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MINI-REVIEW

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Heavy metal-resistant bacteria as extremophiles: molecular physiology and biotechnological use of *Ralstonia* sp. CH34

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Abstract In contrast to thermophilic or psychrophilic organisms, heavy metal-resistant bacteria do not supply enzymes that are active under harsh conditions, but are themselves tools for the evaluation and remediation of heavy metal-contaminated environments. *Ralstonia* sp. CH34 is a gram-negative bacterium with a remarkable set of resistance determinants, allowing this bacterium to live in extreme environments that are heavily contaminated with toxic metal ions. These heavy metal ions are mostly detoxified by inducible ion efflux systems that reduce the intracellular concentration of a given ion by active export. Because all metal resistance determinants in this bacterium are inducible, their regulatory systems can be used to develop biosensors that measure the biologically important concentrations of heavy metals in an environment. Resistance based on metal ion efflux detoxifies only the cytoplasm of the respective cell. Therefore, this resistance mechanism cannot be used directly to develop biotechnological procedures; however, metal ion efflux can protect a cell in a metal-contaminated environment. Thus, the cell can be enabled to mediate biochemical reactions such as precipitation of heavy metals with the carbon dioxide produced during growth or degradation of xenobiotics.

Key words Heavy metal bioprecipitation · Heavy metal biosensors · Heavy metal resistance · Cadmium · Cobalt · Zinc · Nickel · Chromate · *Ralstonia*

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Introduction

Extreme environments are defined by at least one physical (e.g., temperature, radiation) or chemical (e.g., pH value, NaCl concentration, water availability) parameter that is "extreme." Because understanding of bacteria started with the understanding of *Escherichia coli*, it is easy for microbiologists to differentiate "extreme" from "not extreme": thus, the range of parameters *E. coli* is able to tolerate is not considered extreme, whereas parameters well beyond this range may be considered as extreme. An extreme parameter inhibits growth of *E. coli* by acting nonspecifically on a broad range of cellular targets. This mechanism is in contrast to the action of a toxin that inhibits one or a few well-defined physiological processes. Thus, thermophiles, osmophiles, halophiles, acidophiles, or psychrophiles are easily defined and differentiated from bacteria resistant to a toxin that may be of biological origin.

Heavy metal ions are of nonbiological origin and are toxic to cells at high concentrations but with nonspecific action. Some heavy metal cations are essential as trace elements at lower concentrations; some are only toxic (Nies 1999), but depending on the specific metal ion, they completely prevent growth of *E. coli* at concentrations between 10 μM (Hg^{2+}) and 20 mM (Mn^{2+}) (Table 1). The comparison of the minimal inhibitory concentration (MIC) value with the concentration of a metal in seawater, a kind of "average environment" summing up the global availability of a metal, gives a quotient that describes the probability for each metal to become toxic for a "normal" cell (Table 1). This quotient ranks the heavy metal cations in the order $Zn^{2+} > Ag^{+} > Ni^{2+} >> Cu^{2+} > Hg^{2+} >> UO_2^{2-} > Co^{2+} > CrO_4^{2-} > Au^{3+} > Cd^{2+} > Mn^{2+} >>> Pb^{2+}$. Other heavy metals are not available at "normal" conditions, e.g., at a neutral pH value. By using the *E. coli*-based definition, extreme environments contaminated with heavy metals are easily defined, and the ecological importance of heavy metal extreme environments may even be ranked, depending on the metal. Bacteria able to grow in such an environment may thus be designated "metallophile" bacteria.

