Zinc, cadmium and lead resistance mechanisms in bacteria and their contribution to biosensing

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ABSTRACT

Bacteria are abundant and widespread in all kinds of environments, both hospitable and hostile. In order to survive in toxic environments, bacteria need mechanisms which help to resist or detoxify the harmful compounds. Resistance to heavy metals is mainly achieved through active efflux, but also sequestration with proteins or as insoluble compounds is used. Although numerous studies have dealt with zinc, cadmium and lead resistance mechanisms in bacteria, it has still remained unclear how different transporters are integrated into an effective homeostasis/resistance network and whether specific mechanisms for lead sequestration exist. Furthermore, since metals are toxic not only to bacteria but to higher organisms as well, it is important to be able to estimate possible biological effects of heavy metals in the environment. This could be done by determining the bioavailable amount of the metals in the environment with bacterial bioreporters. That is, one can employ bacteria that respond to metal contamination by a measurable signal to assess the property of metals to cross biological membranes and to cause harmful effects in a possibly polluted environment.

In this thesis a new lead resistance mechanism is described, interplay between CBA transporters and P-type ATPases in zinc and cadmium resistance is presented and finally the acquired knowledge is used to construct bacterial bioreporters for heavy metals with increased sensitivity and specificity.

The study of the *pbrTRABCD* gene cluster from *Cupriavidus metallidurans* CH34 revealed that the main transporter component of the operon - P-type ATPase PbrA - exported Zn^{2+}, Cd^{2+} and Pb^{2+}, whereas the second component of the operon - PbrB - was shown to be a phosphatase that specifically increased lead resistance. The new lead resistance model employs a P-type ATPase that removes Pb^{2+} ions from the cytoplasm and a phosphatase that produces inorganic phosphate for lead sequestration in the periplasm. This was the first study where the molecular mechanism of lead sequestration has been described. Although this study concentrated only on the *pbr* operon from *C. metallidurans*, database searches indicated that gene clusters containing neighboring genes for P-type ATPase and phosphatase are present in several different bacterial species suggesting that Pb^{2+} detoxification through active efflux and sequestration could be a widespread resistance mechanism.

Characterization of two P-type ATPases and two CBA transporters in *Pseudomonas putida* KT2440 showed that resistance mechanisms for Zn^{2+} and Cd^{2+} are somewhat different than for Pb^{2+} as these metals cannot be sequestered as insoluble compounds as easily. Resistance to Zn^{2+} was conferred merely by the CBA transporter that could export both cytoplasmic and periplasmic ions; whereas, full resistance to Cd^{2+} required interplay of a P-type ATPase that exported cytoplasmic ions to periplasm and a CBA transporter that further exported periplasmic ions to the outside. Lead resistance in *P. putida* was influenced only by the P-type ATPase; however, even after removal of this transporter the resistance level remained very high suggesting the presence of some additional resistance mechanism. Expression of three studied transporters increased in response to heavy metals, two were

induced nonspecifically by Zn^{2+}, Cd^{2+}, Pb^{2+}, Ni^{2+}, Co^{2+} and Hg^{2+}, whereas a third one was specifically induced in response to Zn^{2+}.

The knowledge on functionality of the transporters and metal-inducible promoters of *P. putida* were exploited in bioreporter technology. A transporter-deficient bioreporter strain that lacked exporters for Zn^{2+}/Cd^{2+}/Pb^{2+} could detect up to 45-fold lower metal concentrations than its wild type counterpart due to the accumulation of metals in the cell. The broad specificity issue of bioreporters was overcome by using Zn-specific promoter as a sensor element, thus achieving Zn-specific bioreporter. The constructed transporter-deficient *P. putida* reporter strain detected Zn^{2+} concentrations about 50 times lower than that possible with other available Zn-bioreporters opening the possibility to detect low-level pollution.

ABBREVIATIONS

ATP	adenosine triphosphate
C_{55}-PP	undecaprenyl pyrophosphate
CDF	cation diffusion facilitator
EPS	exopolysaccharide
GI	genomic island
HGT	horizontal gene transfer
IC	induction coefficient
LOD	limit of detection
MBD	metal binding domain
MFP	membrane fusion protein
MIC	minimal inhibitory concentration
OMF	outer membrane factor
PCR	polymerase chain reaction
RLU	relative light units
RND	resistance-nodulation-division

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals in the text.

I Hynninen A, Touzé T, Pitkänen L, Mengin-Lecreulx D, Virta M. 2009. An efflux transporter PbrA and a phosphatase PbrB cooperate in a lead-resistance mechanism in bacteria. Molecular Microbiology 74:384-394.

II Leedjärv A, Ivask A, Virta M. 2008. Interplay of different transporters in the mediation of divalent heavy metal resistance in *Pseudomonas putida* KT2440. Journal of Bacteriology 190:2680-2689.

III Hynninen A, Tõnismann K, Virta M. 2010. Improving the sensitivity of bacterial bioreporters for heavy metals. Bioengineered Bugs 1(2):1-7.

The author's contribution

The author Anu Hynninen (née Leedjärv) has had an active role in all phases of the research reported in this thesis.

I The author planned the study, conducted all the experiments except phosphatase characterization and complementation assays. She analyzed the data, was responsible for writing the manuscript and acted as the corresponding author of the paper.

II The author planned the research, conducted all the experiments and analyzed the data. She was responsible for writing the manuscript and acted as the corresponding author of the paper.

III The author planned the study and constructed the reporter strains. She performed the calibration of one of the reporter strains, analysis of soil samples and developed statistical method for determining the limit of detection. The author analyzed the data, was responsible for writing the manuscript and acted as the corresponding author of the paper.

1 INTRODUCTION

Although some heavy metals are required as micronutrients, all heavy metals are toxic in excess. In order to avoid toxicity, metals must be quickly and efficiently eliminated from any cell. In general, there are two basic mechanisms of resistance to heavy metal ions: intracellular complexation of toxic metal ions is mainly used in eukaryotes; whereas, reduced accumulation based on active efflux of the cations is the primary mechanism developed in prokaryotes. In bacteria also binding factors and enzymatic transformations (oxidation, reduction, methylation, and demethylation) play a role as defense mechanisms.

Resistance systems in bacteria are abundant and widespread, with frequencies of resistant bacteria ranging from a few percent in pristine environments to nearly all isolates in heavily polluted environments (Silver and Phung, 2005). In this thesis mainly resistance mechanisms for zinc, cadmium and lead are discussed. Similar but distinct mechanisms exist for copper and silver; whereas transport mechanisms for cobalt, chromate, nickel and arsenic are somewhat different (Silver and Phung, 2005). In addition mercury stands on its own as it can be detoxified by reducing mercury ions to gaseous metallic mercury that evaporates from the cell (Barkay et al., 2003).

The knowledge about heavy metal resistance mechanisms generally broadens the insights into the ability of bacteria to inhabit polluted environments, but can also be applied in biotechnology for example for bioreporter-technology or bioremediation purposes. Bacterial bioreporters, which are living microorganisms that produce a specific, quantifiable output in response to target chemicals, take advantage of heavy metal inducible transcription systems and sense bioavailable metals depending on metal flux through the cell. Thus, the bioreporter technology is closely related to the research about heavy metal resistance mechanisms and will be introduced alongside the actual resistance mechanisms.

1.1 Zinc, cadmium and lead resistance in bacteria

Zinc is involved in a wide variety of cellular processes. It is required for maintaining the structural stability of macromolecules and it serves as a cofactor for more than 300 enzymes (McCall et al., 2000). Zn^{2+} also plays a prominent role in gene expression as a structural component in a large number of Zn^{2+}-dependent transcription factors. However, in excess it can inhibit the aerobic respiratory chain, have significant toxicity and act as a potent disrupter of biological systems. Consequently, the ability to maintain the intracellular Zn^{2+} concentration within very narrow limits is a fundamental property of all living cells. In prokaryotes the major mechanisms that maintain cellular Zn^{2+} concentrations are limited to the highly regulated processes of Zn^{2+} import, metal ion sequestration by metallo-chaperones and Zn^{2+} export across the cytoplasmic membrane (Blencowe and Morby, 2003). Cadmium and lead, the chemical “look alikes” for Zn^{2+}, on the other hand, have currently no known biological function in bacteria and may disturb the normal functioning of an organism if bioavailable. Cd^{2+} and Pb^{2+} cause toxicity by interacting with nucleic acids, by binding to essential respiratory proteins (Vallee

and Ulmer, 1972), through oxidative damage by production of reactive oxygen species (Stohs and Bagchi, 1995), and by displacing Ca^{2+} and Zn^{2+} in proteins (Markovac and Goldstein, 1988; Bouton et al., 2001). Cd^{2+} and Pb^{2+} enter bacterial cells via transport systems for essential divalent cations, such as Mn^{2+} (Tynecka et al., 1981; Laddaga et al., 1985) and Zn^{2+} (Laddaga and Silver, 1985; Makui et al., 2000; Grass et al., 2002).

Cells respond to excess Zn^{2+} or the presence of Cd^{2+} and Pb^{2+} by metal-inducible resistance mechanisms. Zn^{2+}, Cd^{2+} and Pb^{2+} resistance in bacteria is mainly based on active efflux of metal ions to prevent toxic effects in the cell. In many cases, Zn^{2+} and Cd^{2+} resistance mechanisms are indistinguishable, i.e. the same genes code both Zn^{2+} and Cd^{2+} resistance. The efflux of Zn^{2+} and Cd^{2+} is facilitated by P-type ATPases, CBA transporters and CDF chemiosmotic transporters. Pb^{2+} resistance is somewhat less studied, but involvement of P-type ATPases and detoxification through sequestration are known. In addition, low level resistance of $Zn^{2+}/Cd^{2+}/Pb^{2+}$ is achieved by binding the ions in inactive form. This binding is mainly nonspecific attachment to cell wall, but also metal-inducible binding factors are known. In the following subchapters these phenomena are discussed in more detail.

1.1.1 Efflux transporters

Three major families of efflux transporters are involved in $Zn^{2+}/Cd^{2+}/Pb^{2+}$ resistance (Figure 1). P-type ATPases span the inner membrane and use ATP energy to pump metal ions from the cytoplasm to periplasm (Nucifora et al., 1989; Rensing et al., 1997); CBA transporters are three-component transenvelope pumps of Gram-negative bacteria that act as chemiosmotic antiporters (Nies and Silver, 1989; Hassan et al, 1999; Franke et al., 2003); and cation diffusion facilitator (CDF) family transporters act as chemiosmotic ion-proton exchangers (Xiong and Jayaswal, 1998; Anton et al., 1999; Grass et al., 2001). In general, P-type ATPases and CDF transporters are commonly found among different bacterial species, whereas the presence of a CBA transporter (an RND protein in Gram-positive bacteria) is exceptional and indicates high-level resistance to heavy metal ions (Nies, 2003). P-type ATPases and CDF transporters export metal ions from the cytoplasm to the periplasm; whereas CBA transporters mainly detoxify periplasmic metal (outer membrane efflux), i.e. CBA transporters further remove periplasmic ions transported there by ATPases or CDF transporters or before ions have entered the cytoplasm. While P-type ATPases and CDF transporters can functionally replace each other, they cannot replace CBA transporter and vice versa (Scherer and Nies, 2009).

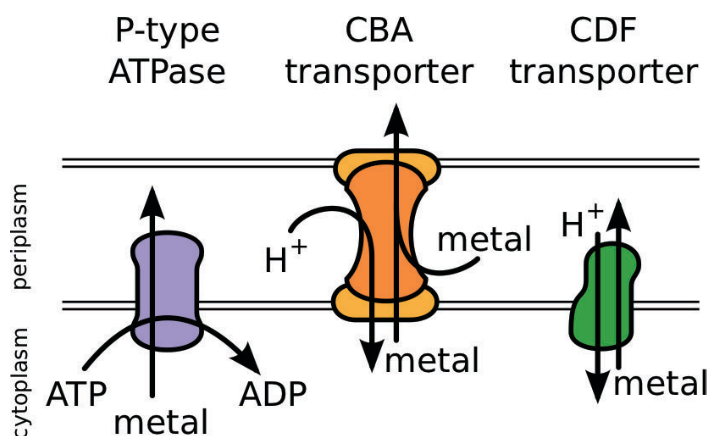

Figure 1. Main transporter families involved in heavy metal resistance. P-type ATPases pump their substrates from cytoplasm to periplasm using energy provided by ATP hydrolysis. CBA transporters are three-component complexes that span the whole cell wall of Gram-negative bacteria and expel ions from cyto- and periplasm to outside using a chemiosmotic gradient. CDF transporters are driven by a proton motive force and they export ions from cytoplasm to periplasm.

