Hayashi Y., Nakagawa C. W., Mutoh N. Isobe M., Goto T.: *Biochem. Cell Biol.* **1991**, *69*, 115.
89. Al-Lahham A., Rohde V., Heim P., Leucher R., Veeck J., Wunderlich K.W., Zimmermann M.: *Yeast* **1999**, *15*, 385
90. Strasdeit H., Duhme A.-K., Kneer R., Zenk M. H., Hermes C., Nolting H.-F.: *J. Chem. Soc., Chem. Commun.* **1991**, *16*, 1129.
91. Dameron C. T., Reese R. N., Mehra R. K., Kortan A. R., Carroll P. J., Steigerwald M. L., Brus L. E., Winge D. R.: *Nature* **1989**, *338*, 596.
92. Bae W., Mehra R. K.: *J. Inorg. Biochem.* **1998**, *69*, 33.
93. Mehra R. K., Mulchandani P., Hunter T. C.: *Biochem. Biophys. Res. Commun.* **1994**, *200*, 1193.
94. Salt D. E., Rauser W. E.: *Plant Physiol.* **1995**, *107*, 1293.
95. Ortiz D. F., Ruscitti T., McCue K. F., Ow D. W.: *J. Biol. Chem.* **1995**, *270*, 4721.
96. Berka T., Shatzman A., Zimmerman J., Strickler J., Rosenberg M.: *J. Bacteriol.* **1988**, *170*, 21.
97. Evans K. M., Gatehouse J. A., Lindsay W. P., Shi J., Tommey A. M., Robinson N. J.: *Plant Mol. Biol.* **1992**, *20*, 1019.
98. Shi J., Lindsay W. P., Huckle J. W., Morby A. P., Robinson N. J.: *FEBS Lett.* **1992**, *303*, 159.
99. Lilius G., Persson M., Büflow L., Mosbach K.: *J. Biochem.* **1991**, *198*, 499.
100. Macek T., Macková M., Kotrba P., Truksa M., Singh Cundy A., Scouten W. H.: *Proc. Int. Symp. Environmental Biotechnology, Oostende, 1997*, (H. Verachtert and W. Verstraete, Eds), p. 263. Technologisch Institute, Oostende 1997.
101. Chen S.-L., Wilson D. B.: *Biodegradation* **1997**, *8*, 97.
102. Chen S.-L., Wilson D. B.: *Appl. Environ. Microbiol.* **1997**, *63*, 2442.
103. Chen S.-L., Kim E.-K., Shuler M. L. Wilson D. B.: *Biotechnol. Prog.* **1998**, *14*, 667
104. Liang Zhu Y., Pilon-Smits E. A. H., Jouanin L., Terry N.: *Plant Physiol.* **1999**, *119*, 73.
105. Howden R., Goldsbrough P. B., Andersen C., Cobbet C. S.: *Plant Physiol.* **1995**, *107*, 1059.
106. Clemens S., Kim E. J., Neumann D., Schroeder J. I.: *EMBO J.* **1999**, *18*, 3325.
107. Lefebvre D. D., Miki B. L., Laliberté J.-F.: *Bio/Technology* **1987**, *5*, 1053.
108. Maiti I. B., Wagner G. J., Yeargan R., Hunt A. G.: *Plant Physiol.* **1989**, *91*, 1020.
109. Pan A., Tie F., Yang M., Luo J., Wang Z., Ding X., Li L., Chen Z., Ru B.: *Protein Eng.* **1993**, *6*, 755.
110. Pan A., Tie F., Duau Z., Yang M., Wang Z., Li L., Chen Z., Ru B.: *Mol. Gen. Genet.* **1994**, *242*, 666.
111. Hasegawa I., Terada E., Sunairi M., Wakita H., Schinmachi F., Noguchi A., Nakajima M., Yazaki J.: *Plant Soil* **1997**, *196*, 277.
112. Truksa M.: *Ph.D. Thesis*. Mendel University for Agriculture and Forestry, Brno 1997.

113. Schroeder J. I., Danuta M., Schachtman D. P., Clemens J. I.: PCT Int. Appl. WO9804700 A1 1998; Chem. Abstr. **1998**, *128*, 176940.