Table 1. Toxicity of heavy metal ions in *Escherichia coli*

Heavy metal ions	MIC (mM)[a]	CSW[b] (nM)	1000 CSW/MIC[c]
Hg^{2+}	0.01	0.149	0.015
Ag^{+}	0.02	2.78	0.139
Au^{3+}	0.02	0.056	0.003
CrO_4^{2-}	0.2	0.962	0.005
Pd^{2+}	0.2		0
Pt^{4+}	0.5	n.d.	0
Cd^{2+}	0.5	0.982	0.002
Co^{2+}	1.0	4.58	0.005
Ni^{2+}	1.0	92.0	0.092
Cu^{2+}	1.0	47.2	0.047
Zn^{2+}	1.0	158	0.158
Tl^{+}	2.0	n.d.	0
UO_2^{2-}	2.0	12.6	0.006
Pb^{2+}	5.0	0.145	0
Mn^{2+}	20.0	36.4	0.002

[a] The minimal inhibitory concentration (MIC) was determined on Tris-buffered minerals salts medium, starting pH 7.0, containing 2 g sodium gluconate/l as carbon source, and 1 g yeast extract/l to complement for *E. coli* auxotrophies, 2 days at 30°C (Nies 1999)

[b] Concentration in seawater (CSW) according to Weast et al. (Weast 1984)

[c] Because toxic effects start at about 1/1000 of the MIC, this quotient gives the toxicity of a given metal in seawater

Heavy metal extreme environments are of anthropogenic or geologic origin. For example, chromate-contaminated soils may contain up to 5 g of chromate/kg soil (Snyder et al. 1997), which equates to 100 mM in solution, the 500-fold MIC value for *E. coli*. Anthropogenic sites contaminated with zinc, cobalt, or copper may contain these metals in a comparable range of concentrations (Diels and Mergeay 1990). As much as 40% of the bacteria isolated from sites such as these, on nonselective media, are β-proteobacteria. *Ralstonia* sp. CH34 [formerly *Alcaligenes eutrophus* (Brim et al. 1999)] is the best known example of these heavy metal-resistant bacterial strains, which could be considered as "metallophiles" under the definition given earlier. This review describes the molecular physiology of metal resistance in this bacterium and how fundamental mechanisms related this topic have led to biotechnological applications.

Molecular physiology of heavy metal resistance in *Ralstonia* sp. CH34: the Czc system

Ralstonia sp. CH34 can grow in media containing up to 20 mM Zn^{2+}, 20 mM Co^{2+}, or 5 mM Cd^{2+} (Mergeay et al. 1985). The genetic ability to survive heavy metal concentrations this high resides on a large plasmid, pMOL30, and was designated *czc* for cobalt-zinc-cadmium resistance (Nies et al. 1987). Primarily, the Czc system detoxifies the cell by cation efflux: the three heavy metal cations, which are taken up into the cell by the fast and unspecific transport system for Mg^{2+} (Nies and Silver 1989a), are actively extruded from the cell by products of the *czc* resistance determinant (Nies and Silver 1989b).

The actual efflux protein complex is composed of three subunits, CzcC, CzcB, and CzcA (Nies et al. 1989b). The Czc efflux complex is not an ABC-ATPase, but cation efflux is instead driven by the chemiosmotic gradient (Nies 1995). CzcA is a RND protein (Tseng et al. 1999), the only protein of the Czc complex that contains more than one transmembrane α-helix and, as shown with the isolated and proteoliposome-reconstituted protein, functions as a cation–proton antiporter (Goldberg et al. 1999). CzcA contains two large hydrophilic domains, which are located in the periplasm (Goldberg et al. 1999), as are the other two subunits of the Czc complex, CzcB and CzcC (Rensing et al. 1997). Although CzcA gives some weak metal resistance by itself, CzcB and CzcC are needed for full function (Nies et al. 1989b; Rensing et al. 1997), and the $CzcCB_2A$ complex may be able to transport the toxic cations across the complete gram-negative cell wall from the cytoplasm to the outside (Fig. 1).