1.1.1.1 Heavy metal translocating P-type ATPases

P-type ATPases constitute a superfamily of transport proteins that transport ions against the concentration gradient using energy provided by ATP hydrolysis. The term "P-type" refers to the formation of a phosphoenzyme intermediate in the reaction cycle. The energy released by the removal of the γ-phosphate from ATP is coupled to the translocation of an ion across biological membranes. Substrates are inorganic cations such as H^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Cu^+, Ag^+, Zn^{2+}, Cd^{2+}, Co^{2+} and Pb^{2+}. Efflux transporters that play a key role in heavy metal homeostasis and resistance belong to the P_{1B}-type subfamily of ATPases (Axelsen and Palmgren, 1998) and can be further divided into the Cu^+/Ag^+-translocating and $Zn^{2+}/Cd^{2+}/Pb^{2+}$-translocating P_{1B}-type ATPase subgroups based on substrate specificity (Argüello, 2003). Heavy metal-translocating ATPases are also called CPx-type ATPases as they carry a conserved proline residue (P), preceded and/or followed by a cysteine residue (C). The physiological functions of these pumps include maintaining the homeostasis of essential metals such as Cu^+, Co^{2+}, and Zn^{2+}, as well as mediating resistance to toxic metals Pb^{2+}, Cd^{2+} and Ag^+. The direction of transport of CPx-type ATPases is mostly to the periplasm without further transport from the periplasm to the outside. The substrates in vivo are likely metal-thiolate complexes rather than the free metals (Sharma et al., 2000).

The specificity of heavy metal translocating ATPases is determined by metal binding domains (MBD). P_{1B}-type ATPases have two MBDs: one in an N-terminal cytoplasmic domain and another composed of transmembrane segments (Argüello, 2003). Characterization of ZntA from *Escherichia coli* has proved that the N-terminal MBD is not essential for the functioning (Mitra and Sharma, 2001), while mutations in the transmembrane MBD render ZntA inactive (Liu et al., 2006). In addition, the replacement of the N-terminus of ZntA with the N-terminus from a Cu-translocating ATPase does not alter the substrate specificity (Hou et al., 2001), confirming that metal ion binding for translocation takes place in the

transmembrane domain of the protein and that this MBD determines the specificity of the transporter. The amino acids responsible for metal specificity of $Zn^{2+}/Cd^{2+}/Pb^{2+}$ P_{1B}-type-ATPases have been identified helping to distinguish Cu^{+}/Ag^{+}-translocating and $Zn^{2+}/Cd^{2+}/Pb^{2+}$-translocating P_{1B}-type ATPase subgroups (Argüello, 2003; Dutta et al., 2006; Dutta et al., 2007).

Divalent metal efflux ATPases are widespread in both Gram-positive and Gram-negative bacteria (Rensing et al., 1999). The prototypes of $Zn^{2+}/Cd^{2+}/Pb^{2+}$-translocating P_{1B}-type ATPases are ZntA from *E. coli* (Rensing et al., 1997) and CadA from *Staphylococcus aureus* (Nucifora et al., 1989). Both of these transporters confer resistance to Zn^{2+}, Cd^{2+} and Pb^{2+} by exporting respective metals from the cytoplasm (Nucifora et al., 1989; Tsai et al., 1992; Rensing et al., 1997 and 1998). Zn^{2+}/Cd^{2+}-translocating ATPases have also been characterized in *Synechocystis* (Thelwell et al., 1998), *Pseudomonas putida* (Lee et al., 2001; Hu and Chao, 2007) and *Cupriavidus metallidurans* (Legatzki et al., 2003; Scherer and Nies, 2009). Probably all the Zn^{2+}/Cd^{2+}-translocating P-type ATPases are also effective in Pb^{2+} export, however Pb^{2+} resistance is very seldom tested and there is no experimental proof for this hypothesis. One of the few characterized Pb^{2+}-translocating P-type ATPases can be found from the plasmid-encoded lead-resistance gene cluster *pbrTRABCD* from *C. metallidurans* (Borremans et al., 2001). This gene cluster consists of six genes that code for the following proteins: PbrR, a transcription factor belonging to the MerR family; PbrT, a putative Pb^{2+} uptake protein; PbrA, a P-type ATPase; PbrB/PbrC, a predicted integral membrane protein and a putative signal peptidase; and PbrD, a putative intracellular Pb-binding protein (Borremans et al., 2001). The Pbr proteins are hypothesized to combine functions involved in uptake (PbrT), efflux (PbrABC) and accumulation (PbrD) of Pb^{2+} (Borremans et al., 2001).

1.1.1.2 CBA transporters

CBA transporters are three-component protein complexes that span the whole cell wall of Gram-negative bacteria. The most important component of the transporter is an RND protein that is located in the inner membrane. The RND protein family was first described as a related group of bacterial transport proteins involved in heavy metal **r**esistance (*C. metallidurans*), **n**odulation (*Mesorhizobium loti*) and cell **d**ivision (*E. coli*) (Saier et al., 1994). The RND protein is usually accompanied by the membrane fusion protein (MFP) and outer membrane factor (OMF). These three proteins form an efflux protein complex that may export its substrate from the cytoplasm, the cytoplasmic membrane or the periplasm across the outer membrane directly to the outside (Nies, 2003). RND-driven export systems are referred to as CBA efflux systems or CBA transporters. This way they can be distinguished from ABC transport systems and this name also reflects the sequence of the genes in the operon encoding for the components of the transporter complex. In bacteria and archaea, CBA transporters are involved in transport of heavy metals, hydrophobic compounds, nodulation factors and proteins. By diminishing not only the cytoplasmic concentration of heavy metal cations but additionally the periplasmic concentration, CBA transport systems could remove cations even before they have the opportunity to enter the cell. Moreover, these efflux systems could mediate

further export of the cation that had been removed from the cytoplasm by other efflux systems (Nies, 2003).

The RND protein is the central and most important component of the CBA efflux system: it mediates the active part of the transport process, determines the substrate specificity and is involved in the assembly of the transenvelope protein complex. OMF and MFP proteins have a rather static function during CBA-mediated transenvelope efflux. In many protein complexes, loss of the MFP or RND protein abolishes the respective resistance, whereas loss of the OMF usually has a relatively moderate influence (Nies et al., 1989; Franke et al., 2003). Since many Gram-negative bacteria contain a variety of OMF-encoding genes, another OMF may take over and complement the loss of the native OMF. Given the current hypothesis about the function of CBA proteins as transenvelope efflux pumps, a CBA transporter should not function in a Gram-positive cell wall. However, RND proteins alone exist in Gram-positive bacteria and might thus function as single-subunit efflux systems in Gram-positive bacteria (Nies, 2003).

The best characterized CBA transporter is the CzcCBA complex from *C. metallidurans* CH34. The *czc* determinant encodes resistance to Cd^{2+}, Zn^{2+} and Co^{2+} by metal-dependent efflux (Nies et al., 1987; Nies and Silver, 1989) driven by the proton motive force (Nies, 1995). CBA transporters responsible for Zn^{2+} and Cd^{2+} efflux can also be found from *Pseudomonas aeruginosa* (Hassan et al., 1999), *P. putida* (Hu and Chao, 2007) and *Alcaligenes* sp. (Kunito et al., 1996). CBA transporters are not known to export Pb^{2+}.

1.1.1.3 CDF family transporters

The cation diffusion facilitator family (CDF) comprises of a group of transporters which can catalyze either influx or efflux of heavy metals. Members of the family have been found from both prokaryotes and eukaryotes. In contrast to other protein families, such as P-type ATPases or CBA transporters, all CDF proteins characterized to date transport only metals (substrate spectrum includes Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+}), and the majority are involved in Zn^{2+} transport (Paulsen and Saier, 1997; Haney et al., 2005). CDF family of chemiosmotic efflux systems was first described with the CzcD Cd^{2+} and Zn^{2+} efflux system of *C. metallidurans* (Nies, 1992; Anton et al., 1999). CDF proteins are driven by a potassium gradient in addition to the proton motive force (Guffanti et al., 2002).

Generally very little is known about the role of CDF transporters in heavy metal resistance. They provide very low level resistance, but it has been assumed that their main role is to function as a kind of heavy metal buffer for the cell at low cytoplasmic metal concentrations (Anton et al., 1999). For example, deletion of *zitB* in *E. coli* has no effect on zinc sensitivity; whereas double disruption of *zitB* and *zntA* renders *E. coli* cells more zinc sensitive than a single disruption in *zntA* alone (Grass et al., 2001). It is possible that ZitB contributes to Zn^{2+} homeostasis at low (non-toxic) concentrations, while ZntA is required for growth at higher and more toxic concentrations (Grass et al., 2001). Alternatively, improvement of metal resistance through regulating the expression of other metal transporters has been speculated to be the role of CDF transporters (Anton et al., 1999).

Zn^{2+} and Cd^{2+}-exporting CDF family transporters have been characterized in *C. metallidurans* (Anton et al., 1999), *S. aureus* (Xiong and Jayaswal, 1998) and *E. coli* (Grass et al., 2001). None of the CDF proteins are known to transport Pb^{2+}.

1.1.1.4 Other transporter proteins

Recently a novel $Zn^{2+}/Cd^{2+}/Ni^{2+}$-transporter distinct from abovementioned transporters was discovered from *P. putida* KT2440 (Haritha et al., 2009). MrdH is a chimeric protein that combines domains of nickel transporter (RcnA domain) and a putative domain of MFP protein (CzcB) of CBA transporters (Haritha et al., 2009). In contrast to CBA transporters, where CzcB is located in the periplasm and its function is to connect CzcA (located in the inner membrane) to the CzcC (located in the outer membrane) and possibly funnel the ions into the transporter, the CzcB domain of the MrdH is located in the cytoplasm (Haritha et al., 2009). It could be hypothesized that the RcnA domain in MrdH could confer the nickel resistance, whereas the domain derived from CzcB accounts for the observed cadmium and zinc resistance by funneling respective ions to the transporter (Haritha et al., 2009).

1.1.2 Binding proteins

In addition to membrane transport pumps that remove metal ions from the bacterial cell, binding factors that detoxify metals by sequestration are involved in creating tolerance to heavy metal ions in some bacteria. These binding factors include intracellular binding proteins (bacterial metallothioneins and metallo-chaperones) and cell wall components (exopolysaccharides). It is arguable whether the primary function of intracellular binding proteins is to increase metal resistance of the cell or to sequester and store micronutrients (Zn^{2+}) in the cell. Also, a cell wall that is capable of binding heavy metal ions cannot be considered to be a specific metal resistance mechanism that is produced in response to metals.

Bacterial metallothioneins are small cystein-rich proteins capable of binding Zn^{2+} and Cd^{2+}. Metallothioneins function as cytoplasmic metal cation-binding proteins, lowering the free ion concentrations within the cytoplasm. Bacterial metallothionein polypeptides (SmtA-like proteins) are not homologous in sequence and are therefore unrelated by evolution to the metallothioneins of the animal cells (Olafson et al., 1988). The first metallothionein characterized in bacteria, SmtA from *Synechococcus* PCC 7942, sequesters and detoxifies Zn^{2+} and Cd^{2+} (Olafson et al., 1988; Turner et al., 1993). Mutants deficient in *smtA* accumulate significantly less zinc than wild-type cells (Turner and Robinson, 1995), intracellular zinc sequestration by SmtA therefore appears to enhance zinc accumulation (Cavet et al., 2003) which is in accordance with theory about metallothioneins as nutrient storage. On the other hand, expression of *smtA* is induced by Zn^{2+} indicating that the protein is produced in response to increased Zn^{2+} concentrations. This, on the contrary, supports the idea that the main function of metallothioneins is to provide tolerance to Zn^{2+} and not to act as Zn^{2+} storage proteins that supply zinc-requiring proteins. SmtA-like proteins have been found, in addition to *Synechococcus* sp., from *Anabaena* PCC 7120, *P. aeruginosa* and *P. putida* (Blindauer et al., 2002).

In addition to metallothioneins, periplasmic proteins that could be classified as Zn^{2+}-translocating metallo-chaperones have been identified in a number of bacterial species (Blencowe and Morby, 2003). Proteins in *E. coli* that have the potential to form Zn^{2+}-binding metallo-chaperones include a periplasmic protein ZraP that is only produced when cells are incubated with high concentrations of Zn^{2+} or Pb^{2+} (Noll et al., 1998; Leonhartsberger et al., 2001). ZraP is a membrane-associated protein that undergoes a specific Zn^{2+}-induced cleavage to release a 12 kDa carboxy-terminal Zn^{2+}-binding region into the periplasm (Noll et al., 1998). The actual roles of such metallo-chaperons are poorly understood, their function could be to sequester toxic metals to protect periplasmic and transmembrane proteins or they might cooperate with other metal resistance proteins by transferring metal ions for further detoxification.

Sequestration is also a detoxification mechanism for Pb^{2+}. Several bacterial species use intra- and extracellular binding of Pb^{2+} to avoid toxicity. *S. aureus*, *Citrobacter freundii* (Levinson et al., 1996; Levinson and Mahler, 1998), *Vibrio harveyi* (Mire et al., 2004) and *Bacillus megaterium* (Roane, 1999) lower the concentration of free lead ions by precipitating lead as a phosphate salt. *Pseudomonas marginalis* avoids lead toxicity by precipitating it as an extracellular polymer (Roane, 1999). However, the molecular mechanisms responsible for the formation of lead precipitates in these strains are not known.