114. Yeargan R., Maiti I. B., Nielsen M. T., Hunt A. G., Wagner G. J.: *Transgenic Res.* **1992**, *1*, 261.
115. Hattori J., Labbé H., Miki B. L.: *Genome* **1994**, *37*, 508.
116. Elmayan T., Tepfer M.: *Plant J.* **1994**, *6*, 433.
117. Sousa C., Kotrba P., Ruml T., Cebolla A., de Lorenzo V.: *J. Bacteriol.* **1998**, *180*, 2280.
118. Kotrba P., Pospíšil P., de Lorenzo V., Ruml T.: J. *Recept. Signal Transduct. Res.* **1999**, *19*, 703.
119. Jacobs F. A., Romeyer F. M., Beauchemin M., Brosseau R.: *Gene* **1989**, *83*, 95.
120. Holan Z. R., Volesky B., Prasetyo I.: *Biotechnol. Bioeng.* **1993**, *41*, 819.
121. Schneider I. A. H., Rubio J.: *Environ. Sci. Technol.* **1999**, *33*, 2213.
122. Brierley J. A., Brierley C. L., Goyak G. M. in: *Fundamental and Applied Biohydrometallurgy* (R. W. Lawrence, R. M. Branion and H. G. Ebner, Eds), p. 291. Elsevier, Amsterdam 1986.
123. Mattuschka B., Junghaus K., Straube G. in: *Biohydrometallurgical Technologies* (A. E. Torma, M. L. Apel and C. L. Brierley, Eds), Vol. 2, p. 125. The Minerals, Metals & Materials Soc., Warrendale 1993.
124. Fourest E., Roux J. C.: *Appl. Microbiol. Biotechnol.* **1992**, *37*, 399.
125. Tobin J. M., Cooper D. G., Neufeld R. J.: *Appl. Environ. Microbiol.* **1984**, *47*, 821.
126. Holan Z. R., Volesky B.: *Biotechnol. Bioeng.* **1994**, *41*, 1001.
127. Holan Z. R., Volesky B.: *Appl. Microbiol. Biotechnol.* **1995**, *43*, 1125.
128. Friis N., Myers-Keith P.: *Biotechnol. Bioeng.* **1986**, *28*, 21.
129. Volesky B., May-Philips H. A.: *Appl. Microbiol. Biotechnol.* **1995**, *42*, 797.
130. Fourest E., Serre A., Roux J. C.: *Toxicol. Environ. Chem.* **1996**, *54*, 1.
131. Fourest E., Volesky B.: *Environ. Sci. Technol.* **1996**, *30*, 277.
132. Beveridge T. J., Murray R. G. E.: *J. Bacteriol.* **1980**, *141*, 876.
133. Fourest E. Roux J. C.: *FEMS Microbiol. Rev.* **1994**, *14*, 325.
134. Jeffers T. H., Bennet P. G., Corvin R. R.: Document No. RI9461. Bureau of Mines, UT, U.S.A. 1993.
135. Brierley C. L., Brierley J. A.: in: *Biohydrometallurgical Technologies* (A. E. Torma, M. L. Apel and C. L. Brierley, Eds), Vol. 2, p. 35. The Minerals, Metals & Materials Soc.,Warrendale 1993.
136. Bedell G. W., Darnall D. W. in: *Biosorption of Heavy Metals* (B. Volesky, Ed.), p. 313. CRC Press, Boca Raton 1990.
137. Sousa C., Cebolla A., de Lorenzo V.: *Nat. Biotechnol.* **1996**, *14*, 1017.
138. Xu Z., Lee S.Y.: *Appl. Environ. Microbiol.* **1999**, *65*, 5142.
139. Mejare M., Ljung S., Bulow L.: *Protein Eng.* **1998**, *11*, 498.
140. Valls M., Gonzales-Duarte R., Atrian S, de Lorenzo V.: *Biochimie* **1998**, *80*, 855.
141. Kjaergaard K., Sorensen J. K., Schembri M. A., Klemm P.: *Appl. Environ. Microbiol.* **2000**, *66*, 10.
142. Samuelson P., Wernerus H., Svedberg M., Stahl S.: *Appl. Environ. Microbiol.* **2000**, *66*, 1243.
143. Kotrba P., Dolečková L., de Lorenzo V., Ruml T.: *Appl. Environ. Microbiol.* **1999**, *65*, 1092.
144. Pazirandeh M., Wells B. M., Ryan R. L.: *Appl. Environ. Microbiol.* **1998**, *64*, 4068.

145. Yamamura T., Watanabe T. in: *Peptide Chemistry* (N. Yanaihara, Ed.), p. 281. ESCOM, Leiden 1993.
146. Yamamura T., Sasaki Y, Ueki M. in: *Peptide Chemistry* (N. Yanaihara, Ed.), p. 284. ESCOM, Leiden 1993.
147. Kotrba P., Dolečková L., Pavlík M., Ruml T.: *Biotechnol. Tech.* **1996**, *10*, 773.
148. Haymore B. L., Bild G. S., Salsgiver W. J., Staten N. R., Krivi G. G.: *Methods Companion Methods Enzymol.* **1992**, *4*, 25.