The amount of the $CzcCB_2A$ efflux complex present in the cell is tightly regulated. There are at least six regulatory proteins (CzcD, CzcR, CzcS, CzcN, CzcI, and an unknown sigma factor, "RpoX") (Fig. 1) involved in regulation of the three structural genes *czcCBA*, which are transcribed as a tricistronic *czcCBA* mRNA (van der Lelie et al. 1997b), a tetracistronic *czcICBA* mRNA, or a pentacistronic *czcNICBA* mRNA (Große et al. 1999). The membrane-bound CzcN protein and the periplasmic CzcI protein, which are encoded upstream of *czcCBA*, may be involved in regulation of "RpoX," a hypothetical sigma factor that could belong to the extracellular function family (ECF) (Lonetto et al. 1994), as indicated by the conserved nucleotide sequences upstream of the *czc* promoters (Große et al. 1999). ECF sigma factors are usually regulated by protein–protein interaction with a membrane-bound antisigma factor and a periplasmic sensing protein (Missiakas and Raina 1998), which could be CzcN (hypothetical membrane-bound antisigma factor) and CzcI (periplasmic sensor), respectively (Große et al. 1999). Thus, binding of heavy metal cations to CzcI may trigger the release of "RpoX" from CzcN, which leads to transcription from the promoters *czcNp, czcIp, czcCp*, and *czcDp* (Fig. 1).

The other three regulatory proteins, CzcD, CzcR, and CzcS, belong to genes located downstream of *czcCBA* and are transcribed in a tricistronic mRNA, *czcDRS*. Transcription of *czcDRS* is inhibited by transcription of *czcCBA* by an overlapping *cis*-acting structure of the *czcAt* terminator and the *czcDp* promoter (Große et al. 1999; van der Lelie et al. 1997b). CzcDRS are probably only needed in case of a sudden exposure of the cell to heavy metals: the two-component regulatory system CzcRS (Nies and Brown 1998) acts on the *czcNp* promoter and may be controlled by cytoplasmic heavy metal cations only, and thus its possible function would be to bring RpoX under CzcN control (Große et al. 1999). CzcD, on the other hand, is itself an efflux system (Anton et al. 1999), but a less efficient one than $CzcCB_2A$, and CzcD transports heavy metal cations only across the cytoplasmic membrane. Because deletion of *czcD* increases the level of *czcCBA* mRNA in induced as well as uninduced CH34 cells, CzcD might transport the