Many bacteria have a cell wall or envelope that is capable of passively adsorbing high levels of dissolved metals, usually via a charge-mediated attraction (Mohamed, 2001). Binding of heavy metals by these organisms takes place mainly through exopolysaccharides (EPSs) (Loaec et al., 1997). Particular heavy metal binding capacity has been observed in uronic acid rich EPSs (Mohamed, 2001; Perez et al., 2008). Although an EPS could act as a biosorbent of free metal ions, it cannot be considered to be an inducible resistance mechanism synthesized and developed in response to metals.

1.1.3 Induced expression of resistance systems

Heavy metal resistance systems are not essential for survival; however, in metal-polluted environment they provide a great advantage for microbial growth. Metal efflux pumps drain the cellular pool of energy (ATP, chemiosmotic gradients) to pump metal ions out of a cell. Therefore, the overall cost of encoding these traits, while advantageous in a contaminated environment, could decrease fitness in a pristine environment as maintenance and expression of tolerance genes carries a measurable energetic cost (Gibbons et al., unpublished results).

The expression of most of the heavy metal transport systems is controlled at the level of transcription and is mediated by metal-responsive regulators (Silver and Phung, 2005). The expression is tightly regulated to prevent inappropriate activation in a pristine environment in order to minimize the associated metabolic burden to the host. Three protein classes of metal-responding transcriptional regulators that sense excess concentrations of metal ions in the cell have been described in detail:

1. Members of the MerR family of homodimeric activator proteins usually bind to RNA polymerase-binding operator regions regardless of the presence of a

bound metal ion. Upon activation by metal binding, the DNA bends and twists into a position that opens the DNA structure and allows mRNA synthesis to initiate (Frantz and O´Halloran, 1990; Brown et al., 2003). The prototype of the family is the MerR protein that regulates the expression of a mercury resistance operon (Summers, 1992).

2. The ArsR/SmtB family of metal-responding regulators consists of repressors that act negatively by binding to the operator region preventing RNA polymerase binding. Members of this family possess a highly conserved DNA recognition helix-turn-helix domain and bind as homodimers to their operator/promoter sequences. When the metal ion binds to the repressor, it is released from the DNA allowing transcription to occur (Busenlehner et al., 2003; Penella and Giedroc, 2005; Silver and Phung, 2005).

3. The third class of transcriptional regulatory proteins has been found mainly to regulate expression of CBA transporters. It is a two component RS-system that includes membrane sensor and intracellular regulatory protein. Transmembrane histidine kinase sensor (S) autophosphorylates in response to the extracellular stimulus (i.e. upon binding of metal ion). The phosphate is then transferred to a cytoplasmic regulatory protein (R-responder), which is then either activated or inactivated for DNA-binding transcriptional control (Silver and Phung, 2005).

Expression of P-type ATPases and metallothioneins is usually controlled by MerR or ArsR/SmtB family regulators that sense intracellular metal concentration (Endo and Silver, 1995; Thelwell et al., 1998; Brocklehurst et al., 1999; Monchy et al., 2006), whereas two component systems that sense periplasmic metal control the expression of CBA transporters (Nies, 1992; Grosse et al., 1999; Hassan et al., 1999) and periplasmic Zn-binding metallo-chaperone ZraP (Leonhartsberger et al., 2001). Regulators acting on CDF genes are poorly studied. Some of the CDF transporter genes are located in the same operon with CBA transporter genes and are therefore controlled by two component regulatory systems (Nies, 1992). Usually the inducer range of the transporter is broader than the actual substrate range of the transporter. For example expression of $Zn^{2+}/Cd^{2+}/Pb^{2+}$-translocating ZntA from *E. coli* is in addition to substrate metals induced by Hg^{2+} (Brocklehurst et al., 1999; Binet and Poole, 2000) and the expression of Zn^{2+}/Cd^{2+}-translocating CadA from *P. aeruginosa* is induced by Zn^{2+}, Cd^{2+}, Hg^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+} (Brocklehurst et al., 2003).

1.1.4 Interplay of different transporters in zinc and cadmium resistance

Despite the broad spectrum of characterized transporters, both on the level of expression and transportation, very little is known about cooperation of different resistance mechanisms in the same bacterial strain. A good model organism for heavy metal resistance studies is *C. metallidurans* CH34 that carries at least 150 genes predicted or known to be involved in metal ion resistance/homeostasis (Monchy et al., 2006). Among these are genes for four Zn^{2+}/Cd^{2+}-translocating P-type ATPases (Legatzki et al., 2003; Scherer and Nies, 2009), for the main $Zn^{2+}/Cd^{2+}/Co^{2+}$ exporter CzcCBA (Nies et al., 1987) and for the CDF transporter

CzcD (Anton et al., 1999). All these transporters are known to influence Zn^{2+} and Cd^{2+} resistance of *C. metallidurans*. Studies about cooperation of these transporters have shown that Zn^{2+} resistance is based only on the action of the CBA transporter CzcCBA (Legatzki et al., 2003). Although the P-type ATPases are able to efficiently efflux Zn^{2+}, their activity is not necessary to achieve the full resistance. According to expression analysis, Zn^{2+} is removed so efficiently by the CBA transporter that the expression from P-type ATPase genes is not induced as insufficient amount of metal ions reach the cytoplasm (Legatzki et al., 2003). The full resistance to Cd^{2+}, on the other hand, requires both the activity of CBA transporter and P-type ATPase (Legatzki et al., 2003; Scherer and Nies, 2009). These results support the theory that CBA transporters and P-type ATPases have distinct functions in metal ion removal from different cellular compartments. CBA transporter efficiently removes excess Zn^{2+} already from the periplasm and additional removal from cytoplasm is not necessary; whereas, some Cd^{2+} escapes the CBA transporter and enters the cytoplasm to be exported by P-type ATPases. The role of CDF transporter in metal resistance remains unclear as it could export Zn^{2+} only when all the other transporters had been removed and had no effect on Cd^{2+} resistance even in transporter-deficient background (Scherer and Nies, 2009).

1.2 Resistance mechanisms to other heavy metals

As already mentioned, resistance mechanisms to other heavy metals are somewhat different than the mechanisms for Zn^{2+}, Cd^{2+} and Pb^{2+}. Monovalent metals Cu^{+} and Ag^{+} are also exported by the CBA transporters (for example CusCBA and SilCBA) and P-type ATPases (for example CopA and SilP), but in addition the resistance mechanisms for Cu^{+} and Ag^{+} involve small periplasmic metal binding proteins (Gupta et al., 1999; Franke et al., 2003; Rensing and Grass, 2003). Similar to divalent metal transporters, the CBA transporters and P-type ATPases for monovalent metals are not specific to one ion. They fail to differentiate between Cu^{+} and Ag^{+} and thus transport both (Solioz and Odermatt, 1995; Stoyanov et al., 2003).

Ni^{2+} and Co^{2+} are, in some occasions, exported by the same CBA transporters as Zn^{2+} and Cd^{2+} (for example NccCBA from *Alcaligenes xylosoxidans* 31A and CzcCBA from *C. metallidurans* CH34) (Schmidt and Schlegel, 1994; Rensing et al., 1997b). However, also efflux proteins not belonging to abovementioned transporter families have been reported to transport Ni^{2+} and/or Co^{2+} (Grass et al., 2001b; Rodrigue et al., 2005).

Resistance to arsenicals (As^{3+} and As^{5+}) and antimonials (Sb^{3+}) is achieved through the action of *ars* operon-encoded proteins (Ji and Silver, 1992; Rosenstein et al., 1992). The operon encodes for ArsB that functions as a chemiosmotic As^{3+} and Sb^{3+} efflux transporter and ArsC that acts as an arsenate reductase reducing As^{5+} to As^{3+} before export. Some operons also encode for ArsA that binds as a dimer to the ArsB protein, converting its energy coupling from the membrane potential to ATP (Rosen et al., 1999). The *ars* operon is widely found among both Gram-positive and Gram-negative bacteria indicating ancient presence of the system (Silver and Phung, 2005).

Another widespread resistance mechanism is the Hg^{2+} resistance system encoded by the *mer* operon. Typical *mer* operon encodes for MerT - a membrane-bound Hg^{2+} uptake protein, and MerA - a mercuric reductase. Broad-spectrum operons encode additionally for MerB - a organomercurial lyase that catalyses the breakdown of the carbon-Hg bond in organic compounds to generate an Hg^{2+} ion and the organic residue (Barkay et al., 2003). The resistance mechanism is based on active transport of Hg^{2+} into the cell and subsequent transformation of ionic Hg^{2+} to metallic Hg^0, which evaporates from the cell (Barkay et al., 2003).

Chromate (CrO_4^{2-}) resistance in bacteria is based on the active extrusion of chromate anion or reduction of Cr^{6+} to the less toxic Cr^{3+} by the action of various enzymatic or nonenzymatic processes. The reductases that act on Cr^{6+} are rather nonspecific, having chromate reduction ability as a secondary function (Ramírez-Díaz et al., 2008). Export of chromate from cells is conferred by the action of a ChrA transporter (Cervantes et al., 1990; Nies et al., 1990) that is not related by sequence to other families of membrane transport proteins (Silver and Phung, 2005).

1.3 Specific bacterial bioreporters and bioavailability

Bacterial bioreporters or whole-cell microbial biosensors are living microorganisms that produce a specific, quantifiable output in response to target chemicals. They provide a biological tool for measuring bioavailability (and in some cases bioaccessibility) of contaminants. Contrary to the chemical analysis methods, which determine the total contaminant concentration in the sample, bioreporters respond only to the biologically relevant fraction that influences the cell and is capable of passing through cellular membranes. Bioavailability assays predict the real exposure of organisms to pollutants and are valuable for risk assessment and for the selection of suitable remediation options (Liao et al., 2006). For example in case of heavy metals, it is speciation and the resulting bioavailability rather than total metal concentration that determines the physiological and toxic effects on biological systems (Rensing and Maier, 2003). The discrepancy between the total and bioavailable amount of the chemical may be significant, especially in the case of contaminants with low dissociation constants or poor aqueous solubility (Tecon and van der Meer, 2008).

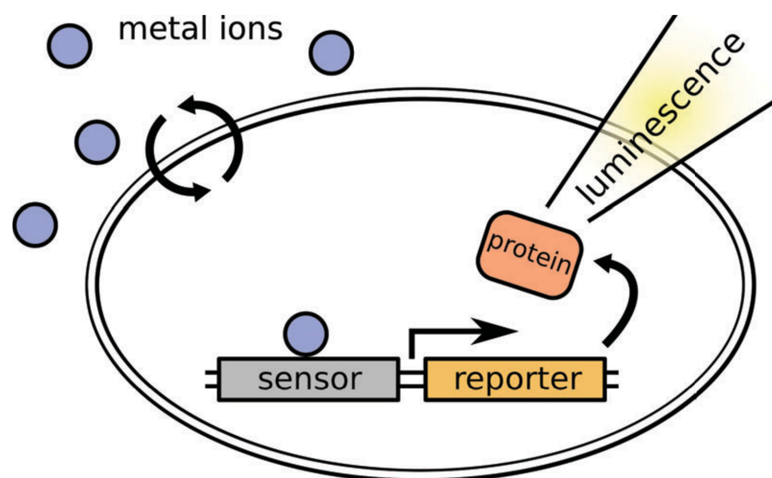

Figure 2. A simplified principle of a luminescent bacterial bioreporter. Metal uptake and efflux mechanisms determine the metal flux through the cell. Upon binding of the metal ion to the sensor element, transcription of the reporter element is initiated and a measurable signal is produced. The signal output correlates with intracellular metal concentration in a certain concentration range.

Specific bioreporters are genetically engineered bacteria where a sensor element that is responsible for the detection of the analyte has been fused to a reporter element (Figure 2). The sensor element usually consists of a gene for a transcription factor and its cognate promoter, whereas the reporter element is a gene or a group of genes that code for an easily measurable signal. The transcription factor and the promoter originate from systems where expression is tightly regulated depending on the presence of a certain compound. Such systems are for example heavy metal resistance operons. Under natural conditions, activation (or derepression) of these operators would lead to the synthesis of proteins, the presence or activity of which would help the cell combat the sensed hazard or adapt to it, but in the recombinant bioreporter strain the selected promoter drives the synthesis of the reporter protein(s). Both fluorescent proteins and enzymes producing easily quantifiable output upon substrate degradation (β-galactosidase, luciferase) are used as reporters (Daunert et al., 2000). Fluorescence is a measure of the presence of the protein, whereas substrate degrading-proteins quantify enzymatic activity. Luciferases are widely used reporter genes in prokaryotic systems as they provide simple and sensitive detection of gene expression and regulation, are easily quantifiable and have no intrinsic expression in the bioreporter strains (Wood and Gruber, 1996).