149. Bianchi E., Folgory A., Wallace A., Nicotra M., Acali S., Phalipon A., Barbato G., Bazzo R., Cortese R., Felici F., Pessi A.: *J. Mol. Biol.* **1995**, *247*, 154.
150. Brown S.: *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8651.
151. Brown S.: *Nat. Biotechnol.* **1997**, *15*, 269.
152. Schreuder M. P., Mooren A. T. A., Toschka H. Y., Verrips C. T., Klis F. M.: *Trends Biotechnol.* **1996**, *14*, 115.
153. Macaskie L. E., Bonthrone K. M., Rouch D. A.: *FEMS Microbiol. Lett.* **1994**, *121*, 141.
154. Basnakova G., Stephens E. R., Thaller M. C., Rossolini G. M., Macaskie L. E.: *Appl. Microbiol. Biotechnol.* **1998**, *50*, 266.
155. Jeong B. C., Hawes C., Bonthrone K. M., Macaskie L. E.: *Microbiology* **1997**, *143*, 2497.
156. Jeong B. C., Poole P. S., Willis A. C., Macaskie L. E.: *Arch. Microbiol.* **1998**, *169*, 166.
157. Hallett D. S., Clark P., Macaskie L. E.: *FEMS Microbiol. Lett.* **1991**, *62*, 7.
158. Finlay J. A., Allan V. J., Conner A., Callow M. E., Basnakova G., Macaskie L. E.: *Biotechnol. Bioeng.* **1999**, *63*, 87.
159. Macaskie L. E.: *J. Chem. Technol. Biotechnol.* **1990**, *49*, 357.
160. Macaskie L. E.: *Science* **1992**, *257*, 782.
161. Macaskie L. E., Dean A. C. R.: *Environ. Technol. Lett.* **1984**, *5*, 177.
162. Macaskie L. E., Dean A. C. R.: *Biotechnol. Lett.* **1985**, *7*, 457.
163. Macaskie L. E., Jeong B. C., Tolley M. R.: *FEMS Microbiol. Lett.* **1994**, *14*, 351.
164. Montgomery D. M., Dean A. C., Wiffen P., Macaskie L. E.: *Microbiology* **1995**, *141*, 2433.
165. Thomas R. A. P., Macaskie L. E.: Environ. Sci. Tech. **1996**, *30*, 2371.
166. Diels L., VanRoy S., Taghavi S., Doyen W., Leysen R., Mergeay M. in: *Biohydrometallurgical Technologies* (A. E. Torma, M. L. Apel and C. L. Brierley, Eds), Vol. 2, p. 133. The Minerals, Metals & Materials Soc., Warrendale 1993.
167. Diels L., VanRoy S., Mergeay M., Doyen W., Taghavi S., Leysen R. in: *Effective Membrane Processes: New Perspectives* (Paterson, Ed.), p. 275. Kluwer Academic Publiskers, Dordrecht 1993.
168. Diels L., Dong Q., van der Lelie D., Baeyens W., Mergeay M.: *J. Ind. Microbiol.* **1995**, *14*, 142.
169. Nies D. H.: *Plasmid* **1992**, *27*, 17.
170. Silver S., Walderhaug M.: *Microbiol. Rev.* **1992**, *56*, 195.
171. Nies D. H.: *J. Bacteriol.* **1992**, *174*, 8102.
172. Grosse C., Grass G., Anton A., France S., Santos A. N., Lawley B., Brown N. L., Nies D. H.: *J. Bacteriol.* **1999**, *181*, 2385.
173. Rensing C., Pribyl T., Nies D. H.: *J. Bacteriol.* **1997**, *179*, 6871.
174. Nies D. H.: *J. Bacteriol.* **1995**, *177*, 2707.
175. van der Lelie D., Schwuchow T., Schwiedetzky U., Wuertz S., Baeyens W., Mergeay M., Nies D. H.: *Mol. Microbiol.* **1997**, *23*, 493.