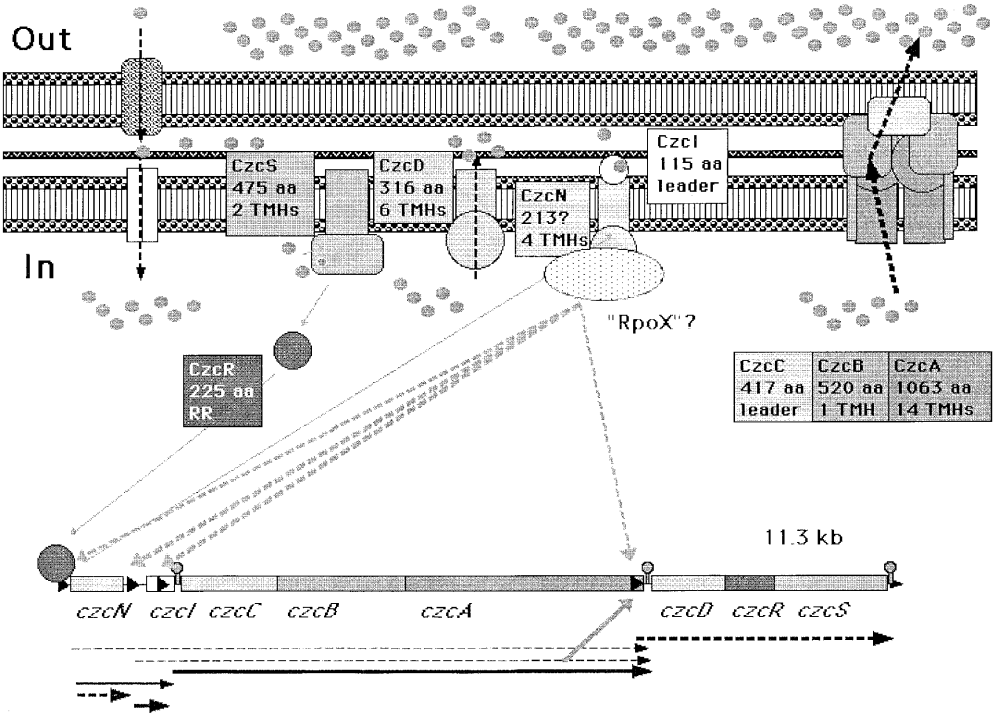

Fig. 1. The Czc resistance system of *Ralstonia* sp. CH34. The *czc* determinant with eight genes from *czcN* to *czcS* is shown at the *bottom*, with *arrows below the genes* indicating the various *czc* mRNAs. Transcription of *czcCBA* inhibits transcription of *czcDRS*, as shown by the *gray arrow pointing upward*. At the *top*, the gram-negative cell wall (with outer membrane, periplasm including murein sacchulus, and cytoplasmic membrane) contains the *czc*- and chromosomally encoded proteins involved in Czc function. Heavy metal cations (*gray dots*) are transported into the cell by facilitated diffusion across the outer membrane and active transport across the cytoplasmic membrane (*left corner at top*) and pumped out by the $CzcCB_2A$-transmembrane efflux system (*right corner at top*). Czc is regulated by two systems: periplasmic cations regulate the availability of an ECF-sigma factor ("RpoX") via CzcNI, and "RpoX" initiates transcription of the *czcNp-*, *czcIp-*, *czcCp-*, and *czcDp* promoters (*gray dashed arrows*) in *middle*. Cytoplasmic cations regulate the specific amount of CzcN by the CzcRS two-component regulatory system (*thin gray arrows in middle*). CzcD transports the cytoplasmic inducers into the periplasm. Thus, cytoplasmic cations regulate the absolute amount of "RpoX" reserved for Czc control and periplasmic cations the actual portion of "RpoX" available for *czc* transcription

cytoplasmic cations, which are inducers of CzcRS, into the periplasm to control RpoX via CzcN and CzcI (Anton et al. 1999). Thus, the complexity of regulation of *czc* may be a direct consequence of cell prevention of starvation in the trace elements Co^{2+} and Zn^{2+}.

Other resistance systems in *Ralstonia* sp. CH34

In addition to *czc*, a variety of other heavy metal resistance systems exist in *Ralstonia* sp. CH34. The structural genes *cnrCBA* of the cobalt–nickel resistance system *cnr* (Liesegang et al. 1993) are related to *czcCBA*. The *cnr* determinant is located on the second megaplasmid of *Ralstonia* sp. CH34, pMOL28 (Taghavi et al. 1997b). $CnrCB_2A$ is likely to form a transmembrane efflux pump for Co^{2+} and Ni^{2+}. Regulation of *cnr*, however, is different from regulation of *czc*. The gene for the ECF sigma factor, *cnrH*, is located directly upstream of *cnrC* and is transcribed in the same direction as *cnrCBA*. Upstream of *cnrH* are additional genes encoding the CnrYX antisigma factor complex involved in regulation of the availability of CnrH (Grass et al. 2000; Liesegang et al. 1993). While Czc is induced best by Zn^{2+} and Co^{2+} (Große et al. 1999), Ni^{2+} induces Cnr with high specificity (Grass et al. 2000; Peitzsch et al. 1998).

Just adjacent to *cnr*, the *chr* determinant (Nies et al. 1989a, 1990; Nies and Silver 1989b) mediates resistance to the toxic oxyanion chromate. Chromate enters the cell by the sulfate uptake system and is effluxed by the ChrAB proteins, which may form a sulfate–chromate antiporter (Nies et al. 1998). Several additional *chr* genes were detected downstream of *chrBA*. These genes encode a superoxide dismutase (ChrC), an ATP-binding subunit of an ABC transporter (ChrD), and a sigma factor of the heat-shock family, ChrH. The genes surrounding the core *chrBA* determinant increase uptake of chromate, reduction of chromate, and probably also the export of the product Cr^{3+} (Peitzsch and Nies, unpublished results). The whole extent of the complicated interaction between the sulfate metabolism, chromate uptake and efflux, and reduction of chromate is not clear at the moment (Peitzsch et al. 1998).