The very first bioluminescent bioreporter was constructed in G. Sayler's laboratory by inserting bacterial luciferase (*lux*) genes into naphthalene catabolic pathway (King et al., 1990). The constructed strain responded to naphthalene and salicylate by increased luminescence and could be used to monitor naphthalene degradation. Subsequentially bioluminescent reporters have been constructed for several heavy metals and organic compounds, for example to assay arsenic and antimony (Tauriainen et al., 1997), cadmium and lead (Tauriainen et al., 1998), mercury (Selifonova et al., 1993; Virta et al., 1995), organomercurials (Ivask et al., 2001), cobalt and nickel (Tibazarwa et al., 2001), zinc (Riether et al., 2001; Ivask et al.,

2002), alkanes (Sticher et al., 1997), benzene and benzene derivatives (Selifonova and Eaton, 1996; Applegate et al., 1998; Willardson et al., 1998), naphthalene and salicylate (King et al., 1990; Heitzer et al., 1992), 2,4-dichlorophenoxyacetic acid and 2,4-dichlorophenol (Hay et al., 2000), polychlorinated biphenyls (Layton et al., 1998) and phenols (Leedjärv et al., 2006). For the expanded list of metal bioreporters and their detection ranges see recent reviews by Harms (2007) and Magrisso et al. (2008). Most of the regulatory units used in the construction of metal-specific sensor bacteria originate from the bacteria that possess precisely regulated natural resistance systems towards heavy metals, whereas sensing elements for sensors that recognize organic compounds originate from the bacteria that are capable of metabolizing the respective compounds.

In contrast to expensive and laborious chemical analysis methods, bacterial bioreporters are relatively inexpensive and easy to use. Bacteria reproduce quickly and normally no pretreatment of the sample is necessary for the assay. The simplest assay procedure involves mixing of the sample with bacteria and recording the signal after reasonable incubation time, which is required for reporter protein synthesis.

1.3.1 Sensitivity and specificity of bacterial bioreporters

Two most important characteristics of bacterial bioreporters are their specificity (the range of detected chemicals) and sensitivity (the limit of detection - LOD).

The specificity of the bioreporter depends on the transcription factor and promoter used as a sensor element. All the chemicals which activate (or derepress) the transcription from the respective promoter cause signal output and are, thus, considered as inducers of a bioreporter in question. Transcription factors for heavy metal resistance operons are regulated by the substrates of the same resistance system, but also other metals might act as activators of the system. No specific transcription regulator for every compound of environmental interest exists. For example, Zn^{2+}, Cd^{2+} and Pb^{2+} are chemically so similar that the majority of the transcription factors do not differentiate between them (Yoon et al., 1991; Brocklehurst et al., 1999; Binet and Poole, 2000; Brocklehurst et al., 2003). The same applies for Cu^{+} and Ag^{+} (Stoyanov et al., 2001; Stoyanov and Brown, 2003). Therefore, the range of truly specific bacterial biosensors is limited: almost all bioreporters sensing Zn^{2+} also respond to Cd^{2+} and Pb^{2+} (Tauriainen et al., 1998; Biran et al., 2000; Riether et al., 2001; Ivask et al., 2002; Shetty et al., 2003; Liao et al., 2006). Also Cu^{+} and Ag^{+} are detected by the same bioreporters (Corbisier et al., 1996; Tom-Petersen et al., 2001; Hakkila et al., 2004).

In a case of co-contamination with several metals, it might be desirable to be able to assess the amount of each metal separately. In such situations, truly specific bioreporters (responding only to one metal) would be of great advantage. The most direct way to influence bioreporter specificity is to modify the sensor element. Metal specificity of transcription factors can be changed by altering the relative location of metal-binding cysteine residues (DeSilva et al., 2002). Single amino acid mutations in $Cu^{+}/Ag^{+}/Au^{+}$-responsive CueR, $Hg^{2+}/Cd^{2+}/Zn^{2+}$-responsive MerR and $Zn^{2+}/Cd^{2+}/Pb^{2+}$-responsive ZntR have changed the response profiles of the

respective transcription factors, although no mutants specific for only one metal have been achieved (Caguiat et al., 1999; Khan et al., 2002; Stoyanov and Brown, 2003). The mutants with altered metal specificity have not yet been used in the construction of bioreporters, but they could represent an important component of bioreporters in the near future (Hynninen and Virta, 2009).

The sensitivity (LOD) is a characteristic of bioreporters that determines how low analyte concentrations they can detect. The better the sensitivity, the lower the concentrations that can be detected. As most bioreporters detect intracellular chemicals and the signal is induced upon binding to the transcription factor, the sensitivity is determined by the homeostasis of the chemical and its affinity to the sensing element (Hynninen and Virta, 2009). The activating concentrations of essential metals (Cu^{+}, Zn^{2+}, Ni^{2+}, Co^{2+}) are not very low, as bacterial response mechanisms to those metals have evolved to balance between metal deprivation and toxicity. In contrast, the requirement for rapid elimination of toxic ions (Cd^{2+}, Pb^{2+}, Ag^{+}, Hg^{2+}) from the cell presumes the ability to detect very low intracellular concentrations of these elements. Thus, bioreporters employing natural transcription factors cannot detect very low levels of essential metals, whereas bioreporters with nanomolar detection ranges can be constructed for nonessential metals (see Harms, 2007; Magrisso et al., 2008). Ultimately, the intracellular metal concentration available for detection by sensing elements is determined by the activity of influx and efflux transporters (see Figure 2). Efflux mechanisms compete with the detection of metal ions and thus lead to a less sensitive response. Also the mechanisms that degrade or modify the analyte may compete with its detection and reduce sensitivity (van der Meer et al., 2004).

Bioreporters have been developed for the analysis of bioavailable metals in the environmental samples, but as bioavailability of metal ions in complex matrices can be below 1% even in highly contaminated samples (Tibazarwa et al., 2001; Ivask et al., 2002 and 2004), improved sensitivities are required for the broader use of bioreporters. One of the strategies for improving the LODs of bacterial bioreporters involves influencing the natural transport chain by enhancing the uptake of contaminant into the cell or by hindering the export of detectable ions from the cell (Hynninen and Virta, 2009). To determine all potentially bioavailable contaminants, bioreporter organisms could be genetically engineered to make their membrane more permeable for the contaminant or to contain additional uptake mechanisms (Selifonova et al., 1993). Another way to achieve more rapid accumulation of metal ions into the cell is to eliminate efflux transporters. If all the metal ions entering the cell were immediately removed, no binding to transcription factors would occur and hence no signal would be generated. However, in the absence of efflux transporters, metal ions accumulate in the cell and expression from metal-regulated promoters occurs at lower extracellular concentrations (Rensing et al., 1998; Stoyanov et al., 2003).

1.3.2 Applications of bioreporters

Taking into account their range of detection and specificity, bacterial bioreporters can be used as an analytical means to quantitatively measure heavy metals in

samples of interest. In contrast to chemical methods, whole-cell bioreporters do not measure the total amount of metal present in the environment; rather they measure the biologically relevant fraction that influences the cell and is capable of passing through cellular membranes. Nearly every research paper on the subject argues that the ability to assess the bioavailability of heavy metal in environmental matrices is a key motivation for the construction of new microbial reporter strains. Although bacterial bioreporters have been used for determining bioavailable amounts of contaminants from very different samples, they do not yet contribute to decisions about contaminated site management (Harms, 2007) nor is the bioavailable fraction included in legislative standards. Bioreporters are still between the proof-of-principle phase and real environmental application (Harms et al., 2006). The possible environmental applications of whole-cell bioreporters, and their advantages and disadvantages compared to chemical analysis have been thoroughly discussed in a recent review by Harms et al. (2006).

Traditionally the concept of whole-cell bioreporters has been validated on water and soil samples. Thus far bacterial bioreporters have proved most useful in testing the presence of As in drinking water in developing countries (Stocker et al., 2003; Harms et al., 2005; Liao and Ou, 2005; Trang et al., 2005) and determining the availability of Fe for cyanobacteria in sea and lake water samples (Durham et al., 2002; McKay et al., 2005; Hassler and Twiss, 2006; Boyanapalli et al., 2007). Bioreporter measurements of solid samples have for the most remained at the level of determining the fraction of bioavailable metal in case of different soil types and contamination levels without further applications for risk assessment (Hansen and Sorensen, 2000; Rasmussen et al., 2000; Tibazarwa et al., 2001; Ivask et al., 2002 and 2004; Flynn et al., 2003; Bontidean et al., 2004; Bernaus et al., 2005; Brandt et al., 2006; Liao et al., 2006; Everhart et al., 2006). More novel bioreporter applications include determination of As content in rice (Baumann and van der Meer, 2007) and Hg content in urine (Roda et al., 2001) and moss (Pepi et al., 2006).

Applications of bacterial bioreporters are not limited to testing contaminated water or soil samples, and determining potential risks of pollution. Bioreporters have also been used to monitor changes in bioavailable metal concentrations in different processes, and when used in parallel with other assays, are particularly useful in evaluating the efficiency of different remediation/immobilization treatments. For example, a Ni-bioreporter has been helpful in assessing the effects of adding beringite and steel shot to contaminated soil, and the effects of liming on Ni bioavailability (Tibazarwa et al., 2001; Everhart et al., 2006). Changes in Zn speciation after remediation with cyclonic ash and compost (Nachtegaal et al., 2005) and changes in Pb bioavailability after treatment with phosphate rock, lime or ash (Geebelen et al., 2003) have also been determined using bacterial bioreporters. In addition, the presence of bioavailable Zn and Cu in runoff waters from roofs (Heijerick et al., 2002; Karlen et al., 2002; Bahar et al., 2008), and release of Cu from antifouling paints into seawater (Sandberg et al., 2007) have been studied with bacterial bioreporters. (Hynninen and Virta, 2009).

2 AIMS OF THE STUDY

The main resistance mechanisms for divalent heavy metals, such as Zn^{2+}, Cd^{2+} and Pb^{2+} are active efflux transporters. Although numerous studies have characterized different heavy metal resistance mechanisms, they have concentrated on the properties of single transporters. Still very little is known about how the different resistance mechanisms interact and how they are integrated into the metal homeostasis/resistance network in a single cell. In general, the resistance to Zn^{2+} and Cd^{2+} is achieved through the same efflux transporters which do not differentiate between those metals. Some of these transporters are also involved in Pb^{2+} efflux, but generally very little is known about lead resistance. There is some evidence that Pb^{2+} could, in addition to active efflux, be detoxified by binding the ion in an inactive form. Despite the demonstration that lead is precipitated by several bacterial species, the actual molecular mechanisms behind the sequestration have remained unclear.

The bioreporter technology is a biotechnological application which greatly depends on the knowledge about mechanisms that regulate and detect intracellular metal concentrations. The existing bacterial sensors often lack the specificity and/or sensitivity. Thus, their use for environmental risk assessment in complex and low-level bioavailable pollution may be of limited use. Better understanding of resistance networks and their regulation would help to improve the sensitivity and specificity of metal-responsive bioreporters.

This study was initiated to address the abovementioned unclear issues and the main aims of this thesis were

- to determine the molecular mechanism behind lead resistance encoded by the *pbrTRABCD* operon from *Cupriavidus metallidurans* CH34
- to explain the cooperation of several efflux transporters in conferring $Zn^{2+}/Cd^{2+}/Pb^{2+}$ resistance
- to demonstrate how the knowledge about the heavy metal resistance mechanisms and their regulation can be exploited in bioreporter technology.

3 SUMMARY OF METHODS

Bacterial strains and growth media

Escherichia coli K12, *Cupriavidus metallidurans* CH34 (Mergeay et al., 1985) and *Pseudomonas putida* KT2440 (Bagdasarian et al., 1981) and their derivatives were used throughout the studies (I, II, III). All the experiments involving heavy metals were carried out in heavy metal MOPS (HMM) medium (LaRossa et al., 1995), which does not contain inorganic phosphates that could possibly bind heavy metals.

pbr-plasmids (Supplementary data I)

Plasmids containing *pbrTRABCD* genes in different combinations were constructed for the functionality analysis of the *pbr* gene cluster. First the whole gene cluster was PCR-amplified from *C. metallidurans* CH34 and cloned into pUC19 yielding pUCpbrTRABCD. This plasmid was modified by PCR and restriction enzymes to omit one or several genes from the *pbr* gene cluster. Each modified gene cluster always included the gene for *pbrR* and the promoter areas for *pbrRT* and *pbrABCD*, thus the expression of *pbr* genes was the same as in original gene cluster. Modified gene clusters were transferred into low-copy-number plasmid pDN18N (Nunn et al., 1990) to mimic the natural expression level of *pbr* genes in the original plasmid pMOL30. Constructed plasmids were electroporated into *C. metallidurans* strains AE104, a plasmid-free derivative of CH34 (Mergeay et al., 1985), and DN440, a derivative of AE104 lacking genes for ZntA and CadA P-type ATPases (Legatzki et al., 2003). The plasmid-bearing strains were subjected to growth inhibition assays with Zn^{2+}, Cd^{2+} and Pb^{2+}.

Growth inhibition assays (I, II)

Growth inhibition assays were performed in 24-well microplates in 1 ml HMM medium containing different concentrations of heavy metals. Bacterial growth was monitored by measuring optical density at 600 nm after 24 h or 48 h incubation on a shaker. The results were presented as relative growth in comparison to growth in metal free medium.