176. Springael D., Diels L., Hooyberghs L., Kreps S., Mergeay M.: *Appl. Environ. Microbiol.* **1993**, *59*, 334.
177. Springael D., Diels L., Mergeay M.: *Biodegradation* **1994**, *5*, 343.
178. White C., Gadd G. M.: *Microbiology* **1996**, *143*, 2197.
179. White C., Gadd G. M.: *Microbiology* **199**8, *144*, 1407.
180. Wood J. M., Wang H. K.: *Environ. Sci. Technol.* **1983**, *17*, 582.
181. Barnes L. J.: *Trans. Inst. Min. Metall., Sec. C* **1996**, *105*, C113.
182. Barnes L. J., Janssen F. J., Sherren J., Versteegh J. H., Koch R. O., Scheeren P. J. H.: *Trans. Inst. Chem. Eng.* **1991**, *69*, 184.
183. Williams J. W., Silver S.: *Enzyme Microb. Technol.* **1984**, *6*, 530.
184. Hamlett N. V., Landale E. C., Davis B. H., Summers A. O.: *J. Bacteriol.* **1992**, *174*, 6377.
185. Moeby A. P., Hobman J. L., Brown N. L.: *Mol. Microbiol.* **1995**, *17*, 25.
186. Uno Y., Kiyono M., Tezuka T., Pan-Hou H.: *Biol. Pharm. Bull.* **1997**, *20*, 107.
187. Sahlman L., Wong W., Powlowski J.: *J. Biol. Chem.* **1997**, *272*, 29518.
188. Hansen C. L., Zwolinski G., Martin D., Williams J. W.: *Biotechnol. Bioeng.* **1984**, *26*, 1330.
189. Blake R. C., Choate D. M., Bardhan S., Revis N., Barton L. L., Zocco T. G.: *Environ. Toxicol. Chem.* **1993**, *12*, 1365.
190. Brunke M., Deckwer W.-D., Frischmuth A., Horn J. M., Lunsdorf H., Rhode M., Rohricht M., Timmis K. N., Weppen P.: *FEMS Microbiol. Rev.* **1993**, *11*, 145.
191. Horn J. M., Brunke M., Deckwer W.-D., Timmis K. N.: *Appl. Environ. Microbiol.* **1994**, *60*, 357.
192. Rugh C. L., Wilde H. D., Stack N. M., Thompson D. M., Summers A. O., Meagher R. B.: *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 3182.
193. Rugh C. L., Senecoff J. F., Meagher R. B., Merkle S. A.: *Nat. Biotechnol.* **1998**, *16*, 925.
194. Gadd G. M.: *FEMS Microbiol Rev.* **1993**, *11*, 297.
195. Thayer J. S.: *Appl. Organomet. Chem.* **1989**, *3*, 123.
196. Gao S. Tanji K. K.: *J. Environ. Qual.* **1995**, *24*, 191.
197. Sreekrishnan T. R., Tyagi R. D.: *Process Biochem.* **1995**, *30*, 69.
198. Kasan H. C.: *C. R. Environ. Sci. Technol.* **1993**, *23*, 79.
199. Caccavo F. Jr., Coates J. D., Rosello-Mora R. A., Ludwig W., Schleifer K. H., Lovley D. R., McInerney M. J.: *Arch. Microbiol.* **1996**, *165*, 370.
200. Lloyd J. R., Macaskie L. E.: *Appl. Environ. Microbiol.* **1996**, *62*, 578.
201. Rittle K. A., Drever J. I., Colberg P. J. S.: *Geomicrobiology* **1995**, *13*, 1.
202. Cantafio A. W., Hagen K. D., Lewis G. E., Bledsoe T. L., Nunan K. M., Macy J. M.: *Appl. Environ. Microbiol.* **1996**, *62*, 3298.
203. Lovley D. R.: *Annu. Rev. Microbiol.* **1993**, *14*, 159.
204. Shen H., Pritchard H., Sewell G. W.: *Biotechnol. Bioeng.* **1996**, *52*, 357.
205. Shen H., Pritchard H., Sewell G. W.: *Environ. Sci. Technol.* **1996**, *30*, 1667.
206. Fuji E., Toda K., Ohtake H.: *J. Ferment. Bioeng.* **1990**, *69*, 365.
207. Komori K., Rivas A., Toda K., Ohtake H.: *Biotechnol. Bioeng.* **1990**, *35*, 951.
208. Koren'kov V. N., Vorobyova L. F. in: *Microbial Methods of Enviromental Pollution Control* (V. A. Lambina, Ed.), p. 50. Academy of Sciences of U.S.S.R., Pushchino 1979.
209. Turick C. E., Graves C., Apel W. A.: *Bioremediation J.* **1998**, *2*, 1.
210. Rege M. A., Petersen J. N., Johnstone D. L., Turick C. E., Yonge D. R., Apel W. A.: *Biotechnol. Lett.* **1997**, *19*, 691.