Mercury resistance in *Ralstonia* sp. CH34 is based on reduction of Hg^{2+} to volatile metallic mercury (Dressler et al. 1991), as it is common in most mercury-resistant organisms (Nies 1999; Summers and Silver 1972). There are three independent mercury resistance transposons in CH34, on the bacterial chromosome and on each of the two

megaplasmids (Diels et al. 1985; Dressler et al. 1991). Resistance to copper in CH34 (Dressler et al. 1991) has just been cloned (van der Lelie 1998), and resistance to lead is based on efflux of Pb^{2+} catalyzed by a inducible P-type ATPase (van der Lelie 1998). Another P-type ATPase transporting Pb^{2+} has just recently been described (Rensing et al. 1998), the CadA protein from *Staphylococcus aureus*. Thus, *Ralstonia* sp. CH34 contains a variety of inducible heavy metal resistance systems. The bacterium can be used to construct biosensors for heavy metals, and it can be used to mediate biochemical reactions in environments contaminated with heavy metals.

Biosensors for heavy metals

Because problems created by heavy metal ions are determined by their impact on living matter, the chemical determination of the absolute amount of a metal in an environmental sample is not sufficient to predict its toxicity in situ. The interaction of a heavy metal cation with low and high molecular weight substances in an ecosystem, with cells and the chemical alteration they create, is so complicated that the "biological" concentration of the metal cannot be calculated from the absolute, or "chemical," concentration. Thus, bioavailability and ecotoxicity must be determined using organisms (Kong et al. 1995) or parts of organisms.

In strain CH34, many of the best characterized systems for metal ion resistance are switched on by the cell when needed. The control system required for this induction may therefore be used for a metal-dependent control of a reporter gene system. By molecular genetics, the control region of a metal resistance was in most cases fused to the *lux* genes, which encode products creating light in a biochemical reaction. This process yields bacterial sensors that produce light in dependence of the "biological" metal ion concentration. The first heavy metal resistance used for such a sensor was resistance to Hg^{2+}. Bacteria harboring *mer-lux* fusions were able to sense Hg^{2+} in the environment down to "chemical" concentrations of 0.5 nM (Selifonova et al. 1993). Other bacteria followed – sensors for antimonite and arsenite (Ramanathan et al. 1997), Zn^{2+} (Erbe et al. 1996), chromate-copper-arsenate (Cai and DuBow 1997), and Cd^{2+} and Pb^{2+} (Tauriainen et al. 1998).

With fusions of *lux* to genes that were not involved in metal detoxification but were influenced in some way by heavy metals, sensors especially for the toxicity of a metal were constructed, as in *Rhizobium* (Paton et al. 1997) and *E. coli* (BenIsrael et al. 1998). Sensors based on *lux* could also be used to measure genotoxicty (van der Lelie et al. 1997a). Methods based on metal-binding proteins (Bontidean et al. 1998; Klein and Mattes 1998; Preininger and Wolfbeis 1996) complete the inventory to determine the bioavailability and toxicity of heavy metals.

All these methods either use different bacteria or proteins for a small range of metals or else the nonspecific response of a general stress system is determined. With *Ralstonia* sp. CH34-based sensor strains, the influence of a variety of heavy metal ions on the same bacterium can be measured, under the same conditions, and by the use of well-characterized resistance systems. The first generation of fusions responded to Cu^{2+} (Corbisier 1997; Corbisier et al. 1996), Zn^{2+}, Cd^{2+}, Co^{2+}, and Pb^{2+} (Corbisier 1997; Corbisier et al. 1996; van der Lelie et al. 1997b), and Ni^{2+} and chromate (Peitzsch et al. 1998). In these strains, activity or regulation of the heavy metal resistance system used is disturbed; thus, bioavailability of a metal can be measured, but not the physiological response of the bacterial cell. In second-generation sensor strains, reporter genes are introduced into heavy metal resistance determinants without disturbing induction or resistance. These new sensor strains, for example, with a reporter gene inserted between *czcC* and *czcB* (Grass et al. 2000; Große et al. 1999), however, are more important for the understanding of heavy metal physiology than for use in biotechnology.