Complementation assays (I)

Complementation of PbrB by other PAP2 phosphatases was tested by constructing plasmids containing *pgpB* from *E. coli* or *pp4813* from *P. putida* KT2440 in the place of *pbrBC*.

The ability of PbrB to act as a phosphatase was confirmed in *E. coli* mutant strain BWTs2bacA. This strain is a conditional thermosensitive mutant with deletions in all chromosomally encoded C_{55}-PP phosphatase genes [*bacA, ybjG, pgpB, lpxT* (formerly *yeiU*)], but carries an intact copy of *bacA* on a plasmid (pMAKbacA) with a thermosensitive replicon (El Ghachi et al., 2005). Such a strain is viable at 42°C only if an external C_{55}-PP phosphatase gene is provided. *E. coli* was transformed with plasmids allowing expression of *pbrB* and *pbrBC* under the control of the IPTG-inducible *trc* promoter. Two parallel cultures were incubated for 16 h, one at 30°C and the other at 42°C, and the ability of plasmids to restore growth to the mutant at the restrictive temperature was recorded.

Phosphatase assay (I)
PbrBC was purified from the *E. coli* strain BWTs2bacA to avoid contamination with endogeneous *E. coli* phosphatases. C_{55}-phosphatase activity of PbrBC was assessed on radioactive [^{14}C]C_{55}-PP. The formation of C_{55}-P was analyzed by thin-layer chromatography. The phosphatase activity of purified PbrBC towards various mono- and pyrophosphate molecules was tested with Malachite green, which allows spectrophotometric detection of released inorganic phosphate.

Construction of *P. putida* transporter-deficient strains (II)
Altogether 15 different knock-out strains containing deletions in *cadA1*, *cadA2*, *czcA1* and/or *czcA2* genes in all possible combinations were constructed in *P. putida* KT2440. Chromosomal disruptions were constructed by using *sacB*-based gene replacement vector system (Hoang et al., 1998), which uses *sacB* for counterselection on sucrose and Flp-recombinase based marker removal. Such a strategy deleted the central part of the gene leaving no marker in the chromosome and thus allowed several subsequent gene deletions to be constructed in the same strain. All the mutant strains were tested for ability to grow in the presence of metals (Zn^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}, Ni^{2+} or Hg^{2+}).

Complementation and overexpression of transporter genes (II)
Plasmids containing *cadA1*, *cadA2*, *czcA1* and *czcA2* genes under a temperature inducible promoter (Jechlinger et al., 2005) were constructed to complement *P. putida* mutant strains and test the functionality of the same transporters in *E. coli*. The plasmids were electroporated into transporter-deficient *P. putida* strains and into *E. coli* TH201, in which both known divalent heavy metal transporters ZntA and ZitB have been disrupted. The achieved strains were, analogously to plasmid-free mutant strains, tested for the ability to grow in the presence of metals; however, two parallel experiments, one at 28°C and another at 37°C, were done.

In vivo measurement of promoter expression profiles (II)
Putative promoter areas of the *P. putida* KT2440 transporters were PCR amplified using chromosomal DNA of *P. putida* KT2440 as a template and ligated into the pSLlux plasmid (Leedjärv et al., 2006) to control the expression of the *luxCDABE* operon. Subsequently, fragments containing the putative promoter areas and *luxCDABE* operons were transferred into a low-copy-number vector pDN18N (Nunn et al., 1990). Resultant plasmids were transformed into *P. putida* KT2440 and the expression level from the different promoters was monitored by measuring luminescence levels upon exposure to metals. The plasmids did not contain the transcription factor acting on the promoter, but it was coded from the chromosome.

For the expression profile analysis bacteria were grown to specific growth phase (different growth phases were tested) and mixed in a 96-well microplate with an aqueous metal solution [$ZnCl_2$, $CdCl_2$, $Pb(CH_3COO)_2$, $CoCl_2$, $NiCl_2$, or $HgCl_2$] using various metal concentrations (from 10^{-8} to 10^{-3} M). The plates were incubated at 30°C to allow gene expression to equilibrate and then luminescence was recorded. Induction of a promoter by metals was expressed as an induction coefficient (IC), calculated as follows: $IC = L_M/L_W$, where L_M is the luminescence in the metal solution and L_W is the background luminescence in water. The plasmid without any promoter sequence in front of *luxCDABE* was used as a control to

distinguish nonspecific changes in luminescence caused by the physiological state of the cell from the changes caused by the effects of a metal on the studied promoter area.

Bioreporter assays (III)
The host strains for bioreporters were *P. putida* KT2440 (wild type) and transporter-deficient KT2440.2431 that lacked the *cadA1*, *cadA2*, *czcA1* and *czcA2* genes. Promoters of *czcCBA1* operon and *cadA1* gene were employed as sensor elements and bacterial luminescence operon *luxCDABE* as reporter element. Bioreporter assays were carried out in 384-well microplates. Bioreporter bacteria grown in HMM medium to exponential growth phase were mixed with equal amount of metal solution and incubated at 30°C for 3h and then the luminescence was measured. Induction of the bioreporters was reported as an IC. Pipetting was done with a pipetting robot.

Aliquots of soil were spiked with known amounts of $ZnCl_2$ to test the performance of bioreporters on environmental samples. Soil suspensions were too heterogeneous to be pipetted by pipetting robot, therefore soil samples were analyzed in 96-well microplates. Otherwise similar protocol as for standard metal solutions was used. Sample specific IC was calculated by dividing luminescence measured in spiked sample with luminescence measured in non-spiked sample at respective dilution.

Determination of LOD for bacterial bioreporters (III)
The limit of detection for bacterial bioreporters was determined as the metal concentration which induced the reporter to above the threshold level LOD_{IC}, calculated according to the equation $LOD_{IC} = (1 + 2\ CV_w) / (1 - 2\ CV_w)$. Here CV_w is coefficient of variation of the signal determined in water (background or blank). An upper estimate of LOD was determined as the mean plus two standard deviations of the mean, calculated from the independent measurements.

4 SUMMARY OF RESULTS AND DISCUSSION

4.1 Novel lead resistance mechanism in bacteria

Although bacterial heavy metal resistance mechanisms have been studied quite thoroughly, the exact mechanisms behind lead resistance have remained vague. Several Zn^{2+}/Cd^{2+} efflux ATPases are known to transport also Pb^{2+} (Rensing et al., 1998; **II**), and in addition lead sequestration is known to be employed as a resistance mechanism (Levinson et al., 1996; Levinson and Mahler, 1998; Mire et al., 2004). However, molecular mechanisms behind lead sequestration have been unclear. One of the few known lead resistance determinants is coded by the *pbrTRABCD* gene cluster from *C. metallidurans* CH34 (Borremans et al., 2001). This gene cluster consists of six genes that code for the proteins that are hypothesized to combine functions involved in uptake (PbrT), efflux (PbrABC) and accumulation (PbrD) of Pb^{2+} (Borremans et al., 2001). However, their exact functions in Pb^{2+} resistance are not known.

In this study, the functions of different Pbr-proteins in divalent heavy metal resistance were examined. In contrast to the preliminary hypothesis that *pbrTRABCD* is a Pb-specific resistance determinant (Borremans et al., 2001), it was shown that the *pbr* determinant per se is not lead specific. The main component of the resistance mechanism, an efflux P-type ATPase PbrA, efficiently exported Zn^{2+}, Cd^{2+} and Pb^{2+} (**I**). In the case of Zn^{2+} and Cd^{2+}, PbrA alone was sufficient for full resistance, whereas for Pb^{2+} resistance, cooperation of PbrA and PbrB was required. PbrA alone was inefficient in lead detoxification, whereas PbrB alone was toxic to the cell in the presence of metal ions (see Figure 1 in **I**). Differently from PbrA, PbrB is not a transporter protein; instead, according to the conserved domain database (Marchler-Bauer et al., 2007), it contains a PAP2_like_2 domain that is characteristic of phosphatases. Further characterizations of PbrB in complementation assays with a PAP2_like_2 protein from *E. coli* and substrate assays with purified protein confirmed that PbrB is an undecaprenyl pyrophosphate (C_{55}-PP) phosphatase (**I**). Although PbrB is expressed as a fusion protein with PbrC - a putative signal peptidase, no PbrC was required for phosphatase activity of PbrB neither in *C. metallidurans* nor in *E. coli*. No effects of other genes belonging to the *pbr* gene cluster (*pbrT* or *pbrD*) were observed on Zn^{2+}, Cd^{2+} or Pb^{2+} resistance (**I**).

4.1.1 PbrB and its role in lead resistance

C_{55}-PP phosphatases are responsible for dephosphorylation of C_{55}-PP to produce C_{55}-P. The C_{55}-P is an important lipid carrier for intermediates for peptidoglycan, O antigen, teichoic acids and other carbohydrate polymers (Bouhss et al., 2008). Orthologues of C_{55}-PP phosphatase families exist in all bacterial species as they are required for peptidoglycan synthesis (Bouhss et al., 2008). The substrate range of C_{55}-PP phosphatases is not limited to C_{55}-PP but includes also various lipid intermediates (Icho, 1988; Dillon et al., 1996; Touzé et al., 2008; **I**).

At first glance it might look surprising that a protein such as a C_{55}-PP phosphatase is involved in lead resistance. However, dephosphorylation of phosphates yields an inorganic phosphate group as a byproduct. Released phosphates can interact with

Pb^{2+} ions and form insoluble lead phosphate salts. Sequestration as a phosphate salt prevents free lead ions from exerting a toxic effect on the cell as nearly insoluble lead phosphate lacks toxicity and does not inhibit bacterial growth (Levinson et al., 1996). In vitro assays with purified PbrB confirmed that this protein releases phosphates (but not pyrophosphates) by acting on a distal phosphate group of C_{55}-PP (see Figure 3 in **I**). Rapid regeneration of C_{55}-PP due to recycling of C_{55}-P (Barreteau et al., 2009) ensures continuous substrate supply for PbrB so that it can produce sufficient amounts of inorganic phosphate for lead sequestration. Increased dephosphorylation of C_{55}-PP does not influence the viability of the cell (El Ghachi et al., 2004 and 2005; Bernard et al., 2005), and rapid regeneration occurs independently of the reason for dephosphorylation, making C_{55}-PP a good substrate for controlled and sufficient inorganic phosphate production for lead sequestration.

PbrB was expectedly found to be a membrane protein and predictive analysis of its membrane topology revealed a periplasmic location of the active site (**I**). Consequently, the phosphate produced by PbrB is released to the periplasm and thus also the lead sequestration takes place there. PbrB cannot sequester cytoplasmic metal ions, which explains why the presence of a P-type ATPase was necessary for resistance - ions removed from the cytoplasm by PbrA are further precipitated by the action of PbrB (**I**). Possibly some ions are precipitated even before entering the cytoplasm, but the main flux of ions must go through the cell as expression of the *pbr* genes is metal-dependent, i.e., the genes are expressed only in the presence of a sufficient amount of cytoplasmic metal (Monchy et al., 2006).

The special contribution of PbrB to lead resistance comes from its lead-induced expression. Although the genome of *C. metallidurans* CH34 contains several other genes for PAP2 phosphatases, they did not complement the activity of PbrB in strains without *pbrB* (**I**). Their expression is probably not induced by lead, and therefore they cannot be produced in response to lead contamination However, the expression from the *pbr* promoter is induced 20-fold in the presence of lead (Monchy et al., 2006). Increased expression is sufficient to achieve increased phosphatase activity, as overexpression of C_{55}-PP phosphatases in *E. coli* and *Bacillus subtilis* cause up to 600-fold greater phosphatase activity in membrane preparations (Dillon et al., 1996; El Ghachi et al., 2004 and 2005; Bernard et al., 2005).

4.1.2 Distribution of *pbr*-like genes in bacteria

Although in this study *pbrTRABCD* genes were characterized only in *C. metallidurans* CH34, similar gene clusters containing genes at least for transcriptional regulator (analog to PbrR), P-type ATPase (PbrA) and C_{55}-PP phosphatase (PbrB) can be found from several other bacterial species (Figure 3) indicating that lead detoxification through active efflux and subsequent sequestration could be widespread resistance mechanism. While *pbrRAB* genes were present in each cluster, the presence and organization of other *pbr* genes (*pbrT, pbrC, pbrD*) seemed rather random, which supported our results that these genes are not necessary for Pbr-facilitated metal resistance (**I**).

It should be noted that the only connection between PbrA and PbrB comes from their expression from the same promoter/localization in the same operon. Otherwise they are completely independent of each other and can functionally cooperate with other P-type ATPases or PAP2_like_2 phosphatases (**I**, unpublished results) (Figure 4). The achievable Pb^{2+} resistance level depends on the efficiency of the P-type ATPase in Pb^{2+} export - all the tested PAP2 phosphatases provided maximal lead resistance (Figure 4A), whereas other P-type ATPases than PbrA together with PbrB were not able to provide maximal resistance (Figure 4B).

It is possible that similar lead resistance mechanisms exist also in bacteria where genes for Pb^{2+}-exporting P-type ATPase and C_{55}-PP phosphatase are distantly located. The only requirement is that the protein levels are suitably high as C_{55}-PP phosphatases expressed on a 'house-keeping' level do not influence lead resistance.