211. Turick C. E., Camp C. E., Apel W. A.: *Appl. Biochem. Biotechnol.* **1997**, *63*, 871.

212. Houba C., Remarcle J.: *Appl. Environ. Microbiol.* **1984**, *47*, 1158.
213. Gould M. S., Genetelli E. J.: *Water Res.* **1984**, *18*, 123.
214. Kodukula P. S., Patterson J. W., Surampalli R. Y.: *Biotechnol. Bioeng.* **1994**, *43*, 874.
215. Chudoba J., Hejzlar J., Doležal M.: *Water Res.* **1986**, *20*, 1223.
216. Hejzlar J., Chudoba J.: *Water Res.* **1986**, *20*, 1217.
217. Zita A., Hermansson M.: *Appl. Environ. Microbiol.* **1997**, *63*, 1168.
218. Jorand F., Boue-Bigne F., Block J. C., Urbain V.: *Water Sci. Technol.* **1998**, *37*, 307.
219. Keiding K., Nielsen P. H.: *Water Res.* **1997**, *31*, 1665.
220. Higgins M. J., Novak J. T. J.: *Environ. Engineer.* **1997**, *123*, 485.
221. Norberg A. B., Persson H.: *Biotechnol. Bioeng.* **1984**, *26*, 239.
222. Atkinson B. W., Bux F., Kasan H. C.: *Water Sci. Technol.* **1996**, *34*, 9.
223. Atkinson B. W., Bux F., Kasan H. C.: *Water SA* **1998**, *24*, 129.
224. Gale N. L. in: *Biotechnology for the Mining, Metal Refining and Fossil Fuel Industries* (H. L. Erlich and D. S. Holmes, Eds), p. 171. John Wiley, New York 1985.
225. Kalin M., Fyson A., Smith M. P. in: *Biohydrometallurgical Technologies* (A. E. Torma, M. L. Apel and C. L. Brierley, Eds), Vol. 2, p. 125. The Minerals, Metals & Materials Soc.,Warrendale 1993.
226. Wieder R. K.: *Wetlands* **1989**, *9*, 299.
227. Webb J. S., McGinness S., Lappin-Scott H. M.: *J. Appl. Microbiol.* **1998**, *84*, 240.
228. Hawkins W. B., Rodgers J. H. Jr., Gillespie W. B. Jr., Dunn A. W., Dorn P. B., Cano M. L.: *Ecotoxicol. Environ. Safety* **1997**, *36*, 238.
229. Hruby T.: *Environ. Manage.* **1999**, *23*, 75.
230. MacDonald J. A: *Environ. Sci. Technol.* **1997**, *31*, 560A.
231. Valls M., de Lorenzo V., Gonzalez-Duarte R., Atrian S.: *J. Inorg. Biochem.* **2000** *79*, 219.
232. Valls M., Atrian S., de Lorenzo V., Fernandez L. A.: *Nat. Biotechnol.* **2000** *18*, 661.
233. Bontidean I., Lloyd J. R., Hobman J. L., Wilson J. R., Csoregi E., Mattiasson B., Brown N. L.: *J. Inorg. Biochem.* **2000** *79*, 225.
234. Bonthrone K. M., Quarmby J., Hewitt C. J., Allan V. J. M., Paterson-Beedle M., Kennedy J. F., Macaskie L. E.: *Environ. Technol.* **2000**, *21*, 123.
235. Kashefi K., Lovley D. R.: *Appl. Environ. Microbiol.* **2000**, *66*, 1050.
236. Francis C. A., Obraztsova A. Y., Tebo B. M.: *Appl. Environ. Microbiol.* **2000**, *66*, 543.
237. Roden E. E., Urrutia M. M., Mann C. J.: *Appl. Environ. Microbiol.* **2000**, *66*, 1062.
238. Macy J. M., Santini J. M., Pauling B. V., O'Neill A. H., Sly L. I.: *Arch. Microbiol.* **2000**, *173*, 49.
239. Hard B. C., Walther C., Babel W.: *Geomicrobiol. J.* **1999**, *16*, 267.
240. Gadd G. M.: *Curr. Opin. Biotechnol.* **2000**, *11*, 271.
241. Cobbett C. S.: *Plant Physiol.* **2000**, *123*, 825.
242. Cobbett C. S.: *Trends Plant Sci.* **1999**, *4*, 335.
243. Cobbett C. S.: *Curr. Opin. Plant Biol.* **2000**, *3*, 211.