Applications of metal resistance for biotechnological processes

Bacteria protected by efflux systems are selfish bacteria; they detoxify their cytoplasm but not the environment for other bacteria, plants, or man. Thus, the heavy metal resistance systems of *Ralstonia* sp. CH34 cannot be used directly for metal remediation; however, they can be applied to protect another catabolic function of CH34, a function now able to perform in an environment with high concentrations of heavy metal cations. Possible functions are the precipitation of heavy metals by metabolic waste products of CH34 and the degradation of xenobiotica in highly contaminated environments.

When *Ralstonia* sp. CH34 grows on the salts of organic acids such as sodium gluconate, lactate, or acetate, the pH value increases as a result of the consumption of protons, carbonate is being produced, and, due to the increase in the pH value, the solubility of carbonate also increases. If heavy metal cations are present, they may be precipitated by the enormous amounts of carbonate produced: e.g., during growth in 3 g sodium acetate/l, 100 mM carbonate is being produced, which is sufficient to precipitate millimolar concentrations of heavy metal cations. These heavy metal cations have solubility products of the carbonates of $5.2 \cdot 10^{-12}$ (Cd^{2+}), $2.5 \cdot 10^{-10}$ (Cu^{2+}), $1.5 \cdot 10^{-15}$ (Pb^{2+}), $1.4 \cdot 10^{-7}$ (Ni^{2+}), and $2 \cdot 10^{-12}$ (Zn^{2+}) (Weast 1984). Thus, the trick is to protect CH34 with its efflux system and to use its metabolic waste products to precipitate heavy metals. This method has been successfully applied in a tubular membrane reactor to treat metal-containing wastewater (Diels et al. 1995a, b; Taghavi et al. 1997a; Van Roy et al. 1997).

Anthropogenic contaminations of the environment are sometimes mixtures of various aromatic xenobiotics and heavy metal cations. *Ralstonia* sp. CH34 is related to a well-known degrader of aromatics, strain JMP134 (Don and Pemberton 1981). Because strain CH34 contains all the genes required for cleavage of aromatic rings on its chromo-

some (Sauret-Ignazi et al. 1996), CH34 is able to express degradation genes for xenobiotics from a variety of CH34-related bacteria. In contrast to the original hosts, CH34 degrades also in the presence of heavy metals (Collard et al. 1994). As an example, CH34 derivatives degrading chlorobiphenyl compounds were constructed (Springael et al. 1994, 1996) and used in a tubular membrane reactor (Diels et al. 1995b).

Conclusions

Ecologically, *Ralstonia* sp. CH34 and related bacteria are the prominent bacterial flora at metal-contaminated sites. The resistance mechanisms are manifold, most have been well characterized, and in the case of cobalt, zinc, and cadmium, even understood on a quantitative physiological level. Biosensors based on strain CH34 can be used to evaluate sample sites in a highly standardized manner because biosensors for a variety of pollutants can be constructed using the same cellular background. With second-generation biosensors, which do not disrupt the physiological function of the cell, an additional and more accurate description of the living circumstances of "metallophilic" bacteria in these sites may be obtained.

In a fermentor and protected by its metal efflux system, strain CH34 is able to perform at metal ion concentrations that inhibit other bacteria. Precipitation of metal ions such as Cd^{2+} is possible in effluents contaminated with xenobiotics; these xenobiotics may even be used as carbon and energy source for metal remediation. Concerning precious metals, strain CH34 may be inferior to other approaches using *Thiobacillus* and sulfate-respiring bacteria, but with the possibility to combine a highly accurate description of an environment with simultaneous remediation of xenobiotics and heavy metals, *Ralstonia* sp. CH34 is unique and has good future prospects.

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