Unfortunately, no genome sequences of bacteria known to precipitate lead as a phosphate salt are available. Therefore it is impossible to tell whether these bacteria harbor similar lead resistance mechanism as that of *C. metallidurans* CH34.

4.1.3 Model of action for Pbr-proteins

Based on the results presented above, the following model of action for Pbr-proteins was developed (Figure 5) (**I**):

1. metal ions enter the cell through the transporters for essential metals
2. expression from the *pbr* promoter is induced and PbrA and PbrB are produced
3. PbrA pumps cytosolic ions back to the periplasm where lead ions are sequestered with the help of inorganic phosphates produced by PbrB
4. eventually expression from the *pbr* promoter is switched off/reduced as the concentration of free metal ions decreases.

This was the first study where the molecular mechanism of lead sequestration has been described. Full Pb^{2+} resistance is based on active efflux and sequestration as both PbrA and PbrB are required. In the case of only PbrA being present, Pb^{2+} ions remain shuttling between the cyto- and periplasm. On the other hand, if only PbrB is present, ions that have once entered the cytoplasm remain there and cause continuous expression from the metal-dependent promoter. An excess of the membrane protein PbrB hinders the growth of the bacteria leaving them in a disadvantageous situation even compared to bacteria without any resistance mechanism (**I**).

Detoxification by precipitation as a phosphate salt is only possible with Pb^{2+} as zinc and cadmium phosphates are more soluble in water. Periplasmic Zn^{2+} and Cd^{2+} can be further removed from the cell with the help of CBA transporters (**II**; Scherer and Nies, 2009).

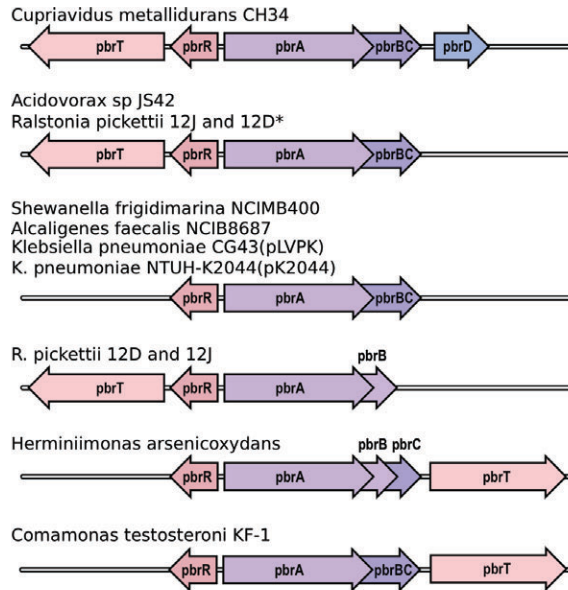

Figure 3. *pbr*-like gene clusters in different bacterial species. *R. pickettii* strains 12D and 12J contain two different *pbr* gene clusters. The sequence contig of one of the *R. pickettii* 12D gene clusters is cut in the middle of *pbrT* (marked with asterisk) and this has not been annotated in the database. The gene clusters were found based on the PbrBC blastp hits in non-redundant GenBank CDS translations.

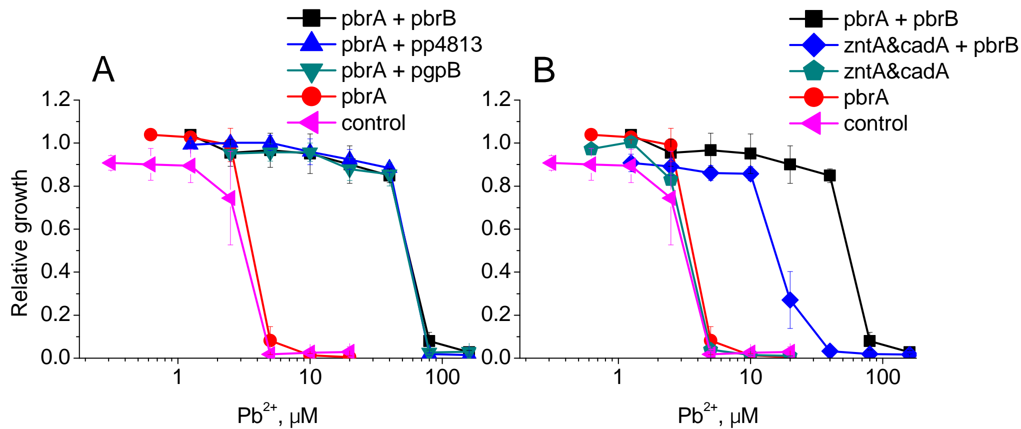

Figure 4. Cooperation of a P-type ATPase and a C_{55}-PP phosphatase in lead resistance. (A) Effect of phosphatase on the lead resistance level of *C. metallidurans*. (B) Effect of P-type ATPase on the lead resistance level of *C. metallidurans*. Dose-response curves of strains expressing either *pbrA*, *zntA* and *cadA* (all from *C. metallidurans*), and/or *pbrB* (*C. metallidurans*), *pgpB* (*E. coli*) or *pp4813* (*P. putida* KT2440) are shown. An empty vector containing no *pbr* genes was used as a control. The data are presented as the mean ± standard deviation of at least three independent experiments.

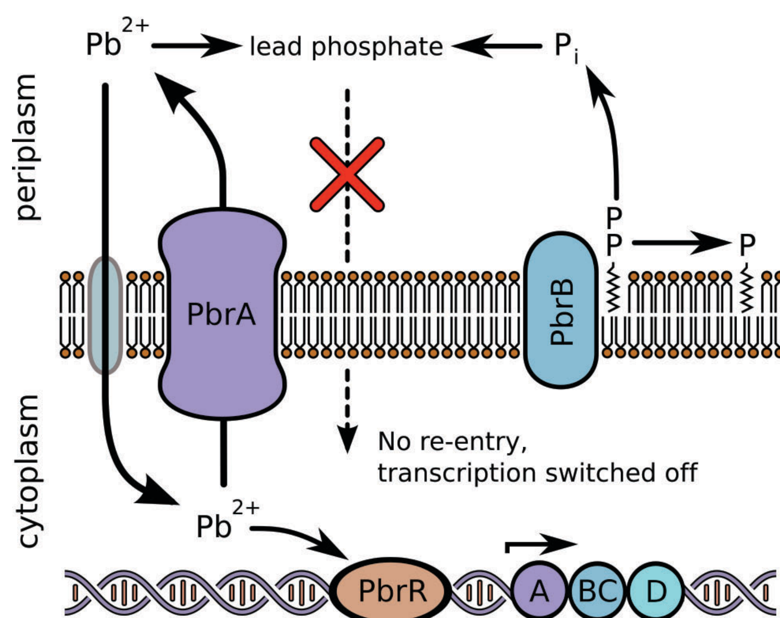

Figure 5. Model of action for the Pbr resistance determinant. Pb^{2+} ions enter the cell through transporters for essential metals. Upon the presence of intracellular metals, transcription of the *pbr* operon is initiated and consequently PbrA and PbrB proteins are produced. PbrA starts to pump Pb^{2+} to the periplasm, while PbrB dephosphorylates its substrates yielding inorganic phosphate. Periplasmic free Pb^{2+} concentration decreases as Pb^{2+} is sequestered as a phosphate salt. The sequestration of lead discontinues the expression of the *pbr* operon, avoiding potentially harmful overexpression of PbrB.

4.2 Interplay of different transporters in mediating resistance to divalent heavy metals

The most widespread efflux transporters, the P-type ATPases and CDF proteins, transport metal ions only to the periplasm, but further removal of the ions is required to completely detoxify the metal. While Pb^{2+} can be detoxified by sequestration as an insoluble salt (**I**), Zn^{2+} and Cd^{2+} require further transport from the periplasm to the outside of the cell. Periplasmic metal can be transported by CBA transporters which span the whole cell envelope and get their substrates probably both from periplasm and cytoplasm while the exact mechanism is still unclear (Nies, 2003). In order to better understand the cooperation between different transporters, the interplay between P-type ATPases and CBA transporters was studied in saprophytic soil bacterium *P. putida* KT2440 (**II**).

According to *in silico* analysis, the chromosome of *P. putida* KT2440 includes genes for two P-type ATPases, CadA1 and CadA2, and for at least two CBA transporters, CzcCBA1 and CzcCBA2 (Canovas et al., 2003). In order to study the functionality of these transporters and reveal their contribution to the overall Zn^{2+}/Cd^{2+}/Pb^{2+} resistance, mutant strains with deletions in the respective genes were constructed and tested for the ability to grow in the presence of heavy metals (**II**). All possible knockout combinations of these genes were constructed to avoid situations where one transporter may compensate for the loss of another. The CBA transporters were disrupted by deleting the *czcA* genes, as it has been shown earlier

that disruption of the RND component (gene A) of a CBA transporter is enough to abolish its function (Nies et al., 1989). The functionality of these transporters was also studied in *E. coli* and the expression profiles from the respective promoters were determined.

In one way or another all *P. putida* transporters proved to be functional metal exporters. The most prominent effect was seen on Cd^{2+} resistance, which was influenced by CadA2 and CzcCBA1. Both transporters were required for full resistance, which indicates that CadA2 and CzcCBA1 have independent functions in the cell and one cannot compensate for the other (**II**). The substrates for P-type ATPases (CadA2) are thiol-bound cations from the cytoplasm (Sharma et al., 2000), whereas CBA transporters (CzcCBA1) can bind their substrates also in the periplasm (Legatzki et al., 2003; Scherer and Nies, 2009). CzcCBA1 could thus serve as a first line of defense by exporting metal ions even before they have entered the cytoplasm but also by further removing the ions already exported from the cytoplasm by CadA2. The effect of CzcCBA1 on Cd^{2+} resistance was moderate in comparison to CadA2, indicating that CadA2 is more efficient in Cd^{2+} transportation and that it is more important for the cell to remove cytoplasmic than periplasmic Cd^{2+} ions (**II**). This finding is in accordance with analysis of 64 bacterial genomes according to which transporters removing cytoplasmic metal and giving the cell the basic ability to endure metals (P-type ATPases and CDF transporters) are widespread (Nies, 2003). The presence of a CBA transporter, on the other hand, provides additional resistance and is restricted to environmental bacteria likely to need higher resistance to metals (Nies, 2003).

In *P. putida* KT2440 Zn^{2+} resistance was affected only by disruption of the CzcCBA1 and CzcCBA2 transporters but not by disruption of P-type ATPases CadA1 or CadA2 (**II**). The effect of the CzcCBA2 transporter on Zn^{2+} resistance could only be seen in a *czcA1*-defective background which means that CzcCBA1 alone was able to confer full Zn^{2+} resistance and CzcCBA2 could only partially complement it. Overexpression of *czcA2* increased Zn^{2+} resistance in a *czcA1*-deficient strain (but not in a wild-type strain) indicating that, when present in sufficient amounts, CzcA2 could substantially confer Zn^{2+} resistance (**II**). In these overexpression experiments, CzcA2 was expressed at about a 3-fold higher level than CzcCB2, but CzcCB1 in turn was expressed at a 100-fold higher level than CzcCB2 (see promoter studies below, Figure 7A). This means that CzcA2 was much more likely to complex with CzcCB1 proteins than with CzcCB2 to form functional transporter (**II**). At the same expression conditions, CzcA1 itself was not able to complement the loss of original *czcA1* gene from the chromosome which indicates that CzcA2 in complex with CzcCB1 was much more efficient in Zn^{2+} export than CzcCBA1 itself (**II**).

It was surprising that the P-type ATPases CadA1 and CadA2 did not confer Zn^{2+} resistance in *P. putida* KT2440 although these transporters were active in Zn^{2+} efflux in *E. coli* (**II**). It is possible that CzcCBA1 could efficiently export periplasmic Zn^{2+} even before it could enter the cell and there was no need for a cytoplasmic transporter. Effective detoxification of Zn^{2+} with only a CBA transporter has been observed in *C. metallidurans* CH34 (Legatzki et al., 2003;

Scherer and Nies, 2009). However, in contrast to *C. metallidurans*, where all excess Zn^{2+} is probably exported before it can enter the cytoplasm (Scherer and Nies, 2009), some Zn^{2+} does escape CzcCBA1 in *P. putida* as there was clear induction by Zn^{2+} from promoters that are activated in response to cytoplasmic Zn^{2+} (**II**). It might be that some transporter other than CadA1 or CadA2 acts as a cytoplasmic exporter or that the CzcCBA1 transporter could, in addition to periplasmic Zn^{2+}, efficiently export cytoplasmic Zn^{2+}. Putative candidates for additional Zn^{2+} exporters include CzcD, belonging to the CDF family, and the recently characterized novel transporter MrdH. CzcD has not been characterized in *P. putida* KT2440, but for example CDF transporters in *C. metallidurans* and *E. coli* confer low level resistance to Zn^{2+} (Anton et al., 1999; Grass et al., 2001; Anton et al., 2004). MrdH confers some resistance to Zn^{2+} and its domain structure presumes cytoplasmic export (Haritha et al., 2009) making it a possible candidate for cytoplasmic Zn^{2+} exporter.

The four studied transporters had a relatively small effect on Pb^{2+} resistance of *P. putida* KT2440. Only a 2-fold decrease in Pb^{2+} resistance was observed in *cadA2* mutant strains (**II**). Deletion of *czcA1* somewhat reduced the maximal growth of the mutant strain in the presence of Pb^{2+}, however, it did not have an effect on actual Pb^{2+} resistance (see Figure 1B in **II**). In *zntA*-deficient *E. coli* CadA2 increased lead resistance by about a factor of 10 confirming its functionality as a Pb^{2+} efflux transporter (unpublished results).

It could be hypothesized that *P. putida* KT2440 has some yet to be discovered resistance mechanism for lead, as even the transporter-deficient strain was very resistant to lead [minimal inhibitory concentration (MIC) of lead 120 µM, whereas MIC was 5 µM for *zntA-cadA-pbr*-deficient *C. metallidurans* and 2 µM for *zntA*-deficient *E. coli* (**I**, **II**, unpublished results)]. One possibility would be a mechanism similar to Pbr in *C. metallidurans* CH34 (**I**), since a PAP2 phosphatase coded by *pp4813* of *P. putida* KT2440 could fully complement PbrB in the *pbr* operon (unpublished results) (see Figure 4A). However, the transporter component of this mechanism has to be some exporter other than CadA1 or CadA2, as these did not have significant effect on lead resistance. Two *in silico* studies on *P. putida* KT2440 metal transporters have revealed no obvious candidates for an additional Pb^{2+} exporter (Canovas et al., 2003, Haritha et al., 2008), but the recent discovery of MrdH (Haritha et al., 2009) suggests that new uncharacterized transporter families might exist. Another possibility is that *P. putida* is intrinsically very resistant to lead without any specific lead resistance mechanism, but rather due to its cell wall composition and biosurfactant production. Rhamnolipids of *P. aeruginosa* are known to efficiently form complexes with heavy metals (Herman et al., 1995); however, *P. putida* KT2440 does not contain the genetic potential required to produce rhamnolipids and no compounds with surfactant properties have been detected in this strain (Matilla et al., 2007). Thus, the actual mechanisms and genetic determinants conferring high level lead resistance in *P. putida* KT2440 remain to be determined.

All the above results confirm that a tight interplay between different proteins exists to confer full resistance and provide backup systems in bacterial strains with several

heavy metal transporters. In both *C. metallidurans* CH34 and *P. putida* KT2440 the resistance to Zn^{2+}, Cd^{2+} and Pb^{2+} is achieved through cooperation of several defense mechanisms (Figure 6) (**I**; **II**; Legatzki et al., 2003; Scherer and Nies, 2009). While the most important step for resistance is the removal of intracellular metal, further (or initial) export from the periplasm and sequestration of metal ions also play important roles. In order to orchestrate such a vast array of resistance mechanisms, control at the gene expression level is necessary.

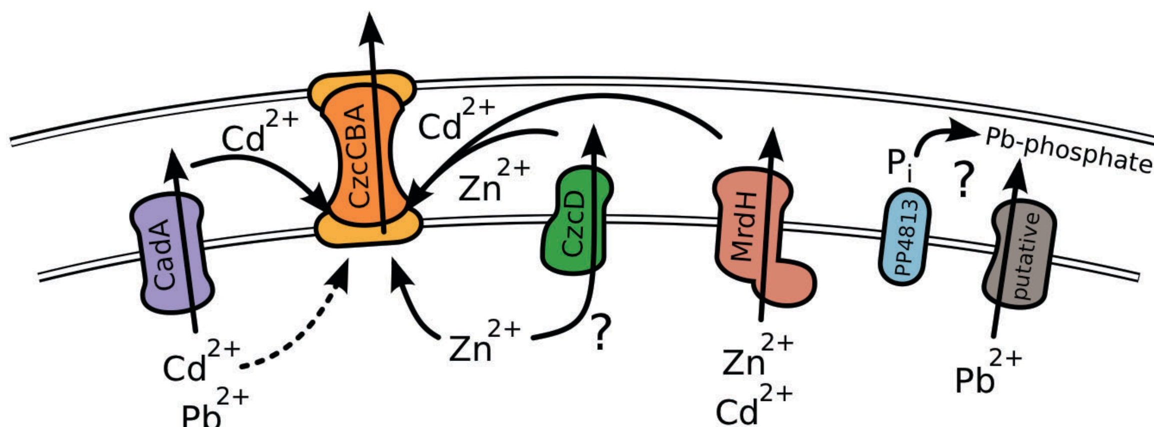

Figure 6. Interplay of different transporters in conferring Zn^{2+}, Cd^{2+} and Pb^{2+} resistance in *P. putida* KT2440. Cd^{2+} is removed from the cell by CadA and CzcCBA, whereas Zn^{2+} is efficiently exported by CzcCBA. Pb^{2+} is exported by CadA2, but probably additional resistance mechanisms exist. MrdH is known to export Zn^{2+} and Cd^{2+}, but its cooperation with other transporters has not been studied. Functions of CadA, CzcCBA and MrdH have been proven experimentally (**II**; Haritha et al., 2009), functions of CzcD and Pb^{2+} detoxification complex are putative.

4.3 Expression of metal transporter genes in *P. putida* KT2440

Heavy metal efflux transporters work against a concentration gradient, and thus they require energy for their activity. As such they are an energetic burden to the cell and regulated expression is necessary to avoid useless presence of metal transporters but at the same time allow quick response to the cellular needs (see Section 1.1.3). In order to determine the expression profiles of *cadA1*, *cadA2*, *czcCBA1* and *czcCBA2* from *P. putida* KT2440, the respective promoter areas were fused with the bacterial bioluminescence operon *luxCDABE* (**II**). In such constructs luminescence level depends on promoter activity and effects of metals can be easily monitored by recording changes in the luminescence.

The data on expression levels gives further insights into the working hierarchy of studied transporters. The four studied promoters exhibited very different expression levels in a metal-free environment. Expression was highest from PcadA2 (promoter of *cadA2*) that was expressed at about a 1000-fold higher level than PcadA1, a 100-fold higher level than Pczc1 and about a 10-fold higher level than Pczc2 (**II**). The relatively high background expression from PcadA2 suggests that CadA2 could be used as a guardian to immediately remove any toxic metals that occasionally enter the cell. The presence of metals (Zn^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}, Ni^{2+} or Hg^{2+}) in the growth

environment induced expression of PcadA2, PcadA1 and Pczc1, but had no effect on Pczc2 activity. Regulation of *cadA2* and *czcCBA1* expression was non-specific, as promoters PcadA2 and Pczc1 were inducible with every tested metal, whereas PcadA1 was relatively specific showing a marked increase in luminescence only in response to Zn^{2+} (**II**). The wide inducer range of PcadA2 and Pczc1 is not surprising, as most heavy metal-responsive transcription regulators/promoters respond to several metals (Brocklehurst et al., 1999; Binet and Poole, 2000; Brocklehurst et al., 2003). Rather, it is surprising that PcadA1 responded only to Zn^{2+}. The *cadA1* gene is not accompanied by any transcription factor gene, and thus the regulator acting on PcadA1 promoter remains unknown.

CadA2 was the most highly expressed transporter for Cd^{2+} and Pb^{2+}, whereas in case of Zn^{2+} CzcCBA1 expression levels exceeded the levels of CadA2 (Figure 7, unpublished results). Also the sensed concentrations followed the same pattern: in the exponential growth phase, expression of PcadA2 was induced at lower Cd^{2+} and Pb^{2+} concentrations than of Pczc1, which, in turn, was activated in response to low Zn^{2+} concentrations that were not sensed by transcription regulator acting on PcadA2. These expression profiles are in accordance with growth inhibition studies which indicated that CadA2 is important in Cd^{2+} and Pb^{2+} resistance and CzcCBA1 is the main transporter in Zn^{2+} resistance (**II**). Although expression of CadA1 increased about 20-fold in response to Zn^{2+}, its expression level remained still very low in comparison to CzcCBA1 (see Figure 7A). The very low expression level of CadA1 in *P. putida* KT2440 might explain why this transporter had no effect on zinc resistance in its original host, although it was found to be a functional Zn^{2+} transporter in *E. coli*. The same holds for CzcCBA2, i.e., its expression level is probably too low to efficiently participate in Zn^{2+} resistance. However, there must be some amount of the functional transporter CzcCBA2 (or CzcCB1A2) present in the cell as it could somewhat complement the loss of CzcCBA1 (**II**).

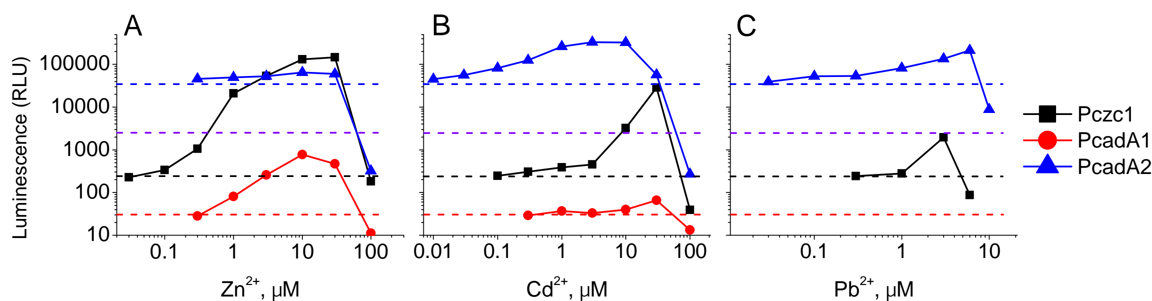

Figure 7. Relative expression from the promoters of *czcCBA1* operon (Pczc1, black), *cadA1* (PcadA1, red) and *cadA2* genes (PcadA2, blue) in response to Zn^{2+} (A), Cd^{2+} (B) and Pb^{2+} (C) in relative light units (RLU). Dashed lines represent the background expression from respective promoters. Promoter of *czcCBA2* operon (Pczc2, violet dashed line) was not induced by any metals and PcadA1 was not induced by Pb^{2+}, only background expressions are presented.

The characteristics of divalent heavy metal efflux transporters and their expression in *P. putida* KT2440 are summarized in Table 1.

The genes for CadA1 and CzcCBA1 have been acquired through horizontal gene transfer (HGT) as they are located in a genomic island (GI) (Weinel et al., 2002, Haritha et al., 2008), whereas the genes for CadA2 and CzcCBA2 are located in the core genome. It might be hypothesized that after having acquired the GI that bears CzcCBA1 the functionality (expression in response to metals) of CzcCBA2 was lost. The disadvantage of CzcCBA2 might lie in its highly efficient zinc export that could in long term lead to deprivation of zinc which despite its toxicity is required as a micronutrient.

Table 1. Characteristics of known divalent heavy metal efflux transporters in *P. putida* KT2440.

Transporter (type)	Exported metals	Inducing metals in minimal medium	Back-ground expression[a]	Putative transc-ription factor	Comments	Refe-rence
CadA1 (P-type ATPase)	Zn^{2+} Cd^{2+}	Zn^{2+} (Cd^{2+})	Very low	unknown	functionality seen in *E. coli*, but not in *P. putida*; acquired through HGT	**II**
CadA2 (P-type ATPase)	Zn^{2+} Cd^{2+} Pb^{2+}	$Cd^{2+}>Pb^{2+}\approx Co^{2+}>Hg^{2+}>Zn^{2+}$	Very high	CadR2	Zn^{2+} transported in *E. coli*, but not in *P. putida*	**II**
CzcCBA1 (CBA transporter)	Zn^{2+} Cd^{2+} Pb^{2+}?	$Zn^{2+}>>Ni^{2+}>Cd^{2+}>>Pb^{2+}\approx Co^{2+}>Hg^{2+}$	Low	CzcRS1 CzcRS2	acquired through HGT	**II**
CzcCBA2 (CBA transporter)	Zn^{2+}	not inducible	Moderate	CzcRS1 CzcRS2	functionality seen only in *czcA1*-defective back-ground; in complex with CzcCB1 very efficient Zn-transporter	**II**
MrdH	Zn^{2+} Cd^{2+} Ni^{2+}	$Zn^{2+}>Ni^{2+}>Cd^{2+}>Co^{2+}$	NA[a]	unknown	acquired through HGT	Haritha et al., 2009

a - relative expression levels of *cadA1*, *cadA2*, *czcCBA1* and *czcCBA2* in metal free environment. The expression level of *mrdH* cannot be compared to others as it was not involved in the same study.

4.4 Applying *P. putida* transporter-deficient strain and metal-inducible promoters for bioreporter technology

Bacterial bioreporters are microorganisms that produce a specific quantifiable output in response to target chemicals. They combine a sensing element, which is responsible for detecting the analyte (for example inducible promoter), and a reporter element, which allows for quantification of the signal (for example luminescence or fluorescence coding genes). The studied *P. putida* KT2440 promoters had two properties that made them very attractive for bioreporter

technology. Namely, Pczc1 responded to very low Zn^{2+} concentrations and exhibited more than 1000-fold inducibility, and PcadA1 was very specific responding only to Zn^{2+} (**II**). Additionally, as the sensitivity of bacterial bioreporters depends greatly on the metal homeostasis/resistance mechanisms that determine the intracellular concentration available for detection (Hynninen and Virta, 2009), transporter-deficient *P. putida* strains could be promising hosts for bioreporters with improved sensitivity. We hypothesized that the removal of efflux transporters causes metals to accumulate in the cell and consequently lower extracellular concentrations could be detected. In order to test the theory, wild type *P. putida* KT2440 and transporter-deficient mutant strain *P. putida* KT2440.2431 (missing *cadA1, cadA2, czcA1* and *czcA2* genes) were transformed with reporter plasmids where the promoters PcadA1 and Pczc1 were fused to the bacterial luminescence operon *luxCDABE* and characterized as metal-responsive bioreporters (**III**).

As hypothesized, a transporter-deficient bioreporter strain could detect lower extracellular metal concentrations than its wild type counterpart (**III**, Table 2). The best improvement in the limit of detection (LOD) for Pczc1-bioreporter

Table 2. Limits of detection of Zn-, Cd- and Pb-sensing bioluminescent bacterial bioreporters.

Bacterial bioreporter	Limit of detection, µM			Reference
	Zn^{2+}	Cd^{2+}	Pb^{2+}	
P. fluorescens OS8::KncadRPcadAlux[a]	4	0.03	0.3	Ivask et al. 2009
E. coli MC1061(pSLzntR/pDNPzntAlux)[a]	5	0.01	0.7	Ivask et al. 2009
E. coli MC1061(pzntRluc)[b]	40	0.06	n.t.	Ivask et al. 2002
E. coli MG1655(pZNT-lux)[b]	1	0.01	0.03	Riether et al. 2001
S. aureus RN4220(pTO024)[c]	1	0.01	0.03	Tauriainen et al. 1998
B. subtilis BR151(pTO024)[c]	1	0.03	n.i.	Tauriainen et al. 1998
P. putida KT2440(pDNPczc1lux)[d]	0.16 (0.08)	1.12 (0.49)	0.90 (0.41)	**III**
P. putida KT2440.2431(pDNPczc1lux)[d]	0.05 (0.02)	0.09 (0.05)	0.02 (0.02)	**III**
P. putida KT2440(pDNPcadA1lux)[d]	0.89 (0.63)	n.i.	n.i.	**III**
P. putida KT2440.2431(pDNPcadA1lux)[d]	0.12 (0.08)	n.i.	n.i.	**III**

a - limit of detection (LOD) determined as a concentration that caused $2 + 6\ SD_w/L_w$ -fold induction
b - LOD determined as a concentration that caused 2-fold induction
c - LOD determined as a concentration that caused $L_w + 2\ SD_w$ -fold induction
d - LOD determined as a concentration that caused $(1 + 2\ CV_w) / (1 - 2\ CV_w)$ -fold induction; upper estimate of LOD (confidence level 97.7 %) and mean LOD (in parenthesis) are presented
L_w - luminescence in water, SD_w - standard deviation of the signal measured in water, CV_w - coefficient of variation of the signal measured in water
n.t. - not tested; n.i. - not inducible

[KT2440(pDNPczc1lux) vs KT2440.2431(pDNPczc1lux)] was achieved with Pb^{2+} (45-fold). The LOD for Cd^{2+} and Ni^{2+} decreased moderately (12-fold and 10-fold, respectively), whereas improvement in Zn^{2+} LOD was relatively small (3-fold) (see Figure 2 in **III**). Although the decrease in LOD for Zn^{2+} was smaller than expected, the Pczc1-bioreporter proved to be still by far the most sensitive luminescent bioreporter for Zn^{2+} (Table 2). The LOD for Cd^{2+} and Pb^{2+} remained on the same level as for previously constructed bioreporters; however, the feasibility of the transporter disruption strategy for LOD improvement indicates that bioreporters with improved sensitivity towards Cd^{2+} and Pb^{2+} could be constructed using already existing bacterial bioreporters. A similar strategy has been shown to work for on Cu/Ag-bioreporters (Stoyanov et al., 2003). The removal of CopA, a Cu^{+}/Ag^{+}-exporting ATPase, from *E. coli* rendered a bioreporter employing the *copA* promoter as a sensor system 15-fold more sensitive to Cu^{+} and 8-fold more sensitive to Ag^{+} (Stoyanov et al., 2003).

When PcadA1 was employed as a sensor element, 8-fold improvement in the LOD of Zn^{2+} was achieved upon disruption of transporter systems of the host strain [KT2440(pDNPcadA1lux) vs KT2440.2431(pDNPcadA1lux)] (Table 2). The improvement in LOD was not restricted only to standard metal solutions as transporter-deficient reporter strain could detect lower Zn^{2+} concentrations also from soil samples (see Figure 4 in **III**). Although the PcadA1-bioreporter could not detect as low Zn^{2+} concentrations as the Pczc1-bioreporter (Table 2), it had the great advantage of being Zn-specific. While the Pczc1-bioreporter cannot differentiate between Zn^{2+}, Cd^{2+} and Pb^{2+}, the PcadA1-bioreporter responds only to Zn^{2+} regardless of other contaminants present in the sample. Specificity of the bioreporter is important when a sample containing several pollutants is analyzed and separate data on bioavailability of each pollutant is required.

4.4.1 Calculating the LOD for bacterial bioreporters

Despite the fact that bioreporter technology has been around for two decades (the first bioreporter was published in 1990 by King et al.) no common method for calculating one of the most important characteristics of bioreporters, the LOD, has been established in the literature. Very different grounds for determining the LOD have been used (see Table 2 footnotes) making comparison of different bioreporters impossible. Several of the methods applied for calculating the LOD lack any statistical basis. However, as bacterial bioreporters are living systems, some variability in the signal within one experiment and between independent experiments is natural. Therefore, a statistically sound method for determining LOD should be used. In this work, a method that considers the variability in the signal for both the blank and also the analyte was used for determining LODs for *P. putida*-based bioreporters (**III**). According to this method the concentration of the analyte where the confidence interval of the signal does not overlap with the confidence interval of the blank signal is considered as the LOD (Biddlecombe and Law, 1996). In addition to considering the variation within the experiment, also the variability between the independent experiments should be taken into account. Thus, in addition or instead of the mean LOD also confidence intervals should be reported. In publication **III** the use of the upper estimate of LOD was suggested.

When only the mean LOD is used for characterizing the bioreporter, 50 % of the experiments actually have a higher LOD than initially reported, which gives false sound to the term 'limit of detection'. However, the upper estimate of LOD guarantees that with a probability of 97.7 % the LOD in a single experiment is below the reported level (for equations see **III**).

4.5 Bioreporters as a method for studying metal distribution in the cell

The data obtained with bioreporters sheds further light on the functionality of the four studied *P. putida* KT2440 metal efflux transporters. The Pczc1- and PcadA1-bioreporters respond to metals in different cellular compartments. Expression from Pczc1 is regulated by the two component transcriptional regulator CzcRS that senses periplasmic metal (**II**, Hassan et al., 1999). The exact transcription factor for PcadA1 is unknown, but by drawing parallels with other P-type ATPases, it is probably regulated by a MerR- or ArsR-family transcription regulator that detects cytoplasmic metal.

In the transporter-deficient strain induction from Pczc1 promoter occurred at 45-fold lower Pb^{2+} concentrations than in the wild type strain (**III**), whereas difference in Pb^{2+} resistance levels was only 2-fold (**II**). This indicates that in the transporter-deficient strain KT2440.2431 Pb^{2+} accumulated into periplasm without causing much toxicity there. As mentioned earlier, the reason for high lead resistance is unknown; however, accumulation into periplasm without causing toxicity could indicate the presence of some binding factor or some sort of lead sequestration. This sequestration cannot be in completely non-bioavailable form since lead could be detected by CzcS.

Cd^{2+} seemed to accumulate both into periplasm and cytoplasm. Periplasmic Pczc1-sensor exhibited a 12-fold reduced LOD (**III**) without fully explaining the 32-fold decrease in resistance to Cd^{2+} in the transporter-deficient strain (**II**). This is in agreement with theory that both CadA2 exporting ions from cytoplasm and CzcCBA1 exporting ions from cytoplasm and periplasm are involved in Cd^{2+} resistance (see Figure 6) (**II**). However, it also indicates that there might still be a functional cytoplasmic Cd^{2+}-exporting protein present in the KT2440.2431 strain, a possible candidate would be MrdH (Haritha et al., 2009).

Contrary to Cd^{2+}, Zn^{2+} seemed to accumulate mainly into cytoplasm as the sensitivity of cytoplasmic PcadA1-sensor improved 8-fold, but sensitivity of periplasmic Pczc1-sensor only 3-fold (**III**). Inducibility of cytoplasmic PcadA1-reporter by Zn^{2+} and accumulation of Zn^{2+} into cytoplasm in transporter-deficient strain confirm that in addition to periplasmic Zn^{2+} CzcCBA1 (and CzcCBA2) exports Zn^{2+} also from the cytoplasm (see Figure 6) and the help of a P-type ATPase (or other cytoplasmic exporter) is not required.

5 CONCLUSIONS AND FUTURE PROSPECTS

In this study zinc, cadmium and lead resistance mechanisms and their regulation in bacteria were studied and the acquired knowledge was applied for construction of bacterial bioreporters with improved sensitivity and specificity.

In publication **I**, the *pbrTRABCD* gene cluster from *C. metallidurans* CH34 was revisited to determine the mechanism behind Pb^{2+} resistance. Only PbrA and PbrB were required for heavy metal resistance, PbrT, PbrC and PbrD were not essential (PbrR is required for the metal-regulated expression from *pbr* promoter). PbrA is a P-type ATPase that conferred resistance to Zn^{2+}, Cd^{2+} and Pb^{2+}; whereas PbrB improved only Pb^{2+} resistance. PbrB was shown to be an undecaprenyl pyrophosphate (C_{55}-PP) phosphatase that contributes to Pb^{2+} resistance by producing inorganic phosphates for lead precipitation in nontoxic form. C_{55}-PP phosphatases are essential enzymes for cell wall synthesis and are, thus, present in all bacterial species. It remains to be determined whether all C_{55}-PP phosphatases have a role in Pb^{2+} detoxification or whether distinct enzymes are involved in cell wall synthesis and Pb^{2+} sequestration. Also, it would be interesting to know whether lead sequestration mechanisms in bacteria, where lead phosphate formation has been observed, are the same as in *C. metallidurans*. Database searches indicate that gene clusters containing genes for P-type ATPase (*pbrA*) and C_{55}-PP phosphatase (*pbrB*) are found in several bacterial species. The presence and location of *pbrT*, *pbrC* and *pbrD* in these gene clusters supports our results that respective proteins are not essential for heavy metal resistance. Further studies are needed to determine whether they have any role at all.

In *P. putida* four different metal efflux transporters were characterized (**II**). Surprisingly, under laboratory conditions only two of them, the P-type ATPase CadA2 and the CBA transporter CzcCBA1 were involved in Zn^{2+}/Cd^{2+}/Pb^{2+} resistance, although all four transporters were functional metal exporters. For full Cd^{2+} resistance both CadA2 and CzcCBA1 were required. CadA2 exported cytoplasmic Cd^{2+} ions, whereas CzcCBA1 was responsible for exporting periplasmic ions before they could enter the cytoplasm or after being transported by CadA2. Expression of both transporters was induced in response to Cd^{2+}. For Zn^{2+} resistance only CzcCBA1 was required. Expression from cytoplasmic promoters in the presence of Zn^{2+} and accumulation of Zn^{2+} into cytoplasm in CzcCBA1-defective strain suggests that CzcCBA1 exported both cytoplasmic and periplasmic ions. Pb^{2+} resistance in *P. putida* was influenced only by CadA2; however, removal of CadA2 reduced Pb^{2+} resistance only 2-fold with the overall resistance remaining quite high suggesting presence of additional lead resistance mechanisms. Putative C_{55}-PP phosphatase (encoded by *pp4813*) from *P. putida* could fully complement PbrB in *C. metallidurans*, but further studies are needed to reveal whether there is additional lead resistance mechanism present in *P. putida* and whether it is similar to lead sequestration in *C. metallidurans*.

In publication **III**, the transporter-deficient *P. putida* strain, which was constructed in study **II**, was applied as a bioreporter strain. Lack of metal exporters caused accumulation of metal ions in the cell and allowed detection of lower extracellular

concentrations. By using the promoter of CzcCBA1 transporter as a sensor element in the transporter-deficient strain, 50-fold lower zinc concentrations could be detected than by previously constructed bioluminescent Zn-bioreporters. Using a similar strategy, new bioreporters with improved sensitivity could also be constructed for other metals by just choosing suitable sensor elements and bacterial strains where resistance mechanisms have been characterized. The current study also proved that one does not have to be content with broad-spectrum sensor elements, but also specific transcription factors exist. Open-minded search for new heavy-metal-regulated promoters would yield bioreporters with different specificities.

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